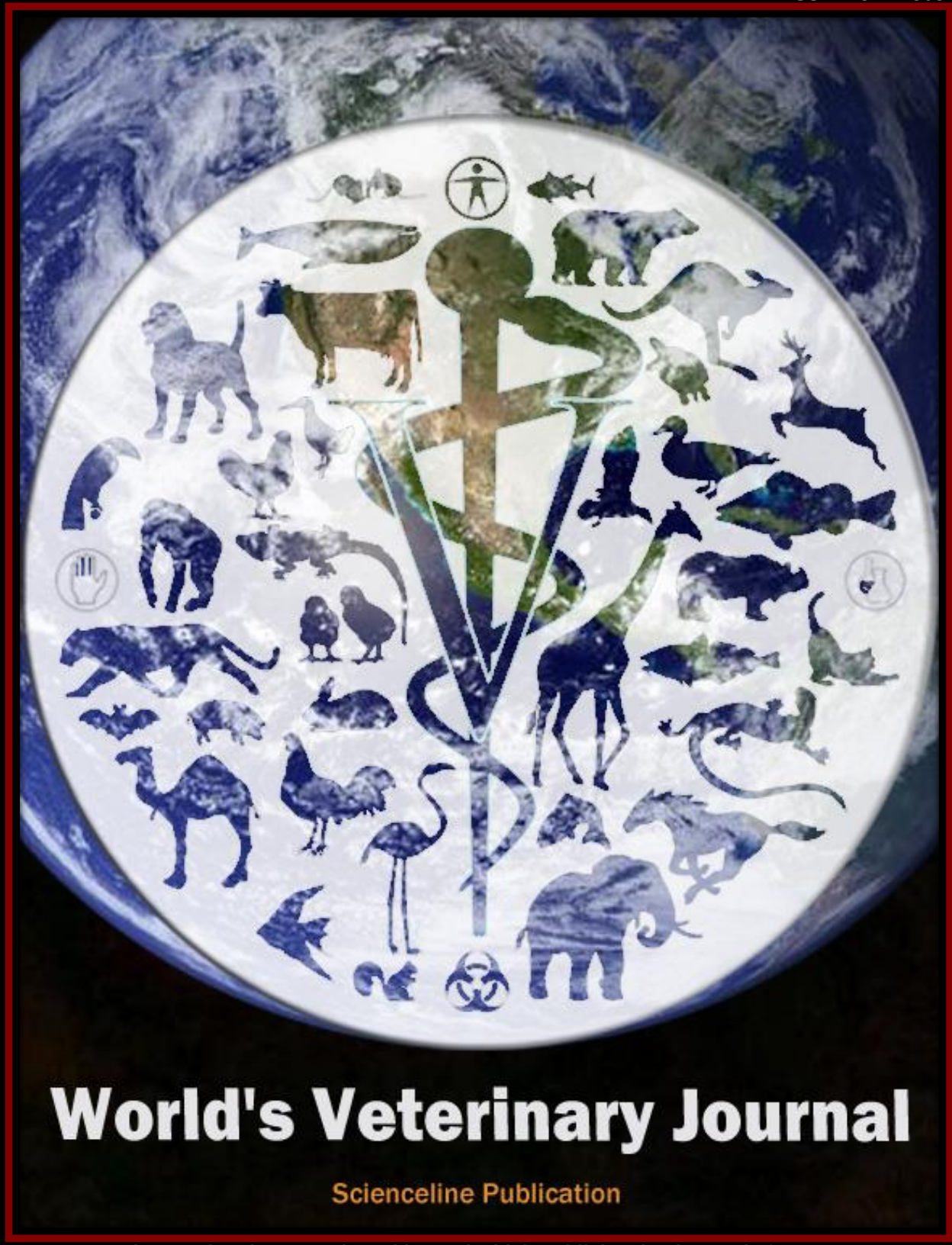
In the Name of God

ISSN: 2322-4568



World's Veterinary Journal

Scienceline Publication

An international peer-reviewed journal which publishes in electronic format

Volume 11, Issue 3, September 2021

WORLD'S VETERINARY JOURNAL

ISSN: 2322-4568

World Vet. J., 11 (3): 317-520; September 25, 2021

Editorial Team

Reviewers

Advisory Board

Join WVJ Team

World's Veterinary Journal is always striving to add diversity to our editorial board and operations staff. Applicants who have previous experience relevant to the position they are applying for may be considered for more senior positions within WVJ. All other members must begin as section reviewer before progressing on to more senior roles. Editor and editorial board members do not receive any remuneration. These positions are voluntary.

If you are currently an undergraduate, MSc or PhD student at university and interested in working for WVJ, please fill out the application form below. Once your filled application form is submitted, the board will review your credentials and notify you within a week of an opportunity to membership in editorial board. If you are PhD, assistant, associate editors, distinguished professor, scholars or publisher of a reputed university, please rank the mentioned positions in order of your preference. Please send us a copy of your resume (CV) or your ORCID ID or briefly discuss any leadership positions and other experiences you have had that are relevant to applied poultry research, Researches or publications. This includes courses you have taken, editing, publishing, web design, layout design, and event planning. If you would like to represent the WVJ at your university, join our volunteer staff today! WVJ representatives assist students at their university to submit their work to the WVJ. You can also, registered as a member of journal for subsequent contacts by email and or invitation for a honorary reviewing articles.

Download WVJ Application Form

Contact us at editor [at] wvj.science-line.com

TABLE OF CONTENTS

Archive

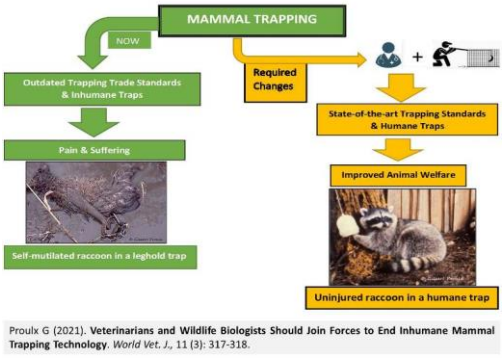
Volume 11 (3); September 25, 2021

Short Communication

Veterinarians and Wildlife Biologists Should Join Forces to End Inhumane Mammal Trapping Technology.

Proulx G.

World Vet. J. 11(3): 317-318, 2021; pii:S232245682100043-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj43



Proulx G (2021). **Veterinarians and Wildlife Biologists Should Join Forces to End Inhumane Mammal Trapping Technology**. *World Vet. J.*, 11 (3): 317-318.

ABSTRACT: Current mammal trapping standards uphold the use of inhumane trapping technology. For example, killing neck snares for the capture of canids, and rotating-jaw traps, and steel-jawed leghold traps for procyonids and mustelids, are being used by trappers despite decades of research showing that they are inhumane, and cause serious injuries and distress in captured animals. Many wildlife biologists unsuccessfully raised concerns about inhumane mammal trappings. This short communication stresses the need for veterinarians and wildlife biologists to work together to improve the fate of mammals captured in killing or restraining traps, and modify mammal trapping standards on the basis of animal welfare science.
Keywords: Humaneness, Mammal trapping, Traps, Trapping standards

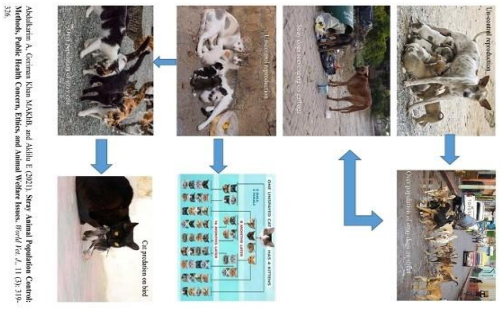
[Full text-PDF] [XML] [Google Scholar]

Review

Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues.

Abdulkarim A, Goriman Khan MAKhB, and Aklilu E.

World Vet. J. 11(3): 319-326, 2021; pii:S232245682100044-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj44



ABSTRACT: Stray animal overpopulation has become one of the most serious global problems with many negative impacts on the community, environment, and public health. Most of the stray animals do not depend on humans for food and shelter, and therefore, can reproduce uncontrollably. The uncontrolled reproduction of stray animals increases their population that leads to a higher chance of predation, road traffic accidents, transmission of zoonotic diseases, and therefore, becoming vectors for some diseases. There are several methods for stray animal population control depending on the situation and the nature of the stray animals. These methods include contraceptives, trap-neuter-return, poisoning, euthanasia, and gun shooting among others. Each of the outlined methods has its advantages and disadvantages as to their practicality, ease of conducting, cost, effectiveness, ethics, and animal welfare issues. In conclusion, to achieve successful control measures of the stray animal population and the problem they create, the concerned authorities need to design and enact animal rights laws, provide medical care (treatment and vaccination), feeding, shelter for the animals, and control their reproduction. Public health and environmental agencies may improve the services by regulating personal and environmental hygiene, prevention, and control of zoonotic and transmissible diseases that can be transmitted from stray animals to the public and other livestock respectively.
Keywords: Animal welfare, Ethics, Population control methods, Public health, Stray animals

[Full text-PDF] [XML] [Google Scholar]

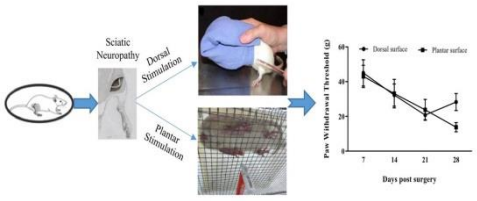
Research Paper

Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain.

Ghanbari A and Mohammadi M.

World Vet. J. 11(3): 327-332, 2021; pii:S232245682100045-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

ABSTRACT: Mechanical and thermal stimuli were used to evaluate neuropathic pain-like behavior in animal models usually. Mechanical stimulation of paw plantar surface is commonly used to determine mechanical allodynia. In the present study, paw withdrawal response to plantar surface stimulation was compared with paw withdrawal response to dorsal surface stimulation. To this end, a total of 30 female Wistar rats (180-220 g), were assigned randomly to three groups as intact (without any manipulation), sham (incision of skin and muscles without nerve injury), and neuropathy (sciatic nerve lesion) with 10 in each group. To induction of neuropathy (chronic constriction injury), four movable ligations were established around the sciatic nerve using catgut chromic suture with a distance of one millimeter apart and then wound incision was closed. In the sham group, the incision site was closed without nerve ligation. Mechanical allodynia was examined by Von Frey filaments for four weeks. The findings indicated that the paw withdrawal threshold following dorsal surface stimulation was significantly reduced compared to the sham group at day 21 post-surgery. Moreover, paw withdrawal threshold following plantar surface stimulation significantly decreased compared to the sham group at day 21 post-surgery. The present results regarding the sham group showed that the paw withdrawal threshold after mechanical stimulation of the plantar surface was not significantly different from that of the dorsal surface paw. In addition, and there was no significant difference between the paw withdrawal response to plantar surface and dorsal one. In conclusion, paw withdrawal threshold to plantar surface mechanical stimulation was not significantly different from one in dorsal surface following neuropathic pain induced by chronic constriction injury.
Keywords: Mechanical allodynia, Neuropathic pain, Paw dorsal surface, Paw plantar surface, Rat



Ghanbari A and Mohammadi M (2021). **Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain.** *World Vet. J.*, 11 (3): 327-332.

[Full text-PDF] [XML] [Google Scholar]

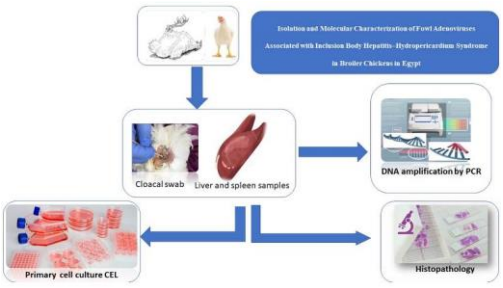
Research Paper

Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt.

Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA.

World Vet. J. 11(3): 333-342, 2021; pii:S232245682100046-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj46

ABSTRACT: Avian adenoviruses are an extremely diversified group of pathogens that recently triggering a variety of problems for poultry production. In particular, Inclusion Body Hepatitis-Hydropericardium Syndrome (IBH-HPS), which has been observed in broiler birds from 3 to 6 weeks of age and is associated with anemia, hemorrhagic disorders, hydropericardium, and high mortality. The disease has been reported worldwide, and recently it was reported in many Egyptian farms, causing severe economic losses. Therefore, the current study aimed to isolate, and genetically type the most common Adenovirus serotypes associated with this syndrome in Egyptian farms. A total of 50 broiler chicken farms (3-6 weeks old) located in different Egyptian governorates were examined. Macroscopically, the diseased flock revealed hydropericardium, enlarged friable livers with ecchymotic hemorrhages, and varying mortality rates (1 to 7.5%). Histopathologically, severe diffuse necrotizing enteritis, hepatitis, pericarditis, and diffuse lymphoid depletion of the spleen were the most prominent lesions. Liver tissues and cloacal swabs were collected from all examined flocks for FAdVs detection by conventional polymerase chain reaction (PCR) targeting the L1 loop in the hexon gene. The PCR products were sequenced for typing of the detected viruses. It was found that 10 out of 50 flocks examined were PCR positive for FAdVs (20%). Phylogenetic analysis of the sequenced genes revealed that the obtained viruses clustered with reference strains belonging to FAdV type D and E serotype 2, 11, and 8a respectively. The isolation of both FAdV type D and FAdV type E were carried out on a primary cell culture chicken embryo liver cell (CEL) and the presence of these viruses was confirmed by PCR after the appearance of cytopathic effect (CPE). From this study, it could be concluded that both FAdVs types D and E are the most common adenoviruses circulating in poultry farms suffering from hydropericardium and inclusion body hepatitis.
Keywords: Broiler chicken, Chicken embryo liver cell, Fowl adenovirus, Hexon gene, Histopathology, Inclusion body hepatitis–hydropericardium syndrome, PCR



Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). **Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt**. *World Vet. J.*, 11 (3): 333-342.

[Full text-PDF] [XML] [Google Scholar]

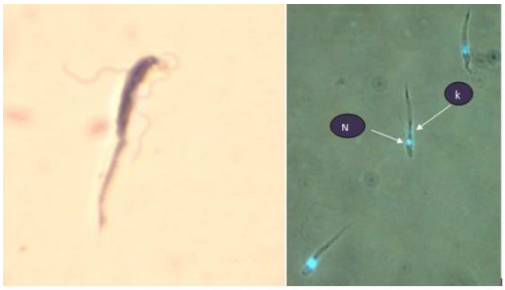
Research Paper

Molecular Identification of Trypanosoma theileri and Biology of Trypanosomes.

Fentahun T and Paeshuyse J.

World Vet. J. 11(3): 343-367, 2021; pii:S232245682100047-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

ABSTRACT: *Trypanosoma theileri* (*T. theileri*) is a non-pathogenic, cosmopolitan, and commensal protozoa of cattle. The main objective of the current study was to investigate the biology and feasibility of *T. theileri* as a model candidate for the discovery of a novel drug. In the present study, the isolates of *T. theileri* obtained from the Institute of Tropical Medicine (ITM) in SDM 79 were cultivated at 26°C. Eight experiments with different inoculum and different times were grown. The growth curve was plotted to check the growth trends. The doubling time in the logarithmic phase was determined to be 17.43 hours. In addition, an experimental infection was done on a 3-month-old Holstein Friesian calf to isolate the blood-streaming shape; however, it was not successful after the blood buffy coat smear and PBMC culture in RPMI 1640 and HMI 9. Furthermore, the viability was determined by quantitative colorimetric Resazurin assay in 96-well fluorescence Microplates containing 0.4 to 2.4 mM of Resazurin. On the other hand, the response to Pentamidine (1-100 ng/mL) showed a strong negative correlation between the fluorescence signal and the highest Pentamidine concentration. IC_{50} was 9.25 ng/mL. Genomic DNA was extracted using the phenol-chloroform method. The gradient PCR amplification using *T. theileri* specific PCR (Tth625-PCR) primers was detected at 465 base pair (bp). In addition, the full-length 18S rDNA sequence was detected at 730 bp. In the silico analysis using common anti-trypanosome drug targets, no significant similarity could be found on either the DNA or the protein level. Nevertheless, homologous sequences have been identified among the drug targets for Ornithine decarboxylase. Therefore, the analysis might show the possibility of using *T. theileri* as a model for the search of new drugs once they have entire genome sequences. Analysis of the whole genome and transcriptome indicated a phylogenetic relationship between *T. theileri* and other pathogenic trypanosomes which can be the basis for novel drug development.
Keywords: Drug model, Novel drug, PCR, Resazurin, SDM 79, *Trypanosoma theileri*



Fentahun T and Paeshuyse J (2021). **Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes.** *World Vet. J.*, 11 (3): 343-367.

[Full text-PDF] [XML] [Google Scholar]

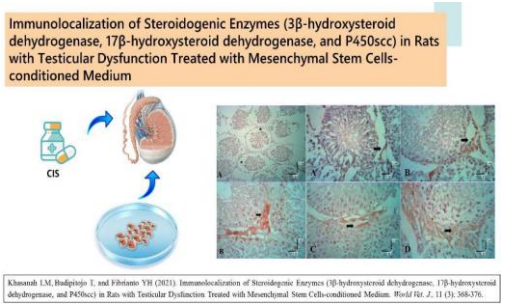
Research Paper

Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium.

Khasanah LM, Budipitojo T, and Fibrianto YH.

World Vet. J. 11(3): 368-376, 2021; pii:S232245682100048-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

ABSTRACT: About 60-80 million couples in the world are suffering from infertility disease. Infertility is a major problem in patients coping with chemotherapy. The chemotherapy process can degenerate non-target organs, especially in testes. Infertility in male or testicular dysfunction is caused by the failure of proliferation and differentiation of the spermatogenic cells. Many studies reported that mesenchymal stem cells-conditioned medium promoted regenerative processes. The present study aimed to investigate the effect of mesenchymal stem cells-conditioned medium on the cisplatin-induced testicular dysfunction by examining the immunolocalization of steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc which are considered as markers of steroid production. All experimental animals were divided into three groups, namely the control group, mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.2 ml/kg body weight (BW, P1), and mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.5 ml/kg BW (P2). Cisplatin was injected into both treated groups to induce testicular dysfunction. The testicular tissues were processed by the paraffin method, then cut to a thickness of 5 µm, followed by immunohistochemical staining. The HSD3B1 immunoreactivities were found only in Leydig cells, and the intensity increased every week after the injection of mesenchymal stem cells-conditioned medium. The variety of weeks and groups was significantly different in the number of immunoreactive cells of HSD3B1. The results indicated a significant difference between one week after the first injection and the one week after the third and fourth injection. The findings showed a significant difference between the treated group with an injection dose of 0.2 ml/kg BW and the control group. The number of immunoreactive cells of HSD3B1 with an injection dose of 0.5 ml/kg BW was greater compared to the group that received an injection dose of 0.2 ml/kg BW. The intensity of HSD3B1 and HSD17B1 increased every week. The p450scc immunoreactive cells were only found in Leydig cells. The intensity of positive cells of p450scc in the treated group with an injection dose of 0.5 ml/kg BW was more intense, compared to the treated group with an injection dose of 0.2 ml/kg BW. The results of the current study showed that the injection of mesenchymal stem cells-conditioned medium can improve the regeneration of spermatogenic cells, and recover spermatogenesis proved by positive cells of HSD3B1, HSD17B1, and p450scc as markers of steroid production.
Keywords: Cisplatin, HSD17B1, HSD3B1, Mesenchymal stem cells-conditioned medium, P450scc, Testicular dysfunction



Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.*, 11 (3): 368-376.

[Full text-PDF] [XML] [Google Scholar]

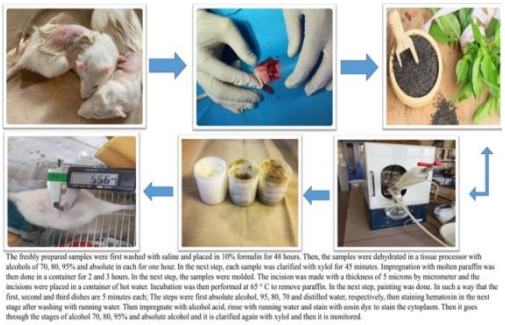
Download GA as PDF

Research Paper

The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats.

Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH.

World Vet. J. 11(3): 377-383, 2021; pii:S232245682100049-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj49



The freshly prepared samples were first washed with saline and placed in 10% formalin for 48 hours. Then, the samples were dehydrated in a tissue processor with alcohols of 70, 80, 95% and absolute in each for one hour. In the next step, each sample was clarified with xylol for 45 minutes. Impregnation with molten paraffin was then done in a container for 2 and 3 hours. In the next step, the samples were molded. The incision was made with a thickness of 5 microns by micrometer and the incisions were placed in a container of hot water. Incubation was then performed at 65 ° C to remove paraffin. In the next step, painting was done. In such a way that the first, second and third dishes are 5 minutes each; The steps were first absolute alcohol, 95, 80, 70 and distilled water, respectively, then staining hematoxin in the next stage after washing with running water. Then impregnate with alcohol acid, rinse with running water and stain with eosin dye to stain the cytoplasm. Then it goes through the stages of alcohol 70, 80, 95% and absolute alcohol and it is clarified again with xylol and then it is monitored.

ABSTRACT: Diabetic wounds and *cutaneous* wounds are important issues in medical science. Basil is an herbaceous plant and has compounds such as terpenes, flavonoids, and antioxidant properties. A total of 50 male Wistar rats were allocated into 10 groups including the healthy group without treatment, the healthy group treated with 1% phenytoin, eucerin, 5% basil seed extract, 10% basil seed, diabetic group without treatment, 1% phenytoin, eucerin. After anesthesia of rats, we made a 4 cm2 wound on the back of the animal, and different histopathological characteristics were examined, and also on days 3, 7, and 21, the area of wounds was analyzed. In the healthy group treated with 10% basil seed extract, it was revealed that the wound size was significantly healed in the research days. In the diabetic rat groups, the decrease in the wound area was not significant and there was no significant difference between groups. Histopathological findings on day 21 in the healthy group treated with 10% basil seed extract revealed proper epidermis formation and relatively dense dermis containing collagen filaments. While in the diabetic groups, healing was slower. The results indicated that basil extract with anti-inflammatory and antioxidant characteristics can quicken the repair of *cutaneous* wounds.
Keywords: Basil, Diabetes, Histopathology, Wound
[Full text-PDF] [XML] [Google Scholar]

Research Paper

Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020.

Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N.

World Vet. J. 11(3): 384-394, 2021; pii:S232245682100050-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). **Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020.** *World Vet. J.*, 11 (3): 384-394.

ABSTRACT: Poultry production has been affected by multiple respiratory diseases triggering serious economic losses in Egypt. The current study aimed to investigate the situation and genetic evolution of respiratory diseases in Egypt during 2020. A total of 53 samples were collected from infected flocks suffering from respiratory signs and variable mortality rates from nine governorates in Egypt during 2020. The collected samples were examined for the detection of respiratory disease viruses (Avian influenza virus (AIV (H5N8, H9N2), Infectious bronchitis virus (IBV), and Newcastle disease virus (NDV)) by rRT-PCR. The single infection was confirmed in 90.6% (37.7% I.B, 30.2% AIV (H5N8), 9.4% I.B and 5.7% NDV) and co-infection of HPAIV (H5N8) + I.BV and LPAIV (H9N2) +IBV were detected in 3.8% of nine governorates. The HA gene of HPAIV (H5N8) was cluster to clad 2.3.4.4.1b in a new branch with characteristic specific mutations especially in T140A in antigenic site A and R72S in the receptor-binding site, compared to A/duck/Egypt/F446/2017 with low A.A identity percent with vaccinal strains of H5N1 and H5N2 reaching to 91.9-94% and 84.6%, respectively. The HA gene of AIV (H9N2) belonged to A/quail/Hong Kong/G1/97-like virus clustered with group B with a specific mutation (212I) that may affect the human transmission of the virus. The HVRs of S1 gene of IBV cluster to GI23 (Egy Var I) clad with multiple mutations in HVR1 and HVR2, compared to IBV/CU/4/2014 and low identity percent (68.3-78.8%) with vaccine strains (H120, M41, 4/91). In conclusion, respiratory disease continues to circulate and rapidly evolve in Egypt during 2020.
Keywords: HPAIV (H5N8), IBV, Genetic characterization, LPAIV(H9N2), Respiratory disease

[Full text-PDF] [XML] [Google Scholar]

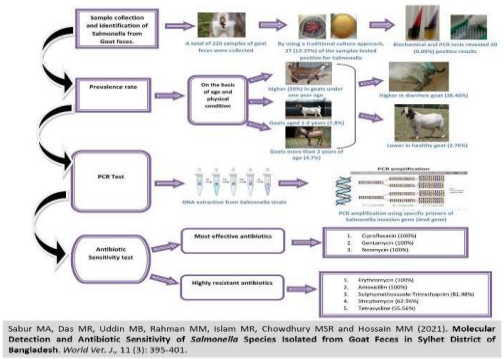
Download GA as PDF

Research Paper

Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh.

Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM.

World Vet. J. 11(3): 395-401, 2021; pii:S232245682100051-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj51



Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). **Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh.** *World Vet. J.*, 11 (3): 395-401.

ABSTRACT: The present study aimed at the molecular detection of *Salmonella* species from feces of goats and the characterization of the isolated *Salmonella* by biochemical and antimicrobial sensitivity techniques. A total of 220 goat feces samples were collected, of which 27 (12.27%) were positive for *Salmonella* by conventional culture methods and 20 (9.09%) by biochemical and PCR techniques. The prevalence was higher in goats under one year of age (20%), compared to older animals aged one to two years (7.8%) and more than two years of age (4.7%), respectively. Moreover, the prevalence of diarrheic goats was significantly higher (38.46%) than healthy animals (2.76%). DNA was extracted from *Salmonella* strains and amplified by PCR using the specific primers of *Salmonella* invasion gene (*invA gene*). The antibiotic sensitivity test indicated that Ciprofloxacin (100 percent sensitivity), Gentamycin (100 percent sensitivity), and Neomycin (100 percent sensitivity) were the most effective antibiotics for the majority of *Salmonella* isolates. On the other hand, *Salmonella* isolates were found to have substantially high resistance to Erythromycin (100%), Amoxicillin (100%), Trimethoprim-Sulfamethoxazole (81.48%), Streptomycin (62.96%), and Tetracycline (55.56 percent). Since the rate of *Salmonella* carriers was relatively high, eating goat meat could increase the risk of foodborne salmonellosis.
Keywords: Antibiotic sensitivity, Goat isolation, PCR detection, *Salmonella*

[Full text-PDF] [XML] [Google Scholar]

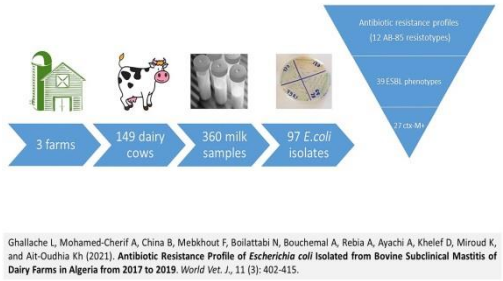
Research Paper

Antibiotic Resistance Profile of Escherichia coli Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019.

Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh.

World Vet. J. 11(3): 402-415, 2021; pii:S232245682100052-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

ABSTRACT: Mastitis in cows is a major problem in dairy farms leading to a decrease in the quantity and quality of milk. The aim of the present study was to examine the association between the presence of *Escherichia coli (E. coli)* in milk and the subclinical mastitis, and to characterize the antibiotic resistance profiles of the isolated *E. coli*. In the current study, a total of 360 cow raw milk samples from three dairy farms of the region of Algiers were analyzed. The analysis period lasted from Spring 2017 to Winter 2019. The California Mastitis Test (CMT) was applied to detect subclinical mastitis. The *E. coli* strains were isolated from milk using conventional bacteriological methods. The antibiotic resistance profile of the isolated *E. coli* strains to 12 different antibiotics was tested using the disk diffusion method. On *β*-lactamase-producing strains, a double diffusion test was applied to identify the Extended-spectrum *β*-lactamase (ESBL) phenotype. Finally, the *ctXx-M* genes were amplified by PCR. Two-thirds (66.4%) of the milk samples were positive for the CMT test. A total of 97 *E. coli* strains were isolated from the milk samples, their resistance to antibiotics was tested, and 3.1% of the strains were resistant to trimethoprim-sulfamethoxazole, 6.2% to chloramphenicol, 12.3% to gentamicin, 13.4% to colistin, 23.3% to amoxicillin/clavulanate, 31.9% to kanamycin, 39.2% to enrofloxacin, 51.5% to cefotaxime, 52% to tetracycline, 57.7% to ampicillin, 74.3% to nalidixic acid, and 75.3% to amoxicillin. Furthermore, most of the *E. coli* strains (92.8%) were resistant to more than one antibiotic with a Multiple Antibiotic Resistance index ranging from 0 to 0.8. The 50 strains resistant to cefotaxime were analyzed for an ESBL phenotype. 39 of them (78%) were positive to the double-disk synergy test. Among the 39 ESBL positive strains, 27 (69.2%) were confirmed for the presence of a CTX-M gene by PCR. The present study showed that multiple drug-resistant *E. coli,* including ESBL-carriers, were frequently isolated from the milk of dairy cows in Algeria. The results underlined that the use of antibiotics on farms must be reasoned to avoid the spread of resistant strains in animals and human populations.
Keywords: Antibiotic Resistance, Cows, CTX-M gene, *Escherichia coli*, Milk, Subclinical Mastitis



Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). **Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019**. *World Vet. J.*, 11 (3): 402-415.

[Full text-PDF] [XML] [Google Scholar]

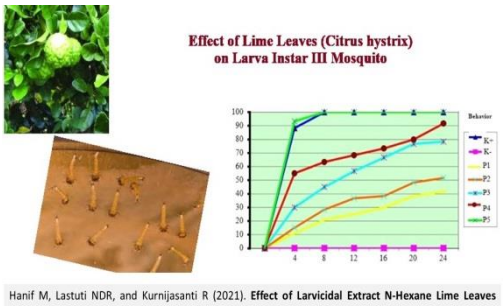
Research Paper

Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*)

Hanif M, Lastuti NDR, and Kurnijasanti R.

World Vet. J. 11(3): 416-421, 2021; pii:S232245682100053-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

ABSTRACT: *Culex quinquefasciatus* mosquitoes are a vector of transmission of several types of diseases, such as filariasis, Japanese encephalitis, and dirofilariasis. Larval control is the key strategy of disease control programs caused by vectors around the world because it can prevent larvae to enter the adult stage. Control of mosquito larvae that are often used is chemically controlled. *Citrus hystrix* is a natural plant and contains chemical compounds that have biological activity, such as flavonoids, carotenoids, and limonoids. The present study aimed to determine the effect of citrus leaf extract (*Citrus hystrix*) on the



Hanif M, Lastuti NDR, and Kurnijasanti R (2021). **Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*).** World Vet. J., 11 (3): 416-421.

mortality of the larva *Culex quinquefasciatus*. The method used in the current study was a laboratory experimental study method with the experimental design using a completely randomized design. The research plot carried out was the rearing larvae of *Culex quinquefasciatus*, the manufacture of citrus leaf extract, the manufacture of larvicidal material, and the observation phase after treatment. Extract N-hexane *omplet name hystrix* leaf has high toxicity because it contains essential oils, flavonoids, alkaloids, terpenoids, saponins, and Limonoids. The Optimal concentration of N-hexane extracts of citrus leaves that lead to larval mortality of *Culex quinquefasciatus* in vitro amounted to 93.33% on 4 hours of observation with a concentration of 4000 ppm. Extract N-hexane lime leaf has a larvicidal effect on the larva *Culex quinquefasciatus* in vitro.
Keywords: *Citrus hystrix*, *Culex quiquefasciatus*, Extract, Mosquitoes, Larvicide

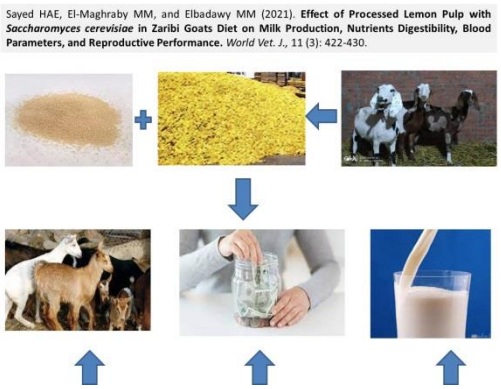
[Full text-PDF] [XML] [Google Scholar] [Crossref Metadata]

Research Paper

Effect of Processed Lemon Pulp with Saccharomyces cerevisiae in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance

Sayed HAE, El-Maghraby MM, and Elbadawy MM.

World Vet. J. 11(3): 422-430, 2021; pii:S232245682100054-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj54



ABSTRACT: The aim of the present study was to evaluate the effect of replacing concentrate feed (CFM) mixture with different levels of treated lemon pulp (TLP) on milk production and composition, nutrients digestibility, and blood parameters of Zaribi goats. A total of 24 Zaribi female goats at the end of the pregnant period were divided into three groups of eight according to live weight. Concentrate feed mixture and clover hay at a rate of 50:50 were offered twice a day. Treatedlemon pulp replaced CFM at rates 0%, 25%, and 50 % in diets of R1, R2, and R3 treatment groups, respectively. The feeding trial lasted 90 days. Dry matter digestibility and Nitrogen free extract digestibility were significantly raised by the increased level of TLP in groups R2 (72.37% and 70.36%) and R3 (72.28% and 70.30%), compared to (70.99% and 68.51%) in R1, respectively. The same trend was observed for organic matter digestibility (OMD), crude protein digestibility (CPD), crude fiber digestibility (CFD), and ether extract digestibility (EED) with R3, compared with either R1 or R2. However, there were insignificant differences between R1 and R2 in terms of OMD, CPD, and EED but CFD was significantly higher in R2, compared to R1. There was a significant increase in the milk yield by the increased level of TLP in the diet and for R2 (1448.4g/h/d) and R3(1558.7g/h/d), while it was reported as 1377.6 g/h/d in the control group. Feeding dams on the R3 diet had a significant effect on improving total antioxidant capacity by 63.5%, compared with control. The results of the present study indicated that the replacement of CFM by TLP decreased the costs of feed and increase milk production for the replacement level by 50% (R3). Moreover, no adverse effects were noticed on nutrients digestibility and blood parameters of the investigated samples.
Keywords: Blood parameters, Lemon pulp, Milk production, Saccharomyces cerevisiae

[Full text-PDF] [XML] [Google Scholar]

Short Communication

The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish

Fuaidah P, Triastuti J, and Pramono H.

World Vet. J. 11(3): 431-438, 2021; pii:S232245682100055-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

ABSTRACT: Scad fish (*Decapterus kurroides*) is the most productive fish species. To provide the added value, fishermen can process scad fish into various forms of processed products, such as preserved fish, which extend the shelf life. In order to extend the shelf life of canned fish, natural preservatives could be used that contain antimicrobial agents, such as bay leaves. The present study aimed to determine the effects of bay leaf extract in different concentrations on the shelf life of preserved scad fish. In the present study, the completely randomized design was used as an experimental research method. The treatments given differed in terms of the concentration of the bay leaf extract. The groups were treated as the scad fish without bay leaf extract (A), scad fish soaked in 6% of bay leaf extract (B), scad fish soaked in 7% of bay leaf extract (C), scad fish soaked in 8% of bay leaf extract (D), and scad fish soaked in 9% of bay leaf extract (E). The analyzed parameters included the total plate count, pH, and water level test using Analysis of Variance (ANOVA). The supportive parameters observed included an organoleptic test. The results indicated that the lowest total bacterial count from the beginning to the end of the experiment (18 hours) occurred on treatment C

(7% of bay leaf extract addition), which was 1.54 × 103 to 5.85 × 106. Regarding the water level test from the beginning to the end of the experiment, treatment A (control) was not significantly different from other treatments. The difference in the concentration of bay leaf extract on scad fish effectively inhibited the growth of bacterial colonies. In conclusion, adding bay leaf extract to scad fish could inhibit bacteria for up to 12 hours. Treatment C (7% of bay leaf extract) gave the best results since this concentration level could inhibit the bacteria on scad fish.
Keywords: Bay leaf, Preservation, Scad fish, Shelf life

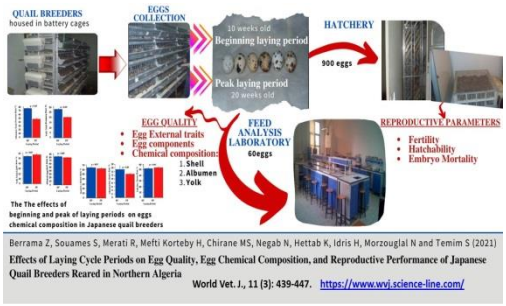
[Full text-PDF] [XML] [Google Scholar]

Research Paper

Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria

Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S.

World Vet. J. 11(3): 439-447, 2021; pii:S232245682100056-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj56



ABSTRACT: Egg quality traits in quail breeders depend on various factors which may influence embryo survival during incubation, affecting the chicks' production and quality. The current study aimed to determine the effect of the laying period on the external and internal quality of the egg, the chemical composition of albumen, yolk, and eggshell as well as the reproductive parameters of quail breeders. A total of 450 quails (*Coturnix japonica*) obtained from the same hatchery, were reared in a battery cage with a sex ratio of 1 male to 3 females. All the quails were subjected to standard breeding conditions and fed a balanced laying diet. A total of 960 eggs were collected at two different periods of the laying cycle, the beginning period of the laying (BP indicating 10 weeks of quail age) and the peak period of laying (PP showing 20 weeks of quail age). At each laying period, 30 eggs were used to analyze the various parameters of egg quality, and 450 eggs were randomly selected to assess the reproductive performances. Overall, no differences in the external quality of eggs, such as egg length, egg width, and the egg shape index, were recorded between the two laying periods. Apart from albumen weight that tended to be higher at the peak laying period, the shell and the yolk weights were not affected by the period of laying. Additionally, Japanese quail tend to deposit similar proportions of shell, albumen, and yolk at the two periods of laying. Likewise, the rate of dry matter of the three egg components, shell mineral concentrations, and yolk fat concentrations did not show any noticeable variation with the laying period. The most significant effect of the laying period was related to the potential reduction in the total protein content of the albumen and the yolk of eggs laid at the peak period of laying. Finally, the laying period did not significantly affect the fertility and hatchability rate of the incubated quail eggs but slightly improved the embryonic mortality rate during the peak laying phase.
Keywords: Albumen, *Coturnix japonica*, Eggshell, Fertility, Quail

[Full text-PDF] [XML] [Google Scholar]

Research Paper

The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB)

Batista FR, Triastuti J, and Pursetyo KT.

World Vet. J. 11(3): 448-455, 2021; pii:S232245682100057-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

ABSTRACT: The decrease in the number of lands for aquaculture will cause the freshwater fish aquaculture, especially tilapia is reared. As one of the efforts to survive, the breeders use the coastal area to anticipate, so the freshwater fish can adapt to the sea waters. Heavy metal pollution near the coasts (or in the coastal waters) has great potential impacts on the environment. The present study aimed to find out the effect of Lead (Pb, heavy-metal) exposure on the Jatimbulan Juvenile tilapia with the different salinity towards histopathology description of the gill, intestine, and the skin tissues. The method used was an experimental method with a completely randomized design using six treatments and three times repetition. The observed parameters included the changes in histopathology description of gill, intestine, and skin organs of Jatimbulan juvenile tilapia. Kruskal-Wallis scoring method was used for data analysis and was continued by Mann-Whitney. The result of the current study showed the effect of salinity towards histopathology description of gill, intestine, and skin of Jatimbulan juvenile tilapia. The effect of changes was proven with the histopathology description in the form of the damage of edema, hyperplasia, and necrosis on the gill tissue; the damage of edema, atrophy, and necrosis on the gill tissue as well as the damage of edema, atrophy, hemorrhagic and necrosis on the gill tissue. From the result of the current study which has been conducted, it can be concluded that the heavy-metal exposure by lead was 0.03 ppm on the salinity of 10 ppt and 20 ppt. given the significant effect on the histopathology description of gill, intestine, and skin of Jatimbulan juvenile tilapia.
Keywords: Histopathology, Lead, Metal, Oreochromis niloticus, Salinity

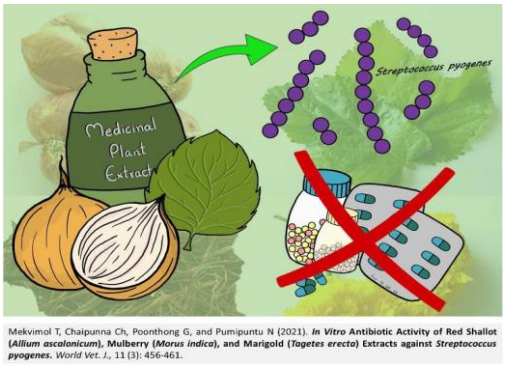
[Full text-PDF] [XML] [Google Scholar]

Research Paper

In Vitro Antibiotic Activity of red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against Streptococcus pyogenes

Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N.

World Vet. J. 11(3): 456-461, 2021; pii:S232245682100058-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj58



Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). ***In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*.** *World Vet. J.*, 11 (3): 456-461.

ABSTRACT: Bacterial infection is a major global health concern. One of the critical problems is the widespread of antimicrobial-resistant bacteria from inappropriate and prolonged use of antimicrobial agents in both humans and animals. Plant extracts might afford the chance to replace antibiotic drugs and reduce the emerging of antimicrobial-resistant bacteria. This study aimed to examine the antibiotic activity of ethanolic crude extracts of some Thai medicinal plants grouping in their parts as whole onions of red shallot (*Allium ascalonicum*), petals of marigold (*Tagetes erecta*), mulberry leaves, and root barks (*Morus indica*) to inhibit the growth of *Streptococcus pyogenes*. The antibiotic activities of the crude extract of three Thai medicinal plants using absolute ethanol were trialed against *Streptococcus pyogenes* using the disk diffusion method. Erythromycin and Ceftriaxone discs were chosen to be positive control standards as the representative of antibiotic drugs. Each dried plant extracts was prepared to test the inhibition with a concentration at 25, 50, and 75 mg/mL stock solution. The results showed that three groups from all testing groups of Thai medicinal plant extracts had the potential of antibiotic activity against *S. pyogenes*. The highest antibiotic activity against *S. pyogenes* was detected from whole onion extract red shallot followed by the extract of the mulberry leaves and root barks of mulberry strain Nakhon Ratchasima 60 (Nak 60) while the extract of marigold petal did not present antibiotic activity. The results revealed that crude extract of those two Thai medicinal plants, including red shallot and mulberry, had antibiotic activity against bacterial growth of *S. pyogenes* in the experiment and these medical Thai plants had potential benefits for developing as alternative treatment agents for *S. pyogenes* infections in both humans and animals in the future.
Keywords: Antibiotic activity, Ethanolic crude extract, Thai medicinal plants, *Streptococcus pyogenes*

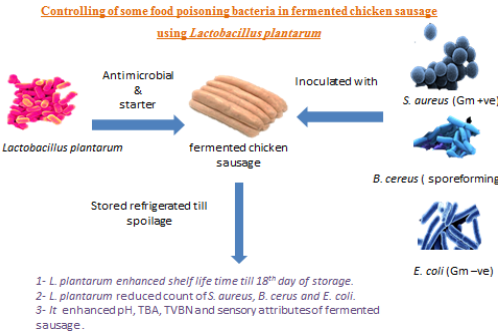
[Full text-PDF] [XML] [Google Scholar]

Research Paper

Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using Lactobacillus plantarum

Elsabagh R, Nada ShM, and Abd-Elaaty EM.

World Vet. J. 11(3): 462-468, 2021; pii:S232245682100059-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj59



ABSTRACT: *Lactobacillus plantarum* (*L. plantarum*) bacteria is generally recognized as safe and widely used in the food industry. The current study aimed to study the antimicrobial effects of *L. plantarum* against some food poisoning microorganisms, such as *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), and *Escherichia coli* (*E. coli*) in oriental fermented chicken sausage for 18 days of storage at 4°C. The *L. plantarum* has broad-spectrum antimicrobial effects that enhance the quality and safety of food products. *L. plantarum* reduced the count of *S. aureus, B. cerus,* and *E. coli* to 1.54, 4.26, and 3.03 Log_{10}, respectively, after 18 days of refrigerated storage. Moreover, there were significant effects of *L. plantarum* on pH, thiobarbituric acid, total volatile basic nitrogen, and sensory attributes of fermented sausage samples during storage time. It was revealed that *L. plantarum* enhanced the physic-chemical, sensory attributes, and shelf life of fermented chicken sausage. Moreover, *L. plantarum* inhibited the inoculated food poisoning bacteria in fermented chicken sausage. In conclusion, it is recommended to use *L. plantarum* in fermented meat products as a starter and a bio-preservative to enhance the quality of the fermented chicken sausage.
Keywords: Chicken sausage, Food safety, *Lactobacillus plantarum*, Probiotic

[Full text-PDF] [XML] [Google Scholar]

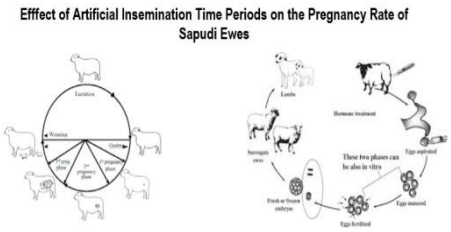
Short Communication

The Effect of the Different Artificial Insemination Time Periods on the Pregnancy Rate of Sapudi Ewes

Putri CD, Ismudiono, and Poetranto ED.

World Vet. J. 11(3): 469-473, 2021; pii:S232245682100060-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj60

ABSTRACT: Brucellosis Artificial insemination is required to increase the reproduction rate in ruminant breeding. The artificial insemination success rate in sheep only reaches 47.6%, whereas the proposed ideal rate is 70%. One of the factors influencing the artificial insemination success rates in sheep is improper estrus detection, resulting in no fertilization. The present study aimed to determine the effect of different artificial insemination time periods on the pregnancy rates of Sapudi ewes. The research design was based on a completely randomized design. A total of 20 female Sapudi sheep were divided into four treatment groups with five repetitions (for each group). In addition, the observed variables were artificial insemination time in Sapudi ewes. Estrus synchronization in ewes was conducted by injecting PGF2α. The results of the research indicated that ewes subjected to artificial insemination 6, 12, 18, and 24 hours after estrus had a pregnancy rate of 20%, 100%, 60%, and, 60%, respectively. It can be concluded that the time differences in artificial insemination significantly influence the pregnancy rate in Sapudi ewes'.



Keywords: Artificial insemination, Estrus, Pregnancy rate, Sapudi ewes

[Full text-PDF] [XML] [Google Scholar]

Research Paper

Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt

Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR.

World Vet. J. 11(3): 474-483, 2021; pii:S232245682100061-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

ABSTRACT: The study aimed to identify the culling reasons of Holstein cows raised in a large commercial herd in Egypt with emphasis on the performance of retained and culled cows. A total of 31534 complete lactation records for 10994 cows calved from 2008 to 2019 were used. The overall rate of culling per lactation was 61.1%. Involuntary culling represented 92% of all culling cases. The reasons for culling included mastitis and udder problems (24.2%), reproductive disorders (18.7%), metabolic and digestive disorders (13.6%), lameness (13%), endemic diseases (10.8%), low milk yield (8.1%), respiratory diseases (4.3%) and unknown causes (7.3%). Means of 305-day milk yield and daily milk yield were significantly lower in culled cows than the retained ones. On the other hand, no significant differences were observed between culled and retained cows for days open and the number of services/conception. The high involuntary culling rate of Holstein under the Egyptian conditions revealed that management practices regarding mastitis prevention and reproductive efficiency should be improved.

Keywords: Culling reasons, Egypt, Holstein, Milk production, Reproductive performance

[Full text-PDF] [XML] [Google Scholar]

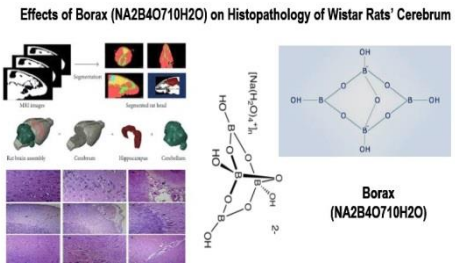
Short Communication

The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*)

Elfan F, Kuncorojakti S, and Triakoso N.

World Vet. J. 11(3): 484-488, 2021; pii:S232245682100062-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

ABSTRACT: The present study aimed to determine the effects of borax (Na2B4O7.10H2O) addition on the changes of histological cerebrum imaging in the brains of white mice (Rattus norvegicus). The current research was an experimental study with randomization of 24 white mice that were divided into four treatment groups with five replications. Borax was dissolved for each treatment with a dose of 19 mg/mouse/day, 26 mg/mouse/day, and 37 mg/mouse/day, and it was administered orally for 14 days. Then, it was analyzed statistically using the Kruskal-Wallis test. The statistical analysis results suggested that there were significantly different results in each treatment group. The control treatment with an administration dose of 26 mg/rat/day had a significantly different result in the worst cloudy swelling degeneration of cerebrum in histopathology imaging on Wistar rats (Rattus norvegicus). Using the Mann-Whitney test, it was found that the dose of borax at 37 mg/rat/day led to significant difference, compared to the other treatment groups, which means that 37 mg/rat/day of borax caused the worst pyramidal cell necrosis in histopathology imaging of the cerebrum on white



mice. Borax exposure on Wistar rats (Rattus norvegicus) can cause cloudy swelling at a dose of 26mg/head/day, and pyramidal cell necrosis at a dose of 37 mg/head/day.
Keywords: Borax, Cerebrum, Cloudy swelling, Necrosis

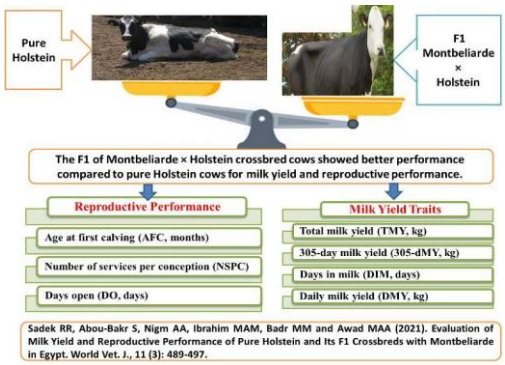
[Full text-PDF] [XML] [Google Scholar]

Research Paper

Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt

Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA.

World Vet. J. 11(3): 489-497, 2021; pii:S232245682100063-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj63



ABSTRACT: The present study was carried out to compare the milk yield and reproductive performance of pure Holstein (HO) cows with those of their first generation (F1) crossbreds with Montbeliarde cows (MO) in four commercial dairy herds under Egyptian conditions. Data used in the current study comprised 2268 records for the first four lactations of 531 HO and 536 MO × HO F1 crossbred cows during the period between 2012 and 2020. Data were analyzed using the least squares method by XLSTAT software. The MO × HO crossbred cows were significantly superior compared with pure HO cows for 305-day milk yield, scoring 9210 ± 96 kg versus 7987 ± 149 kg. Moreover, MO × HO F1 crossbred cows had a significantly higher daily milk yield (30.0 ± 0.45 kg) than pure HO cows (25.9 ± 0.52 kg). However, pure HO cows had significantly greater days in milk (399 ± 6 days) than MO × HO crossbred cows (341 ± 5.2 days). With regard to reproductive performance, MO × HO F1 crossbred cows had significantly less number of services per conception and days open than pure HO cows (2.6 ± 0.16 vs. 3.7 ± 0.18) and (132 ± 5.2 days vs. 190 ± 6 days), respectively. However, the statistical difference between MO × HO F1 crossbred cows and pure HO cows for age at first calving was not significant (22.9 ± 0.11 vs. 23.1 ± 0.15 months, respectively). It can be concluded that under Egyptian subtropical conditions, the first generation of MO × HO crossbred cows exhibit better performance, compared to pure HO cows in milk yield and reproductive traits. These findings could provide an effective strategic option for the genetic improvement of dairy cattle in hot subtropical regions.
Keywords: Crossbreeding, Egypt, Holstein, Milk Yield, Montbeliarde, Reproduction

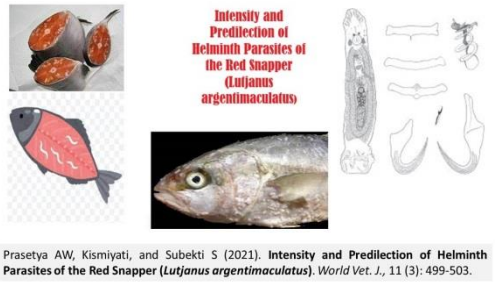
[Full text-PDF] [XML] [Google Scholar]

Short Communication

Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*)

Prasetya AW, Kismiyati, and Subekti S.

World Vet. J. 11(3): 498-503, 2021; pii:S232245682100064-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj64



ABSTRACT: Marine fish, particularly the red snappers, are often exposed to helminth parasitic infestation. As a result of the parasitic infestation, the fish population, the fish weight, and the morphological changes in the fish are shrinking. The present research aimed to find out the intensity and predilection of the helminth ectoparasites over the infection of the red snapper (Lutjanus argentimaculatus) and employed the survey method for this purpose. The sampling was carried out by the purposive sampling technique. The sample obtained consisted of 30 fish, 20% of the total red snapper population of 150 fish reared in the floating net cages of Balai Besar Perikanan Budidaya Laut Lampung. The intensity of the fish infestation by a mixture of Haliotrema epinepheli and Benedenia epinepheli was 132.5 individuals/fish. Neobenedenia girellae and Haliotrema epinepheli infected fish with an intensity of 149.41 individuals/fish. The 66.7% of Benedenia epinepheli had a predilection for the dorsal fin, and 33.3% for the anal fin. In Neobenedenia girellae, 57.1% had a predilection for the body surface, 37.2% for the head surface, and 5.7% for the dorsal fin. In conclusion, all sampled fish were positively infected with helminth ectoparasites, including Neobenedenia girellae, Haliotrema epinepheli, and Benedenia epinepheli.
Keywords: Ectoparasite, Helminths, Infestation, Red snapper

[Full text-PDF] [XML] [Google Scholar]

Research Paper

Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique

Asmorowati RW, Kusnoto, and Eliyani H.

World Vet. J. 11(2): 504-509, 2021; pii:S232245682100065-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

ABSTRACT: In the adult stage, *Haemonchus contortus* worms infect the abomasum host causing anemia and even death in animals. However, identifying the *H. contortus* protein can be used as a reference for the diagnosis of diseases. The diagnosis is performed by serological cross-reaction between *H. contortus* protein and anti-L2 *Toxocara vitulorum* (*T. vitulorum*) serum using the western blot technique. The main purpose of the current research was to identify the cross-reaction between *H. contortus* proteins and anti-L2 *T. vitulorum* serum using the western blot technique. *T. vitulorum* worms were collected from the intestine of cattle and *H. contortus* worms were collected from the abomasum of goats. The first step was making antibodies by oral infection of rats with infective eggs (L2) of *T. vitulorum*. The blood was taken 21 days after infection. Then, the blood was centrifuged at 1500 rpm for 10 minutes to get the serum. The second step was making homogenates from the whole worm extract of *H. contortus*. After crushing the worms, it was centrifuged at 5000 rpm for 15 minutes and the supernatant was taken. The supernatant was then analyzed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with coomassie brilliant blue staining. The third step was the analysis of *H. contortus* protein with serum anti-L2 *T. vitulorum* using the western blot technique. From the *H. contortus* homogenates analysis using SDS-PAGE, 16 protein bands were obtained. The cross-reactions were 141.3, 81.3, 64. 6, 51.3, 46.8, and 38 kDa. The data from cross-reactions suggested that the *H. contortus* protein cannot be used as a diagnostic material. It is serologically Haemonchosis because it caused false positives with diagnostic Toxocariasis.
Keywords: Cross reaction, *Haemonchus contortus*, SDS-PAGE, *Toxocara vitulorum*, Western blot

Asmorowati RW, Kusnoto, and Eliyani H (2021). **Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique.** *World Vet. J.*, 11 (3): 504-509.

[Full text-PDF] [XML] [Google Scholar]

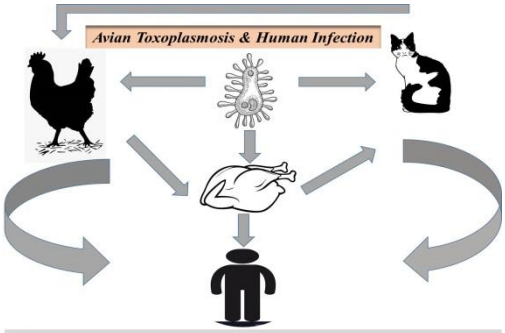
Review

A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation

Abd El-Ghany WA.

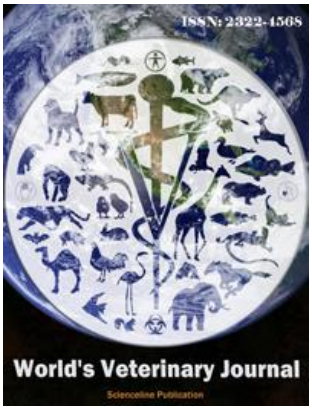
World Vet. J. 11(2): 510-520, 2021; pii:S232245682100066-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

ABSTRACT: This review article was developed to the infection of avian species with *Toxoplasma gondii* (*T. gondii*), diagnosis, pet bird and human infection, and control methods with a special reference to the current status of infection among the Egyptian poultry farms and population. Toxoplasmosis is a zoonotic disease caused by a unicellular, protozoan parasite *T. gondii*. Different domesticated and wild animals, as well as birds can harbor *T. gondii* and may be a potential source of infection to humans. Avian species could be infected with *T. gondii* through the ingestion of contaminated food, soil, and water with oocysts shed in the excreta of infected animals, especially cats. Poor sanitation and hygienic conditions increase the risk of infection. Consumption of food or water, as well as undercooked poultry meat or meat products containing the oocysts of the parasite, are the main sources of human infection with *T. gondi*. Diagnosis of *T. gondii* in the infected host depends on the serological detection of specific antibodies and molecular detection of the parasite. Microscopic demonstration of the oocysts and other developmental stages of the parasite in the intestine, liver, brain, and skeletal muscles tissues is another means for rapid diagnosis. Generally, a high prevalence of the disease is also reported in pet birds. Toxoplasmosis in humans is associated with abortion, congenital disorders, stillbirth, and other complications, especially in immunocompromised patients. Application of hygienic measures, as well as public awareness, are essential for the prevention and control of toxoplasmosis. In different Egyptian governorates, a high prevalence of *T. gondii* has been detected in animals, birds, and humans. High incidence of infection was recorded due to the contact with *Toxoplasma* oocysts shed mainly from infected cats or other carriers. Egyptian chicken and turkey flocks and backyard birds revealed the presence of different developmental stages of the parasite and even its antibodies. In addition, human populations showed signs of toxoplasmosis with severe complications.
Keywords: Bird, Egypt, Human, *Toxoplasma gondii*, Zoonosis



Abd El-Ghany WA (2021). **A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation.** *World Vet. J.*, 11 (3): 510-520.

[Full text-PDF] [XML] [Google Scholar]



World's Veterinary Journal

ISSN: 2322-4568

Frequency: Quarterly

DOI: https://dx.doi.org/10.54203/scil.2020.wvj

Current Issue: 2021, Vol: 11, Issue: 3 (September 25)

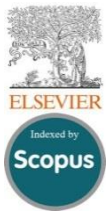
Publisher: SCIENCELINE

www.wvj.science-line.com

World's Veterinary Journal (ISSN 2322-4568) is an international, English language, peer reviewed open access journal aims to publish the high quality material from veterinary scientists' studies ... View full aims and scope

Editors-in-Chief:

- WVJ indexed/covered by SCOPUS, NLM Catalog (NLM ID: 101688928), ScopeMed, RICeST-ISC, Ulrich's™/ProQuest, NAAS (Score: 3.96), UBTIB, SHERPA/RoMEO, Genamic, INFOBASE, Index Copernicus International (ICV 2019= 115.91) (full index information)



- Open access full-text articles is available beginning with Volume 1, Issue 1.
- Full texts and XML articles are available in E-Journals Database (RICeST).
- This journal is in full compliance with Budapest Open Access Initiative and International Committee of Medical Journal Editors' Recommendations (ICMJE).

Editorial Offices:
Atatürk University, Erzurum 25100, Turkey
University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
University of Maragheh, East Azerbaijan, Maragheh 55136, Iran
Homepage: www.science-line.com
Phone: +98 914 420 7713 (Iran); +90 538 770 8824 (Turkey); +1 204 8982464 (Canada)
Emails: administrator@science-line.com ; saeid.azar@atauni.edu.tr

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 317-318, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj43

Veterinarians and Wildlife Biologists Should Join Forces to End Inhumane Mammal Trapping Technology

Gilbert Proulx

Alpha Wildlife Research & Management Ltd., 229 Lilac Terrace, Sherwood Park, Alberta, T8H 1W3, Canada

*Corresponding author's Email: gproulx@alphawildlife.ca; ORCID: 0000-0002-6980-8927

Short Communication
pii: S232245682100043-11
Received: 21 June 2021
Accepted: 01 August 2021

ABSTRACT

Current mammal trapping standards uphold the use of inhumane trapping technology. For example, killing neck snares for the capture of canids, and rotating-jaw traps, and steel-jawed leghold traps for procyonids and mustelids, are being used by trappers despite decades of research showing that they are inhumane, and cause serious injuries and distress in captured animals. Many wildlife biologists unsuccessfully raised concerns about inhumane mammal trappings. This short communication stresses the need for veterinarians and wildlife biologists to work together to improve the fate of mammals captured in killing or restraining traps, and modify mammal trapping standards on the basis of animal welfare science.

Keywords: Humaneness, Mammal trapping, Traps, Trapping standards

INTRODUCTION

Trapping standards developed by the International Organization for Standardization (ISO) in the 1990s, the Agreement on International Humane trapping Standards (AIHTS) in 1997, and the USA Best Management Practices (White et al., 2021) are industrial standards to save the fur industry from trade bans (Proulx et al., 2020). These are sub-standard which do not meet, and have never met, state-of-the-art trapping technology and protocols associated with animal welfare (Proulx et al., 2020).

Red foxes (*Vulpes vulpes*), coyotes (*Canis latrans*), and grey wolves (*Canis lupus*) are snared annually in the fur trade or predator control programs in Canada and the United States (Iossa et al., 2007). However, nearly 40 years ago, Canadian researchers assessed the "humaneness" of manual killing neck snares, where the animal provides the energy necessary to tighten the noose and strangle itself, to quickly render red foxes irreversibly unconscious. They found that they could not cut off the airflow when the snare was held tight around the trachea (FPCHT, 1981). Less than a decade later, Proulx and Barrett (1990) showed that red foxes captured in power killing neck snares, where springs provide the energy to tighten the noose on an animal's neck, did not quickly lose consciousness. Finally, Proulx (2018) monitored a working trapline with remote video cameras and showed that a coyote and a wolf lost consciousness 14 hours 16 minutes, and 3 hours 39 minutes, respectively, after being captured in a manual killing neck snare. In both cases, animals struggled intensively and frequently, cut their tongue and gums, damaged their teeth, bled, and suffered from extreme swelling of the neck and head.

Killing neck snares are only examples of traps that are allowed in North America. Despite extensive research showing that rotating jaw-traps do not have the ability to quickly kill northern raccoons (*Procyon lotor*) (Proulx and Drescher, 1994), these traps have been certified (e.g., the Fur Institute of Canada, 2020) or accepted as Best Management Practices (AFWA, 2014). Likewise, steel-jawed leghold traps that seriously injure animals and cause them to self-mutilate (Proulx et al., 1993; White et al., 2021) are still being used in the United States.

Inadequate Mammal Trapping Standards

It is puzzling to see that traps that have been repeatedly found unacceptable for decades are still being used to capture mammals. Also, in most countries, animal anti-cruelty legislation is blatantly inadequate for wildlife. In the past, a handful of scientists have expressed opinions against inadequate mammal trapping standards and the use of trapping devices causing unacceptable pain and suffering (Iossa et al., 2007; Proulx and Rodtka, 2017; Proulx et al., 2020). Some environmental organizations have raised concerns but most of them are readily dismissed by government agencies (White et al., 2021).

In an effort to reform mammal trapping technology, and minimize harm to animals, veterinarians should join forces with wildlife biologists (Cattet, 2013). Wildlife biologists are responsible for the professional conservation and management of wildlife species and populations, and the wellbeing of individuals (Paquet and Darimont, 2010). The list

To cite this paper: Proulx G (2021). Veterinarians and Wildlife Biologists Should Join Forces to End Inhumane Mammal Trapping Technology. *World Vet. J.*, 11 (3): 317-318. DOI: https://dx.doi.org/10.54203/scil.2021.wvj43

of injuries associated with trapping devices that are technologically outdated suffices to illustrate that animal welfare groups' concerns are neither exaggerated nor overemotional. When wildlife biologists and veterinarians remain passive and do not stand against cruelty in mammal trapping, they fail to act as professionals dedicated to the sound management of wildlife populations and the wellbeing of animals.

CONCLUSION

Inadequate mammal trapping standards uphold the use of inhumane trapping technology. Both veterinarians and wildlife biologists should take the lead in examining and modifying mammal trapping standards on the basis of animal welfare science.

DECLARATION

Acknowledgments

I thank Dr. Daryoush Babazadeh and Ali Sadeghi for their comments on an earlier version of this manuscript.

Competing interests

I declare no conflict of interest.

Consent to publish

I agree with the publication of this paper.

REFERENCES

Association of Fish and Wildlife Agencies (AFWA) (2014). Best management practices for trapping raccoons in the United States. Mimeograph. Available at: https://www.dec.ny.gov/docs/wildlife_pdf/trapbmpsraccoon.pdf

Cattet MRL (2013). Falling through the cracks: Shortcomings in the collaboration between biologists and veterinarians and their consequences for wildlife. Institute for Laboratory Animal Research, 54: 33-40. DOI: https://www.doi.org/10.1093/ilar/ilt010

Federal Provincial Committee for Humane Trapping (FPCHT) (1981). Report of the federal provincial committee for humane trapping. Federal-Provincial Wildlife Conference, Ottawa, Ontario, Canada.

Iossa G, Soulsbury CD, and Harris S (2007). Mammal trapping: A review of animal welfare standards of killing and restraining traps. Animal Welfare, 16: 1-18. Available at: https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.973.5675&rep=rep1&type=pdf

Paquet P, and Darimont CT (2010). Wildlife conservation and animal welfare: Two sides of the same coin?. Animal Welfare, 19: 177-190. Available at: http://www.raincoast.org/wp-content/uploads/Paquet-and-Darimont-2010-Wildlife-Conservation-Animal-Welfare.pdf

Proulx G (2018). Intolerable cruelty – The truth behind killing neck snares and strychnine. Alpha Wildlife Publications, Sherwood Park, Canada, p. 96. Available at: https://cwbm.ca/product/intolerable-cruelty-the-truth-behind-killing-neck-snares-and-strychnine/

Proulx G, and Barrett MW (1990). Assessment of power snares to effectively kill red fox. Wildlife Society Bulletin, 18: 27-30. Available at: https://alphawildlife.ca/wp-content/uploads/2015/03/25-1990-snare-red-fox.pdf

Proulx G, and Drescher RK (1994). Assessment of rotating-jaw traps to humanely kill raccoons. Journal of Wildlife Diseases, 30: 335-339. DOI: https://www.doi.org/10.7589/0090-3558-30.3.335

Proulx G, and Rodtka D (2017). Steel-jawed leghold traps and killing neck snares: Similar injuries command a change to agreement on international humane trapping standards. Journal of Applied Animal Welfare Science, 20: 198-203. DOI: https://www.doi.org/10.1080/10888705.2017.1286989

Proulx G, Cattet M, Serfass TL, and Baker SE (2020). Updating the AIHTS trapping standards to improve animal welfare and capture efficiency and selectivity. Animals, 10: 1262. DOI: https://www.doi.org/10.3390/ANI10081262

Proulx G, Onderka DK, Kolenosky AJ, Cole PJ, Drescher RK, and Badry MJ (1993). Injuries and behavior of raccoons (*Procyon lotor*) captured in the Soft Catch™ and the EGG™ traps in simulated natural environments. Journal of Wildlife Diseases, 29: 447-452. Available at: file:///C:/Users/Owner/Downloads/0090-3558-29.3.447.pdf

White HB, Boggess EK, Brown CL, Butfiloski JW, Decker TA, Erb JD, Fall MW, Hamilton DA, Hiller TL, Hubert GF Jr et al. (2021). Best management practices for trapping furbearers in the United States. Wildlife Monographs, 207: 3-59. DOI: https://doi.org/10.1002/wmon.1057

To cite this paper: Proulx G (2021). Veterinarians and Wildlife Biologists Should Join Forces to End Inhumane Mammal Trapping Technology. *World Vet. J.*, 11 (3): 317-318. DOI: https://dx.doi.org/10.54203/scil.2021.wvj43

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 319-326, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues

Abubakar Abdulkarim, Mohd Azam Khan Bin Goriman Khan*, and Erkihun Aklilu

Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, 16100 Pengkalan Chepa, Locked Bag 36, Kota Bharu, Kelantan, Malaysia

*Corresponding author's Email: azamkhan@umk.edu.my; ORCID: 0000-0001-5282-435X

REVIEW ARTICLE
pii: S232245682100044-11
Received: 19 June 2021
Accepted: 05 August 2021

ABSTRACT

Stray animal overpopulation has become one of the most serious global problems with many negative impacts on the community, environment, and public health. Most of the stray animals do not depend on humans for food and shelter, and therefore, can reproduce uncontrollably. The uncontrolled reproduction of stray animals increases their population that leads to a higher chance of predation, road traffic accidents, transmission of zoonotic diseases, and therefore, becoming vectors for some diseases. There are several methods for stray animal population control depending on the situation and the nature of the stray animals. These methods include contraceptives, trap-neuter-return, poisoning, euthanasia, and gun shooting among others. Each of the outlined methods has its advantages and disadvantages as to their practicality, ease of conducting, cost, effectiveness, ethics, and animal welfare issues. In conclusion, to achieve successful control measures of the stray animal population and the problem they create, the concerned authorities need to design and enact animal rights laws, provide medical care (treatment and vaccination), feeding, shelter for the animals, and control their reproduction. Public health and environmental agencies may improve the services by regulating personal and environmental hygiene, prevention, and control of zoonotic and transmissible diseases that can be transmitted from stray animals to the public and other livestock respectively.

Keywords: Animal welfare, Ethics, Population control methods, Public health, Stray animals

INTRODUCTION

Stray animals are un-owned domestic animals, particularly pets, such as cats and dogs (Sandøe et al., 2019). They mostly live independently, reproduce uncontrollably, overpopulate the environment, and often become a source of nuisance and global challenge to public health and environmental safety (Voslářová and Passantino, 2012). There have been many debates on the nature of stray animals especially animals' welfare and their problems in the community, such as hunting behavior, nuisance, and predating other livestock and poultry farms (Crowley et al., 2019).

It has been estimated that approximately 7.6 million pet animals enter into stray every year, of which about 3.4 million are cats (Statistics - National Kitten Coalition, 2020). According to the World Health Organization (WHO), the estimated number of stray dogs was approximately 200 million or more worldwide between 2009 and 2010 (Patti, 2011). With this number of strayed animals, four to five million are euthanized every year (Fournier and Geller, 2004). The abandoned pet animals neither affect their welfare nor pose a significant risk to public health and the environment. This condition makes them vulnerable to starvation, road transport accidents, diseases, and even death. Under some conditions, they may also harm the environment and native species through competition, predation, and spreading infectious diseases (Jessup, 2004). It has been observed that the stray animals in some communities are catered to by the residents who take care of and provide food and shelter for them (Centonze and Levy, 2002).

Animal professionals, such as veterinary doctors, animal scientists, and animal welfare officers have considered pet animal overpopulation as a people problem rather than an animal problem (Gerhold and Jessup, 2013). For the achievement of effective management of stray animals in an environment, there is a need to understand the relationship between the population of the strayed aminal and their impacts on a different dimension of the environments, and to know the way their relationship will be affected by other menace and environmental factors (such as climates) (Legge et al., 2017). It is difficult to estimate the number of stray animals in a community due to their high reproductive capacity. Under normal conditions, animals like dogs and cats with their puppies and kitten can make a population of more than a hundred in a year, this means that despite high mortality rates, their population can still be sustained (Stoskopf and Nutter, 2004). Highly adaptable, these animals thrive in environments ranging from villages up to metropolitan urban cities.

Different methods have been used to control stray animal populations for several years. These methods include poisoning, conducting Trap-Neuter-Return (TNR) programs, performing euthanasia using a high dose of anesthetic agents, such as a barbiturate, and administering some hormone-based contraceptive agents, such as progesterone (progestin) and testosterone propionate. Each of the outlined methods has its own advantages and disadvantages in terms

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

of their practicality, ease of conducting, cost, effectiveness, ethics, and animal welfare issues. Therefore, this literature review summarizes the impact of stray animals on the environment, the effect on the community and public health concern, and methods of stray animal population control with the ethics and animal welfare issues related to the different methods of stray animal population control.

Stray animals

Stray animals are free-roaming pet animals, especially dogs and cats that survive without human care and with an uncontrollable population increase. This condition leads to significant health and welfare problems for the animals concerning their public health and safety with environmental concerns (Fournier and Geller, 2004). Stray animals, such as dogs and cats, due to their predation nature may attract other wild predators, such as fox and coyotes, into society or community (Seimenis, 2004; Turner et al., 2012).

Stray animals are considered as one of the major public health problems worldwide in terms of their welfare and society. This concern mainly arises from the possible interactions of these strayed animals in many countries due to their interaction with people, especially children (Slater, 2001). Some may cause injuries to children through bites and scratches and may transmit zoonotic diseases. Interaction between humans and animals has been a common incidence over the past several centuries. Dogs play different roles in society, they act as pets, guards against thieves, crime detectors, runners, and hunters in champions, similarly, cats are also kept as pets and used for hunting rodents and reptiles that destroy properties at home (Bernstein, 2007).

It is essential to establish a long-term and sustainable approach to deal effectively with stray animal populations. The idea is to protect the people against those animals and safeguard the welfare and health of the animals. Experiences from the efforts in stray animal control show that effective control measures involve the adoption of more than one strategy. Among the strategies, western societies' concept of "ownership" is the most popular. This strategy focuses on a comprehensive, coordinated, and progressive program of owner education, mandatory registration and identification, environmental management, reproduction control of pets, and management of the animal population through regulated breeding and marketing (OIE, 2019). All these rules should be regulated by effective and enforced legislation. Implementing these rules successfully requires the cooperation and collaboration of more than one agency which in turn depends upon the willingness of government departments, municipalities, veterinary agencies, and non-government organizations (NGO's) to work together (Tasker, 2007).

Source of stray animals

In developing countries, many pet animals especially, cats and dogs, are abandoned on the streets and become part of a stray population. While in developed countries, stray cats and dogs are taken to animal shelters (Voslářová and Passantino, 2012).

Abandoned pet animals are increasing in number and have become a problem in many countries. Even though the proportion of the stray dog and cat population is not frequently measured, evidence shows that their numbers are increasing daily (OIE, 2019). The population density of the strayed animals may differ from the culture, habitat, and socio-economic conditions of the nation.

Stray dogs

Stray dogs are free-roaming dogs in communities that have become one of the major problems in society. They harm the environment and human health. Many studies have shown the important roles that pet animals are playing in the life of human being and dog is considered as one of the animals that kept as a pet in the past centuries (Ghasemzadeh and Namazi, 2015), but most of the pet dogs are going to stray due to owners cannot afford to take care of them. Many stray dogs roaming on the streets at night cause nuisance, defecate everywhere, scavenging on garbage, cause or get involved in road accidents, transmit diseases, and may die on the streets (Lyu, 2015). Stray dogs can cause an accident when running into the road, which might either result in injury to other people or themselves. Besides, stray dogs have become a threat to human lives and health by spreading deadly diseases, such as rabies. It had estimated by WHO that there are more than 200 million stray dogs worldwide, and every year 55000 people die from rabies transmitted by rabid dogs' bites (Chandran and Azeez, 2016).

Most of the dogs were considered as stray due to abandonment by their owners for some reason such as family immigration, diseases, and unbearable physiological behaviors of pets, no fund for treatment or feeding. Strayed dogs are facing many problems concerning their management. Sheltering alone cannot be an option for stray dog management because many conditions, such as feeding, disease treatment, annual vaccination and deworming, and appropriate quarantine require a lot of money to settle the conditions. In this regard, money and resources will never be sufficient to meet the sufficient demands for stray dog management (Lyu, 2015).

Dog population management (DPM) is an adaptable concept that aims to promote the health and well-being of stray dogs by minimizing the problems caused by the dogs while targeting to reduce their population size. Dog

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

population management can be achieved for many animal welfares, economic purposes, public health, and safety. These may be regulated through eradicating the transmission of infectious diseases and zoonotic diseases (especially rabies) by controlling dog bites or injuries to human beings and other animals, minimizing the occurrence of road or transport accidents, and reducing nuisance and fecal contamination of the environment caused by stray dogs during breeding (Taylor et al., 2017). In this regard, DPM programs have some goals depending on the conditions which may or may not be permanently limiting the size of the stray dog population. The means to achieve DPM objectives are planned to make a long-term positive impact on stray dog populations, in comparison to dog culling (Jackman and Rowan, 2007).

Stray cats

Stray cats, also known as feral cats, are free-roaming unowned cats mainly independent (Turner et al., 2012). The Feral domestic cat is one of the most challenging invasive species worldwide. The stray cat population has contributed to the extinction of some species of birds, such as Socorro dove (*Zenaida graysoni*) and Manx shearwater (*Puffinus puffinus*) among others, mammals, and reptiles in the environment which represent about 14% of modern extinctions in these vertebrate groups (Razgūnaitė et al., 2019). Many stray cats are roaming along the street, sometimes seen in restaurants, institutions, places of worship, and refuse dump looking for food. The same problem has also been observed in other developing countries (Legge et al., 2017).

It is difficult to estimate the number of stray cats in a community due to their high reproductive capacity. Cats are sexually mature by 5 to 6 months of age. Therefore, even with high mortality rates cat population can be sustained (Stoskopf and Nutter, 2004). The reproductive approaches, such as early sexual maturity, seasonally polyestrous cycling, and multiparous pregnancies, contribute to the prolific nature of the cat population (Kutzler, 2007). Highly adaptable, cats have thrived in environments ranging from villages, towns, and metropolitan urban cities (Legge et al., 2017).

Currently, the rate of cat eradication from society does not indicate significant changes in many societies where invasive cats are likely to challenge native wildlife (Medina et al., 2011). Therefore, to expand their biodiversity benefits, a new approach to cat eradication needs to be designed and implemented effectively. Furthermore, conservation biologists have a good understanding of and play an important role in the way stray cats impact native species of the environment (Medina et al., 2011).

Effect of stray animals on the community

The life activities of domestic animals mainly depend on their owners, which can be in form of personal ownership or a group of people in a community. Owners play an important role in the community or environments by regulating the management of pets (Taylor et al., 2017). In many cases, some of the owners may not afford to take care of the animals. These may pose any risk to the communities since they cannot maintain the well-being of the animals.

Due to the above conditions, pet animals are abandoned while roaming freely and join the stray population in the community and become a common source of spread of diseases to humans (zoonotic) and other pet animals across the community (Robertson, 2008). The main challenges and effects associated with stray animals on the community include predation, road traffic accident, defecation, urine spraying, nuisance, running and fighting during breeding, scavenging on open garbage and scattering all sealed refused, attacking people as in case of a rabid dog, soiling and threatening pet animals by attacking and biting (Trotman and Brown, 2007).

Stray animals and public health

Public health as a field of medicine is important because it comprises training in many fields and experience of working in teams with familiarity with interdisciplinary research. Stray animals are the major cause of public health problems and animal welfare concerns in any part of the world. Abandoned animals living in mass can reproduce and increase the number of the new stray animal population (Seimenis and Tabbaa, 2014). Public health plays an important role in minimizing stray animal overpopulation by controlling their reproduction, improving their health and well-being by vaccinating and treatment of sick ones among the animals, and increasing the responsibility by providing ownership to the animals.

The impact of public health on the occurrence of stray animals in both rural and urban areas includes the emergence and endemicity of many zoonotic diseases. Disease transmission from stray animals to humans and livestock depends on the nature of the countries, the climatic regions, and the health status and density of the human population (Robertson, 2008). Public health and epidemiology as disciplines are essential in understanding the impact of the stray animal population. The field of public health and epidemiology is critical in understanding the demographics, potential interventions, types of the problems, and required assessment of stray animal population control (Slater, 2001).

The Public health program will design control measures on stray animal populations and the spread of zoonotic diseases among the human population. These can be through public awareness of the harmful existence of stray animals in a society, public and environmental hygiene, controlling vectors that transmit the causative agents of the diseases, educating the public on zoonotic diseases, and performing measures to prevent and control the disease. The zoonotic

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

diseases of public health importance that can transmit to humans via stray animals include brucellosis, toxoplasmosis, rabies, campylobacteriosis, leishmaniasis, bartonellosis, salmonellosis, Pasteurellosis, and leptospirosis with others (Ghasemzadeh and Namazi, 2015).

The stray animal population control can be achieved by applying the aims of public health to determine why the animals are on strayed? Where are their sources? What are their populations? What are the problems they cause to people and livestock? Which way to evaluate and intervene in the situation and what are the costs to confront the situations (Slater, 2001)?.

Impact of stray animals on the environment

The impact of stray animals, especially dogs and cats, is more visible than other pet animals concerning the problems they create in the environment (Trotman and Brown 2007). Some species become tamed without showing fear of contact with humans and other domestic animals, which gives the chance of becoming the source of causing an effect on the environment and spread of infectious diseases (Seimenis and Tabbaa, 2014).

Stray animals also play an important role in transmitting diseases to humans and domestic animals through environmental pollution. These issues happen due to poor environmental hygiene and either directly from contact with feces (Traversa et al., 2014), urine, and other excretions from stray animals, or indirectly via infected or contaminated foods and food products obtained from diseased animals such as milk, and poorly prepared meat and meat products (Seimenis, 2004). Some Stray dogs and cats are scavenging around slaughterhouses for feeding, which may serve as an important source of disease transmission. In this regard, besides the control of scavenging stray animals, strict measures are to be taken by providing incinerators or disposal sites to prevent their access to the sources of infections, particularly the abattoirs and butcheries (Seimenis and Tabbaa, 2014).

Stray animals can become opportunistic predators (Medina et al., 2011) by attacking small animals, such as lizards, rats, and birds, and also by attracting other wild predators, such as fox, jackals, and coyotes into the environment (Turner et al., 2012) which can spread the impacts of predation across the environment (Dickman and Newsome, 2015). In an initial way, that was not the natural habitat of the ecological equations.

Ethics and animal welfare issues related to stray animal population control

To address the ethical issues and welfare of stray animals, there is a need to consider some factors, such as feeding, shelter, health care, and in some instant, their interaction with people (Briggs, 2013). The caretakers are expected to show sympathy and ethical concerns by providing the necessary care for the animals (Centonze and Levy, 2002). Neutering of stray animals improves their health and body score, minimizes roaming, and becomes friendly. It also observed that some offsprings of strayed animals die or disappear within some months of birth with trauma from children or other stray animals, starvation, disease, and mobile accident (Stoskopf and Nutter, 2004). Such conditions depend on geographical locations and other factors including weather and human population density. It is observed that the mortality rate of some stray animals was high, and the causes of death ranged from mobile accidents, poisoning, disease conditions, and attack from other stray animals that correspond with a poor standard of life (Aguilar et al., 2015).

The welfare of stray animals can vary noticeably. Some communities consider the elimination of stray animals as humane than allowing them to live a miserable life. Humanely considering stray animal population control, one method does not fit all the conditions due to their differences (Stoskopf and Nutter, 2004). Campaigning to educate and enlighten the public on domestic animal ownership responsibilities and their effects and implications of abandoning should be highlighted routinely (Param, 2016). Animal welfare agencies are the agencies that care and concern about the health and well-being of animals (domestic, wild, and stray). Some of these agencies include the American Society for the Prevention of Cruelty to Animals (ASPCA), Humane Society of the United States (HSUS) (Rowan and Kartal, 2018), International Fund for Animal Welfare (IFAW), Animal Welfare Institute, Animal Welfare Organization, Ipoh Society for the Prevention of Cruelty to Animals (ISPCA) (Param, 2016), World Animal Protection (WAP), International Animal Rescue, Global Alliance for Rabies Control, National Animal Welfare Trust among others. These agencies aim to control stray animal populations through enacting and enforcing appropriate policies that regulate humane animal population control and provide animal-control facilities. These include sterilization of all adopted animals. And the procedures require proper training, surveillance, and dedication from the staff and volunteers (Fournier and Geller, 2004).

The ethical approach for stray animal population control from both human and animal welfare points of view. The human welfare view usually focuses on the spread of zoonotic diseases and other effects caused by stray animals. The animal welfare view rests on the expectation that disease and death in uncontrolled populations of strayed animals cause suffering that prevails over the associated control programs (Kathy, 2006). These opinions are influenced by different views about the fundamental values related to suffering, freedom, and the existence of stray animals. The most used ethical way of resolving this issue is either by the humane killing of strayed animals (mostly euthanasia) or by controlling their reproduction (sterilization or neutering, OIE, 2019).

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

Methods of controlling the population of stray animal

Contraception

Contraception is the method used to prevent the occurrence of pregnancy in female animals and can be achieved through the use of a medication, device, or procedure. Choices of contraceptive methods should address factors, such as efficacy, safety, availability, acceptance, and affordability (Bansode et al., 2019). Contraceptive agents can be used in different forms depending on the types (pills or injectable forms) and the method of use. Some contraceptive agents induce contraception by altering the hormonal activities, especially the hormone-based contraceptive like diethylstilbestrol, progestin, medroxyprogesterone acetate (MPA), proligestone (PROL), and mibolerone (Asa, 2018). While the use of implants is impaired through the use of an intrauterine device (IUD), such as the Depo-proveras and melengestrol acetate (MGA) implants (Boutelle and Bertschinger, 2010).

The use of the non-surgical contraceptive method is more accurate when owners request to breed their animals in the future (Massei and Miller, 2013) unless in the case of some diseases, such as rabies, where permanent sterilization is preferable. Most of the contraception techniques should be implemented by trained veterinarians, except for oral contraceptives. Most of the new devices are not licensed and require expert and trained personals to use them, and their cost is very high (Taylor et al., 2017).

Female animals treated with hormone-based contraceptives may experience some side effects, such as hormonal in-balance, uterine infection, pyometra, mammary tumor, diabetes mellitus, and other potentially life-threatening side effects that may arise from the drugs. Therefore, animals treated with hormone-based contraception require daily monitoring by veterinarians for the prevention of the mentioned complications (Asa, 2018).

The advantages of contraception include a longer duration before reverse, fewer side effects, suppression of sexual behavior, easy administration, a low cost, and also applicable for humans (Cathey and Memon, 2010). On the other hand, the disadvantages of this method are the need for repetition, quick reverse when discontinued using the repeated dose, possible slow onset of activity in some drugs, possible chance of a reaction at the injection site (Cathey and Memon, 2010).

Trap-neuter-return

Stray animal control population must require safety considerations for non-target animals and humans, be supported by municipal agencies or charitable organizations, including plans to reduce continuous stray dog and cat migration and reproduction, and be aesthetically acceptable to the public (Levy et al., 2003).

The considerable argument concerning the methods of controlling stray animals, particularly identifying the alternatives that are practicable, effective, and humane, TNR programs are designed to pause reproduction without causing harm to the animals (Scott et al., 2002).

In this method, animals are trapped, neutered, and returned to their previous habitat. The procedure is performed by veterinary surgeons through spaying the female animal or castration in a male animal. The concept of TNR as a humane method for stray animal population control is recommended by welfare organizations (Centonze and Levy, 2002). However, this method requires a regular surveillance and maintenance program that can be costly. The expenses of the TNR method depend on the level of resources that the stakeholders execute for the management of the problems.

Mostly, the ongoing costs of stray animal control through the TNR method will be high. The technique is expensive, inappropriate for large-scale implementation, and time-consuming, as well as requiring many expert veterinary surgeons (DEWHA, 2008). Other disadvantages of this method that may be encountered, in case of poor management after the surgery, there's a chance of some complications such as urinary incontinence and there will be an increase in the incidence of cystic, hematologic, and osteo-tumors (Scott et al., 2002). However, it has many advantages, such as producing complete sterilization, minimizing the incidence of sexual behaviors, and reducing the incidence of mammary, ovarian, and uterine tumors (Levy et al., 2003).

Euthanasia

The word euthanasia derived from a Greek called "safe killing" or "good death" is a humane method of killing animals through medical measures. It can achieve by administering a large volume of anesthetic agents such as barbiturates (Materstvedt et al., 2003), ketamine, a compound containing potassium (Harris, 2001) beyond accurate dosage. It is painless and induces rapid unconsciousness followed by respiratory and cardiac arrest, and finally causes death. In other words, it is the painless inducement of a quick death. The World Society for the Protection of Animals (WSPA) advocates that the killing of pet animals through euthanasia is a humane method and should be carried out only by trained professionals. The organization also accepts that euthanasia is humane and acceptable if the animal facing the hardship of life due to illness, injury, or behavioral problems, but WSPA avoided the use of euthanasia in healthy animals and considered it as cruelty (Veikune, 2014).

This method costs very high in many respects, concerning financing the officials that handle the activities, professionals that perform the euthanasia, the ideal way of disposing of the carcasses, and the workers that disposing of the carcasses. For many years, euthanasia has been desired as an alternative to deal with the overpopulation of pet

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

animals (Purswell and Kolster, 2006). Regardless of the technique used, it is good to reduce distress, anxiety, and pain by ensuring that the person who performs the euthanasia is well trained. Some of the euthanasia must use in combination with sedation before euthanizing the animal. The chemical agents used for euthanasia include Chloral hydrate, Formalin, Chloroform, Nitrous oxide, Ether, pentobarbital, and some neuromuscular blocking agents such as nicotine, potassium chloride, and magnesium sulfate (OIE, 2019). Based on the above conditions, euthanasia is a challenging method and is not suitable for controlling the stray animal population. It may help in reducing their populations, but should not be considered as an active measure for the control of the stray animal population. Besides, euthanasia is necessary for old age, seriously injured, and dying pets suffering from severe pain, but not suitable for healthy animals (Bartlett et al., 2005).

The advantages of euthanasia (OIE, 2019) as a method of control for stray animal populations include, it induces painless death without cruelness, not like poisoning, it is accepted ethically worldwide, the chemical agents are of low cost, and minimal materials are required.

Regarding the disadvantages, this method must be performed by an expert (OIE, 2019), is difficult for a large population, requires some chemical agents which are not always available, demands agents like barbiturate that have long withdrawal action may cause sedation or other complications to any predator that fed on the euthanized carcass, needs a large volume of the chemical agents for inducing euthanasia which may be expensive to perform (Purswell and Kolster, 2006).

Poisoning

Poisons are chemical substances that cause damages or harm to the organs, the system, or the body. It happens due to chemical reactions occurring when an animal absorbs or is exposed to the chemicals by a sufficient quantity. Poisoning has been one of the methods for the control of the stray animal population. The most commonly used chemicals are anticoagulants and Sodium monofluoroacetate (Sherley, 2004). The major problem associated with poisoning is that it is cruel, and animals suffer a painful and slow death (Robertson, 2008). The clinical signs associated with poisoning include excessive salivation, vocalization, vomiting, snapping and biting, diarrhea, nystagmus, twitching of facial muscles, pulmonary congestion, tremor, tonic convulsions with paddling of the limbs, and finally death due to cardiac and respiratory arrest (Sherley, 2004). Regarding risks of poisoning, it may affect other non-targeted species that may directly or indirectly be exposed to the poison in the targeted environment. Likewise, there will be a risk of human exposure to the poison, especially children who are close to animals on playgrounds (Lyu, 2015), for whom there will be a chance of being exposed to the poison, for this reason, it is not an ideal way of practice and must be abandoned. However, poisoning as a method of stray animal population control can be advantageous since it is easy and efficient, cheaper, and can be used in a large population, it has less labor-intensive than other techniques (Mitchell, 2011). However, it causes painful death, it is cruel compared to other methods, there is a risk to other non-target animals and human beings, mainly children, it has animal welfare implications (Mitchell, 2011).

Shooting

Shooting is another method of stray animal population control (McLaughlina and Aitken, 2011) that is quick, painless, and efficient. It can be achieved if the bullets hit the targeted animals. There is a challenge of handling a gun by everyone without government permission. Shooting stray animals on the street is dangerous and has many risks if the target missed and mistakenly hits people (Mitrofanova et al., 2018). In other cases, some stray animals may not die on shooting and stay alive with pain and injury for long. These cases are mostly considered inhumane and brutal acts. (Lyu, 2015).

The use of shooting as a method of stray animal control is beneficial since it is considered humane if used on target animals, is target-specific, can be used as clean-up techniques, is the fastest method of controlling rabid dogs, and it can be applied to other animals with the same conditions (Lyu, 2015). However, it requires a skilled and trained person to shoot, is dangerous to people and non-targeted animals, needs proper equipment, and may damage the brain if the animal was shot by the head, especially as in case of a rabid dog where the brain is required for the rabid test, can cause severe wounds and pain if the animal does not die by shot instantly, and it needs government approval before carrying out the operation.

CONCLUSION

Stray animal overpopulation has become a global challenge that requires immediate solutions to manage their population. The most common methods that are used for stray animal population control include surgical neutering (sterilization) and non-surgical contraception techniques. The non-surgical contraception methods entail immunocontraceptives agents and hormone-based contraceptives, while the surgical methods of controlling stray animals are through spying and castration of female and male animals, respectively. Other available control methods are trap neuter return, euthanasia, poisoning, trap and relocate, and gun shooting among others. To achieve successful control measures of stray animal populations and the problems they cause in a community or environment, the

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

concerned authorities have to design and enact animal rights laws. The law needs to address the feeding, shelter, medical care (treatment and vaccination), management, welfare, and legal rights of stray animals. To establish a prominent system of monitoring and management of the stray animal population, the community has to be educated about the problems that the animals are causing if they were on free-roaming and to provide a possible solution to the problem that may cause by the stray animals. Public health and environmental agencies may improve the services by regulating personal and environmental hygiene, prevention, control of zoonotic and transmissible diseases that can be transmitted from stray animals to the public and other livestock respectively.

DECLARATION

Authors' contributions

All authors contributed equally to this work.

Competing interests

The authors declare that they have no conflict of interest.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, double publication and/or submission, and redundancy) have been checked by the authors.

Acknowledgments

The authors gratefully acknowledged the effort of all who reviewed and provided a helpful comment on the manuscript. For the images used for the graphical abstract, the authors also acknowledged the attributions of images by Ingrid E. Newkirk, The Canadian Press, Patti Strand, and Healthy Homemade.

REFERENCES

Aguilar G, Farnworth M, and Winder L (2015). Mapping the stray domestic cat (Felis catus) population in New Zealand: Species distribution modelling with a climate change scenario and implications for protected areas. Applied Geography, 63: 146-154. DOI: https://www.doi.org/10.1016/j.apgeog.2015.06.019

Asa CS (2018). Contraception in dogs and cats. Veterinary Clinics of North America - Small Animal Practice, 48(4): 733-742. DOI: https://www.doi.org/10.1016/j.cvsm.2018.02.014

Bansode OM, Sarao MS, and Cooper DB (2019). Contraception. In *StartPearls*. StartPearls Publishing. Available at: https://www.ncbi.nlm.nih.gov/books/NBK536949/

Bartlett PC, Bartlett A, Walshaw S, and Halstead S (2005). Rates of euthanasia and adoption for dogs and cats in Michigan animal shelters. Journal of Applied Animal Welfare Science, 8(2): 97-104. DOI: https://www.doi.org/10.1207/s15327604jaws0802_2

Bernstein PL (2007). The human-cat relationship. Springer, pp. 47-89. Available at: https://link.springer.com/chapter/10.1007/978-1-4020-3227-1_3

Boutelle SM, and Bertschinger HJ (2010). Reproductive management in captive and wild canids: Contraception challenges. International Zoo Yearbook, 44(1): 109-120. DOI: https://www.doi.org/10.1111/j.1748-1090.2009.00107.x

Briggs J (2013). Contraception and fertility control in dogs and cats report. Available at: http://carodog.eu

Cathey M, and Memon MA (2010). Nonsurgical methods of contraception in dogs and cats: where are we now? Veterinary Medicine, 105: 12-17. Available at: https://www.dvm360.com/view/nonsurgical-methods-contraception-dogs-and-cats-where-are-we-now

Centonze LA, and Levy JK (2002). Characteristics of free-roaming cats and their caretakers. Journal of the American Veterinary Medical Association, 220(11): 1627-1633. DOI: https://www.doi.org/10.2460/javma.2002.220.1627

Chandran R, and Azeez PA (2016). Stray dog menace: Implications and management. Economic and Political Weekly, 51(48): 58-65. Available at: https://www.epw.in/journal/notes/stray-dog-menace

Crowley SL, Cecchetti M, and McDonald RA (2019). Hunting behaviour in domestic cats: An exploratory study of risk and responsibility among cat owners. People and Nature, 1(1): 18-30. DOI: https://www.doi.org/10.1002/pan3.6

Department of the Environment, Water, Heritage and the Arts (DEWHA) (2008). Threat abatement plan for predation by feral cats. Department of the Environment, Water, Heritage and the Arts. Available at: http://www.environment.gov.au/biodiversity/threatened/tap-approved.html

Dickman CR, and Newsome TM (2015). Individual hunting behaviour and prey specialisation in the house cat Felis catus: Implications for conservation and management. Applied Animal Behaviour Science, 173: 76-87. DOI: https://www.doi.org/10.1016/j.applanim.2014.09.021

Fournier AK, and Geller SE (2004). Behavior analysis of companion-animal overpopulation: A conceptualization of the problem and suggestions for intervention. In Behavior and Social Issues, 13: 51-69. DOI: https://doi.org/10.5210/bsi.v13i1.35

Gerhold RW, and Jessup DA (2013). Zoonotic diseases associated with free-roaming cats. Zoonoses and Public Health, 60(3): 189-195. DOI: https://doi.org/10.1111/j.1863-2378.2012.01522.x

Ghasemzadeh I, and Namazi SH (2015). Review of bacterial and viral zoonotic infections transmitted by dogs. Journal of Medicine and Life, 8(4): 1-5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28316698

Harris NM (2001). The euthanasia debate. Journal of the Royal Army Medical Corps, 147(3): 367-370. DOI: https://www.doi.org/10.1136/jramc-147-03-22

Jackman J, and Rowan A (2007). Free-roaming dogs in developing countries. Animals, 8(5): 55-78. Available at: https://www.wellbeingintlstudiesrepository.org

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

Jessup DA (2004). The welfare of feral cats and wildlife. Journal of the American Veterinary Medical Association, 225: 1377-1383. DOI: https://www.doi.org/10.2460/javma.2004.225.1377.

Kathy N (2006). 'Street dog' Population control case study. Vetwork - Resource Centre - Animal Health and Welfare. Available at: http://www.vetwork.org.uk/abc.htm

Kutzler MA (2007). Estrus induction and synchronization in canids and felids. Theriogenology, 68(3): 354-374. DOI: https://www.doi.org/10.1016/j.theriogenology.2007.04.014

Legge S, Murphy BP, McGregor H, Woinarski JCZ, Augusteyn J, Ballard G, and Zewe F (2017). Enumerating a continental-scale threat: How many feral cats are in Australia? Biological Conservation, 206: 293-303. DOI: https://www.doi.org/10.1016/j.biocon.2016.11.032

Levy JK, Gale DW, and Gale LA (2003). Evaluation of the effect of a long-term trap-neuter-return and adoption program on a free-roaming cat population. Journal of the American Veterinary Medical Association, 222(1): 42-46. DOI: https://www.doi.org/10.2460/javma.2003.222.42

Lyu P (2015). Proposal on solutions to stray dog problem in American cities. Journal of Political Sciences and Public Affairs, 3(3): 1-3. DOI: https://www.doi.org/10.4172/2332-0761.1000175

Trotman M, and Brown HJ (2007). Regional realities: Impact of stray dogs and cats on the community Impact on economy, including tourism impact on livestock, wildlife and the environment. Available at: https://www.hsi.org/wp-content/uploads/welfareresources/regional_realities.pdf

Massei G, and Miller LA (2013). Nonsurgical fertility control for managing free-roaming dog populations: A review of products and criteria for field applications. Theriogenology, 80: 829-838. DOI: https://www.doi.org/10.1016/j.theriogenology.2013.07.016

Materstvedt LJ, Clark D, Ellershaw J, Førde R, Boeck Gravgaard AM, Müller-Busch HC, and Rapin CH (2003). Euthanasia and physician-assisted suicide: A view from an EAPC Ethics Task Force. Palliative Medicine, 17(2): 97-101. DOI: https://www.doi.org/10.1191/0269216303pm673oa

McLaughlina EA, and Aitken RJ (2011). Is there a role for immunocontraception? Molecular and Cellular Endocrinology, 335: 78-88. DOI: https://www.doi.org/10.1016/j.mce.2010.04.004

Medina FM, Bonnaud E, Vidal E,Tershy BR, Zavaleta ES, Donlan CJ, Keitt BS, Corre ML, Horwath SV, and Nogales M (2011). A global review of the impacts of invasive cats on island endangered vertebrates. Global Change Biology, 17(11): 3503-3510. DOI: https://www.doi.org/10.1111/j.1365-2486.2011.02464.x

Mitchell J (2011). Poisoning of feral pigs. NQ Dry Tropics, Townsville. Available at: https://sugarresearch.com.au/sugar_files/2017/02/Poisoning_of_feral_pigs.pdf

Mitrofanova SY, Fedorchuk KV, Grigoryeva EV, Buslaev SI, Kurbanov RA, Gurbanov RA, and Belyalova AM (2018). Student environmental attitude towards stray domestic animals. Ekoloji, 27(106): 1895-1902. Available at: http://www.ekolojidergisi.com

Param S (2016). Need to look at humane ways to reduce the stray animals situation. Available at: https://www.malaysiakini.com/letters/360169

Patti S (2011). The Global Stray Dog Population Crisis. National Animal Interest Alliance. Retrieved November 14, 2020. Available at: http://www.naiaonline.org/articles/the-global-stray-dog-population-crisis-and-humane-relocation#sthash.O9Kc3L4P.xlQzPm3O.dpbs

Purswell BJ, and Kolster KA (2006). Immunocontraception in companion animals. Theriogenology, 66: 510-513. DOI: https://www.doi.org/10.1016/j.theriogenology.2006.04.018

Razgūnaitė M, Radzijevskaja J, Sabūnas V, Karvelienė B, and Paulauskas A (2019). Vector-borne zoonotic pathogens in cats. Biologija, 65(2): 140-149. DOI: https://www.doi.org/10.6001/biologija.v65i2.4030

Robertson SA (2008). A review of feral cat control. Journal of Feline Medicine and Surgery, 10(4): 366-375. DOI: https://www.doi.org/10.1016/j.jfms.2007.08.003

Rowan A, and Kartal T (2018). Dog population and dog sheltering trends in the United States of America. Animals, 8: 68. DOI: https://www.doi.org/10.3390/ani8050068

Sandøe P, Jensen JBH, Jensen F, and Nielsen SS (2019). Shelters reflect but cannot solve underlying problems with relinquished and stray animals—A retrospective study of dogs and cats entering and leaving shelters in Denmark from 2004 to 2017. Animals, 9(10): 1-14. DOI: https://www.doi.org/10.3390/ani9100765

Scott KC, Levy JK, and Crawford PC (2002). Characteristics of free-roaming cats evaluated in a trap-neuter-return program. Journal of the American Veterinary Medical Association, 221(8): 1136-1138. DOI: https://www.doi.org/10.2460/javma.2002.221.1136

Seimenis A, and Tabbaa D (2014). Stray animal populations and public health in the South Mediterranean and the Middle East regions. Veterinaria Italiana, 50(2): 131-136. DOI: https://www.doi.org/10.12834/VetIt.48.134.3

Seimenis AM (2004). Urban veterinary hygiene in developing countries. The 29th Congress of the World Small Animal Veterinary Association. Available at: https://www.vin.com/apputil/content/defaultadv1.aspx?pId=11181

Sherley M (2004). The traditional categories of fluoroacetate poisoning signs and symptoms belie substantial underlying similarities. Toxicology Letters, 151: 399-406. DOI: https://www.doi.org/10.1016/j.toxlet.2004.03.013

Slater MR (2001). The role of veterinary epidemiology in the study of free-roaming dogs and cats. Preventive Veterinary Medicine, 48(4): 273-286. DOI: https://www.doi.org/10.1016/S0167-5877(00)00201-4

Statistics - National Kitten Coalition (2020). Available at: https://kittencoalition.org/news-events/statistics/

Stoskopf MK, and Nutter FB (2004). Analyzing approaches to feral cat management-one size does not fit all. Journal of the American Veterinary Medical Association, 225: 1361-1364. DOI: https://www.doi.org/10.2460/javma.2004.225.1361

Tasker L (2007). Stray animal control practices (Europe). World Society for the Protection of Animals (WSPA) and the Royal Society for the Prevention of Cruelty to Animals International (RSPCA International) Report. Available at: http://www.stray-afp.org

Taylor LH, Wallace RM, Balaram D, Lindenmayer JM, Eckery DC, Mutonono-Watkiss B, and Nel LH (2017). The role of dog population management in rabies elimination-A review of current approaches and future opportunities. Frontiers in Veterinary Science, 4: 1-15. DOI: https://www.doi.org/10.3389/fvets.2017.00109

Traversa D, Frangipane Di Regalbono A, Di Cesare A, La Torre F, Drake J, and Pietrobelli M (2014). Environmental contamination by canine geohelminths. Parasites and Vectors, 7: 1-9. DOI: https://www.doi.org/10.1186/1756-3305-7-67

Turner P, Berry J, and Macdonald S (2012). Animal welfare bien-être des animaux animal shelters and animal welfare: Raising the bar. Canadian Veterinary Journal, 53: 893-896. Available at: https://pubmed.ncbi.nlm.nih.gov/23372200/

Veikune C (2014). Companion animal overpopulation on o'ahu. Available at: https://scholarspace.manoa.hawaii.edu

Voslářová E, and Passantino A (2012). Stray dog and cat laws and enforcement in Czech Republic and in Italy. Annali Dell'Istituto Superiore Di Sanita, 48: 97-104. DOI: https://www.doi.org/10.4415/ann_12_01_16

World Organization for Animal Health (OIE). (2019). Stray dogs population control. Terrestrial Animal Health Code, pp. 1-12. Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_aw_stray_dog.pdf

To cite this paper: Abdulkarim A, Goriman Khan MAKhB, and Aklilu E (2021). Stray Animal Population Control: Methods, Public Health Concern, Ethics, and Animal Welfare Issues. *World Vet. J.*, 11 (3): 319-326. DOI: https://dx.doi.org/10.54203/scil.2021.wvj44

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 327-332, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain

Ali Ghanbari[1*] and Mohadeseh Mohammadi[2]

[1]*Research Center of Physiology, Semnan University of Medical Sciences, Semnan, Postal Cod: 351989995, Iran*
[2]*Student Research Committee, Semnan University of Medical Sciences, Semnan, Postal Cod: 351989995, Iran*

*Corresponding author's Email: ghanbari@semums.ac.ir; ORCID: 0000-0002-7568-3681

ORIGINAL ARTICLE
pii: S232245682100045-11
Received: 28 May 2021
Accepted: 19 July 2021

ABSTRACT

Mechanical and thermal stimuli were used to evaluate neuropathic pain-like behavior in animal models usually. Mechanical stimulation of paw plantar surface is commonly used to determine mechanical allodynia. In the present study, paw withdrawal response to plantar surface stimulation was compared with paw withdrawal response to dorsal surface stimulation. To this end, a total of 30 female Wistar rats (180-220 g), were assigned randomly to three groups as intact (without any manipulation), sham (incision of skin and muscles without nerve injury), and neuropathy (sciatic nerve lesion) with 10 in each group. To induction of neuropathy (chronic constriction injury), four movable ligations were established around the sciatic nerve using catgut chromic suture with a distance of one millimeter apart and then wound incision was closed. In the sham group, the incision site was closed without nerve ligation. Mechanical allodynia was examined by Von Frey filaments for four weeks. The findings indicated that the paw withdrawal threshold following dorsal surface stimulation was significantly reduced compared to the sham group at day 21 post-surgery. Moreover, paw withdrawal threshold following plantar surface stimulation significantly decreased compared to the sham group at day 21 post-surgery. The present results regarding the sham group showed that the paw withdrawal threshold after mechanical stimulation of the plantar surface was not significantly different from that of the dorsal surface paw. In addition, and there was no significant difference between the paw withdrawal response to plantar surface and dorsal one. In conclusion, paw withdrawal threshold to plantar surface mechanical stimulation was not significantly different from one in dorsal surface following neuropathic pain induced by chronic constriction injury.

Keywords: Mechanical allodynia, Neuropathic pain, Paw dorsal surface, Paw plantar surface, Rat

INTRODUCTION

Neuropathic pain is chronic pain, caused as a result of a lesion or disease of the central or peripheral nervous system. Spontaneous pain, allodynia (pain sensation following non-noxious stimuli), and hyperalgesia (exaggerated pain following noxious stimuli) are some of its signs (Ji and Suter, 2007; Popiolek-Barczyk and Mika, 2016; Carrasco et al., 2018). Despite several studies toward neuropathic pain treatment, no successful treatment has been presented so far (Kingery, 1997; Chanchal et al., 2016). Due to the ethical issues and the impossibility of creating a human model of pain, most of the available information is the result of research on animal models (Mogil, 2009; Deng et al., 2021), and a few available human studies have addressed people who have suffered from the pain caused by traumatic events. Since pain is a subjective matter and animals are unable to express it, the animal's response to the stimulus with behaviors, such as withdrawing the limb from the stimulus, does not necessarily mean pain-like behavior (Deuis et al., 2017), therefore, various methods have been introduced to evaluate pain-like behavior in animals, each of which has some advantages and disadvantages. However, there is a need to consider the benefits and drawbacks of each method to improve our understanding of pain.

There are different methods to determine pain-like responses in animals, including putting foot or tail in hot water, infrared radiation to paw plantar surface or tail, putting the animal on a hot plate device, clamping the skin with a certain amount of force, stimulating the plantar or dorsal surface of the paw or cheeks of the animal with polyethylene filaments, and applying pressure or pushing a sharp point object on the skin (Jensen and Finnerup, 2014; Deuis et al., 2017). Among the mentioned methods, mechanical stimulation of the paw plantar surface with polyethylene filaments is one of the most common methods used to evaluate mechanical pain in animals (Yam et al., 2020). In this method, the animal is placed in a cage with meshed metal floor and the researcher triggers the pain response by applying the Von Frey filament to the plantar surface of the paw (Jensen and Finnerup, 2014) which the animal responds by lifting and moving its foot away from the stimulus.

Recently, researchers have also used the dorsal surface of the foot to examine the response to mechanical stimulation. In each case, the animal is placed on a table and the researcher holds the animal with one hand and places

To cite this paper: Ghanbari A and Mohammadi M (2021). Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain. *World Vet. J.,* 11 (3): 327-332. DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

the filament on the animal's foot (the skin between the toes 2 and 3) to trigger a pain-like response by considering raising the leg as the criterion for pain (Ren, 1999; Safakhah et al., 2017; Pop et al., 2021). According to common methods, the plantar surface but not the dorsal surface, is used to determine thermal pain that may be different from the dorsal surface in point of withdrawal response to mechanical stimulus. Therefore, it is necessary to clarify whether the plantar surface response is the same as the dorsal surface response. Therefore, due to the fact that in some studies, the plantar surface of the foot is used to evaluate mechanical allodynia, and in others, the dorsal surface of the foot is used, so in the present study, the pain-like response of paw plantar surface was compared with that of dorsal surface in the chronic constriction injury-induced neuropathic pain in rats.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Ethics Committee of the Faculty of Medicine, Semnan University of Medical Sciences, Iran (Certificate Number: IR.SEMUMS. REC.1398.162). All experiments were performed in accordance with National Institutes of Health guidelines for working with laboratory animals. To minimize diurnal variations, all experiments were carried out between 2 and 5 PM.

Animals

In the present study, female Wistar rats, weighing 200 ± 20 g were housed in a place with controlled temperature (22 ± 2°C), and 12 hours light-dark cycles were used. The rat fed and watered ad libitum. A total of 30 rats were divided into three groups of intact, sham, and neuropathy with 10 in each group. It should be mentioned that animals in the intact group did not receive the intervention (for comparison with the sham group to show the effect of incision on pain-like behavior). In the sham group (as the control group for neuropathy) only skin and muscles were incised while in the neuropathy [chronic constriction injury (CCI)] group, skin and muscles at the sciatic nerve were incised and sciatic nerve was sutured.

Neuropathic pain induction

Chronic constriction injury of the sciatic nerve was made using the method described by Bennett and Xie (1988). Animals were anesthetized using intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine (80 and 10 mg/kg, respectively, Vafaei et al., 2020) and the upper right thigh was shaved and then a 2 cm incision was made in the place of the sciatic nerve. The sciatic nerve was exposed and separated from surrounding tissues. The nerve was ligated using four catgut chromic sutures 4/0, with a 1 mm distance between each and then the incision site was closed using silk suture 4/0. Animals in the sham group received surgery without nerve ligation. The rats were housed in individual cages until full consciousness was regained.

Evaluation of mechanical pain-like behavior

Mechanical allodynia was evaluated using Von-Frey filaments in the plantar and dorsal surface of the foot using methods described by Ren (1999), and Bennett and Xie (1988), respectively. Von-Frey filament is a polyethylene hair, which is calibrated according to its diameter. Each filament applies a certain amount of force to the surface on which it is pressed. The filaments are used increasingly from low strength toward stronger ones. Each filament was used 5 times with 10 seconds interval between each stimulation. If the animal responded to three consecutive stimulations, this force would be considered as the response threshold; and otherwise, the stimulation would be continued with the stronger filament. The experiments were performed first with the plantar surface as described by Bennett and Xie (1998) and then continued one hour later with the dorsal surface following the method proposed by Ren (1999). Pain-like behavior assessments were performed at the end of the first, second, third, and fourth weeks post-surgery. A force of 60 g was considered as the cutoff point.

Statistical Analysis

All data were analyzed using GraphPad Prism version 8.0 software (GraphPad, San Diego, CA, USA). Regarding the normal distribution of data (using the Kolmogorov–Smirnov test), two-way analysis of variance (ANOVA) and then Sidak's multiple comparisons test were used. All data were expressed as mean ± SEM of the examined variable. $P < 0.05$ was considered statistically significant.

RESULTS

The obtained result showed that there was no significant difference between the intact and sham groups regarding the pain-like response to mechanical stimulation of the plantar and dorsal surfaces of the paw ($p > 0.05$). Moreover, the

To cite this paper: Ghanbari A and Mohammadi M (2021). Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain. *World Vet. J.*, 11 (3): 327-332. DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

withdrawal threshold following plantar surface stimulation was similar to the response of dorsal surface in both intact and sham groups (data not shown).

The current findings indicated that chronic constriction injury (CCI) significantly reduced paw withdrawal threshold following dorsal surface stimulation, compared to the sham group. The findings indicated that the paw withdrawal threshold significantly decreased during 21 days post-surgery ($p < 0.05$) and increased on day 28 in the CCI group, compared to the sham group ($p < 0.05$, Figure 1). Regarding plantar surface, the present results showed that paw withdrawal threshold following plantar surface stimulation significantly reduced in the CCI group during day 21 post-surgery, compared to the sham group, and continued to decrease until day 28 post-surgery ($p < 0.05$, Figure 2). Paw withdrawal threshold following mechanical stimulation of the plantar surface was not significantly different from that of the dorsal surface in the sham group (Figure 3).

In addition, in the sciatic nerve lesion group (CCI), there was no significant difference between the withdrawal responses induced by the mechanical stimulation of the plantar surface, compared to the dorsal surface response (Figure 4). Withdrawal response diagram following mechanical stimulation of the paw plantar and dorsal surface showed a downward trend from the seventh day following the injury and continued until the end of the experiment.

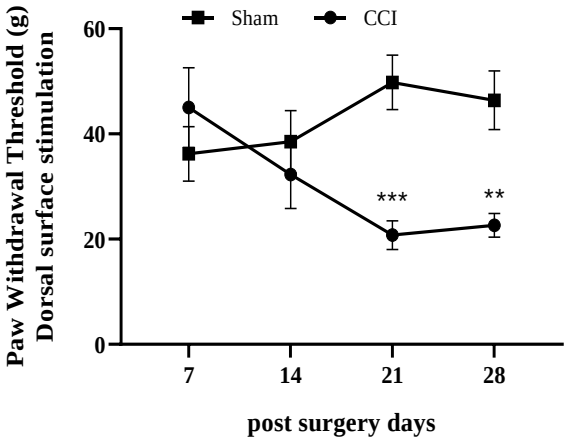


Figure 1. Withdrawal threshold following mechanical stimulation of the dorsal surface of the paw. Withdrawal response following mechanical stimulation of dorsal paw significantly reduced at third weeks in the neuropathy group, compared to the sham group, and continued to the end of the experiment (fourth week). Data are expressed as mean ± SEM with 10 rats in each group. ** $p < 0.01$, *** $p < 0.001$

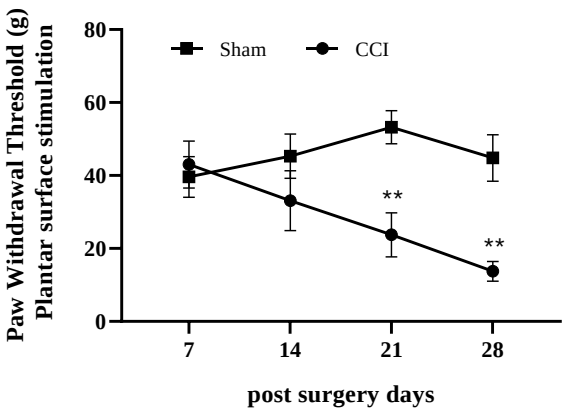


Figure 2. Withdrawal threshold following mechanical stimulation of the plantar surface of the paw. Withdrawal response following mechanical stimulation of paw plantar surface significantly reduced at third weeks in the neuropathy group, compared to the sham group and continued to the end of the experiment (fourth week). Data are expressed as mean ± S.E.M. with 10 rats in each group. ** $p < 0.01$

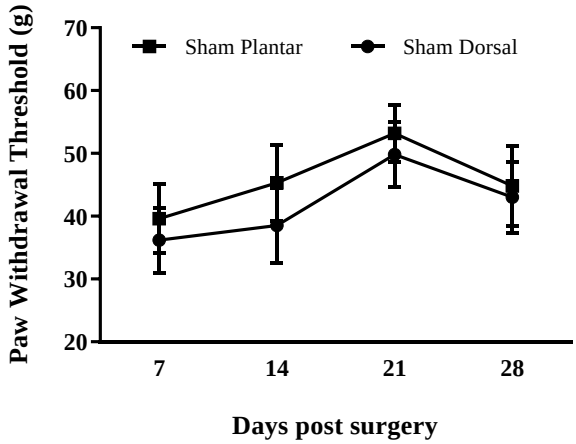


Figure 3. Comparison of withdrawal response threshold following mechanical stimulation of the plantar surface and dorsal surface of the foot in the sham group. Paw withdrawal threshold following mechanical stimulation of the plantar surface did not show a significant difference, compared to the dorsal surface in the sham group. Data are expressed as mean ± SEM with 10 rats in each group.

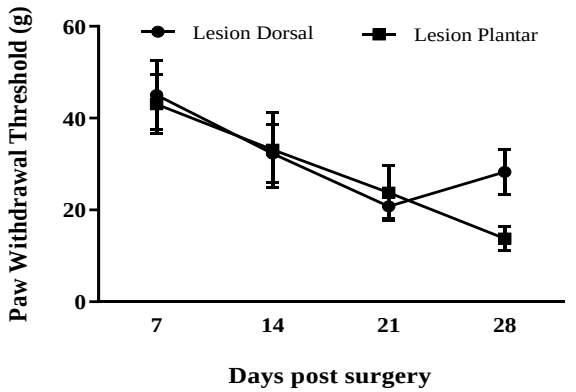


Figure 4. Comparison of withdrawal response threshold following mechanical stimulation of the plantar surface and dorsal surface of foot in the chronic constriction injury-induced neuropathy group. Paw withdrawal threshold following mechanical stimulation of the plantar surface did not show a significant difference compared to the dorsal surface in the neuropathy group. Withdrawal response diagram following mechanical stimulation of plantar and dorsal surface showed a downward trend from the seventh day following the injury and continued until the end of the experiment. Data are expressed as mean ± SEM with 10 rats in each group.

To cite this paper: Ghanbari A and Mohammadi M (2021). Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain. *World Vet. J.,* 11 (3): 327-332. DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

DISCUSSION

In the present study, the responses to mechanical stimulation of the plantar surface and dorsal surface of the foot following chronic constriction injury of the sciatic nerve were evaluated.

Comparison of animals' painful responses following mechanical stimulation of the paw plantar surface and dorsal surface in intact group and sham group showed that incision of skin and tissue around the nerve had no significant effect on the response to mechanical stimulation.

According to the present results, there was no significant difference between the withdrawal response of the plantar surface and dorsal surface due to mechanical stimulation of the plantar and dorsal surfaces following sciatic nerve injury. Several studies have been performed on different methods of determining pain in laboratory animals (Taiwo et al., 1989; Santos-Nogueira et al., 2012; Jensen and Finnerup, 2014; Deuis et al., 2017), however, there is no report about possible difference between the pain-like response due to plantar surface stimulation and dorsal surface stimulation in CCI-induced neuropathic pain rats.

The results of the current experiments showed that the withdrawal threshold following plantar surface stimulation was significantly different from the control group on day 21 after CCI, which was similar to the dorsal surface response. This result was in agreement with previous results indicating that two to three weeks after CCI surgery, the response threshold to mechanical stimulation significantly reduced, compared to the control group (Safakhah et al., 2016; Safakhah et al., 2017). The present results were also consistent with studies that showed two to three weeks after spinal cord injury; the paw withdrawal threshold significantly decreased, compared to the control group (Masri et al., 2009). Withdrawal response during plantar surface mechanical stimulation was not significantly different compared to dorsal surface stimulation in both the sham group and CCI group. This result was inconsistent with the results of Soignier et al (2011) indicating complete Freund's adjuvant (CFA)-induced pain which showed withdrawal threshold following dorsal surface stimulation was higher than the plantar surface threshold (Soignier et al., 2011). However, there were differences between the findings of the current study and those reported by Soignier et al (2011). Unlike neuropathic pain that was evaluated in the current study, Soignier et al (2011) assessed nociceptive pain. Furthermore, the current study examined pain-like behavior over a long period of time after the injury (at least 1 week), while Soignier et al (2011) evaluated pain behavior for a few hours following CFA administration. Finally, the method of pain induction in the current study was different from the method used by Soignier et al (2011).

Although there was no difference between the pain-like response following stimulation of the plantar surface and the dorsal surface of the foot, the determination of the pain-like behavior with the plantar surface method was associated with problems suggesting that the dorsal surface method was preferable. Pitcher et al. (1999) reported that the surface on which the animal stand can affect the animals' response by affecting sensory processing. They showed that if the animals' foot is on a wire mesh surface, determining the pain-like response from the plantar surface is associated with a lot of variation, whereas if a Plexiglas surface is used, this variation will be reduced. Given that in most cases, to assess the mechanical pain-like response, the animal is placed in cages with a wire mesh floor leading to a variety of responses, and then ambiguous and incorrect interpretations. Moreover, it has been reported that wire mesh floor possibly leads to tactile hyperstasis (Mizisin et al., 1998) which in turn affects the data.

It has also been reported that weight-bearing, which can be considered as pressure on the plantar surface of the foot, can be a confounding factor in assessing the pain-like response through the plantar surface (Kauppila et al., 1998).

According to the above reports, withdrawal response may be due to plantar surface stimulation could not be a result of von-Frey filament stimulation entirely, so part of which possibly is a result of tactile hyperstasia due to wire mesh floor contact. Possibly, discomfort due to the weight-bearing of the hind paws that are placed on the wire mesh can cause a change in sensory processing and create a non-pain response and thus mislead evaluating the pain-like response (Kauppila et al., 1998). Therefore, the observed reaction following the stimulation of the plantar surface of the foot is somewhat suspicious in comparison with dorsal surface stimulation. The following reasons, which are disadvantages of the plantar stimulation method for determining pain-like behavior, can further clarify the issue.

First, detection of withdrawal response through plantar surface stimulation takes a long time to stabilize the animal (in mice, it may take up to an hour, Minett et al., 2011), and therefore prolonged contact of the plantar surface of the foot with the wire mesh causes the animal to move. Second, the desired area for detecting withdrawal response may be covered by the wire mesh and thus the determination of pain from the same area may not be recorded in all animals (Pitcher et al., 1999). Third, moving the foot after one stimulation may cause the different area to be accessible and so the stimulation of the next time at the previous area is impossible and therefore creates a different response. In this regard, it has been reported that the thickness of the epidermis and CGRP-releasing fibers in the medial and lateral regions of the plantar surface are different from each other, and especially that surgical intervention increases the effect of filaments from the medial to the lateral (Duraku et al., 2012) which can lead to a different response. Moreover, there was a need to contact the filament perpendicular to the surface of the plantar surface of the animals' foot, so insufficient visual control of the desired position makes it impossible to stimulate the same area in all cases. In addition, the filament

To cite this paper: Ghanbari A and Mohammadi M (2021). Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain. *World Vet. J.,* 11 (3): 327-332. DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

may come in contact with the plantar surface of the foot suddenly or diagonally, which in both cases can cause scratches, and therefore the animal moves its foot away from the stimulus and this movement may consider as a pain-like response. Moreover, it is possible that repetitive stimuli may cause the animal to learn to move its foot away to prevent further stimulation and the inexperienced researcher may mistakenly consider this answer as a pain-like behavior. The fifth reason is that, in this method, a strong filament can move (raise) the foot and the researcher considers the observed response to be a pain-like behavior in the animal (Tal and Bennett, 1994; Xiao and Bennett, 1994; Ren et al., 1995). On the other hand, in the dorsal surface stimulation method, the withdrawal response following the stimulation is quite obvious and creates more confidence in the observed response as a pain-like reaction. This idea is consistent with Soignier et al (2011), who stated that the stimulation on the dorsal surface shows a much more accurate correct painful response (Soignier et al., 2011). According to the above-mentioned points, determining the pain-like behavior following stimulation of the dorsal surface can give a more obvious and acceptable criterion than stimulation of the plantar surface of the foot and increase the validity of the obtained data.

CONCLUSION

The pain-like response due to mechanical stimulation of the plantar surface of the foot is not significantly different from the response due to the stimulation of the dorsal surface, but assessing the pain-like response from the dorsal surface of the foot is easier and more reliable than the method of determining the response from the plantar surface.

DECLARATIONS

Authors' contribution

Ali Ghanbari designed the overall study and wrote the paper. Ali Ghanbari and Mohadeseh Mohammadi performed the experiment, collected, and processed the data. All authors discussed the results and commented on the manuscript. The final draft of the manuscript and statistical analysis were confirmed by the authors.

Conflict of interests

The authors declare no conflicts of interest in the present study.

Ethical consideration

Plagiarism, consent to publish, misconduct, data fabrication and or falsification, double publication and or submission, and redundancy have been checked by all the authors.

Consent to publish

Hereby, the authors agreed to publish the article.

Acknowledgments

The present study was funded by the Vice-Chancellor for Research and Technology of Semnan University of Medical Sciences (ethical code. IR.SEMUMS.REC.1398.162), and the authors would like to express their appreciation.

REFERENCES

Bennett GJ and Xie YK (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain, 33(1): 87-107. DOI: https://www.doi.org/10.1016/0304-3959(88)90209-6

Carrasco C, Naziroğlu M, Rodríguez AB, and Pariente JA (2018). Neuropathic pain: Delving into the oxidative origin and the possible implication of transient receptor potential channels. Frontiers in Physiology, 9: 95: 1-15. DOI: https://www.doi.org/10.3389/fphys.2018.00095

Chanchal SK, Mahajan UB, Siddharth S, Reddy N, Goyal SN, Patil PH, Bommanahalli BP, Kundu CN, Patil CR, and Ojha S (2016). *In vivo* and *in vitro* protective effects of omeprazole against neuropathic pain. Scientific Reports, 6: 1-10. DOI: https://www.doi.org/10.1038/srep30007

Deng J, Han J, Chen J, Zhang Y, Huang Q, Wang Y, Qi X, Liu Z, Leung EL, Wang D et al. (2021). Comparison of analgesic activities of aconitine in different mice pain models. PLoS One, 16(4): e0249276. DOI: https://www.doi.org/10.1371/journal.pone.0249276

Deuis J R, Dvorakova L S, and Vetter I (2017). Methods used to evaluate pain behaviors in rodents. Frontiers in Molecular Neuroscience, 10: 1-17. DOI: https://www.doi.org/10.3389/fnmol.2017.00284

Duraku LS, Hossaini M, Hoendervangers S, Falke LL, Kambiz S, Mudera VC, Holstege JC, Walbeehm ET, and Ruigrok TJ (2012). Spatiotemporal dynamics of re-innervation and hyperinnervation patterns by uninjured CGRP fibers in the rat foot sole epidermis after nerve injury. Molecular Pain, 8: 61: 1-13. DOI: https://www.doi.org/10.1186/1744-8069-8-61

Jensen TS, and Finnerup NB (2014). Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. The Lancet. Neurology, 13(9): 924-935. DOI: https://www.doi.org/10.1016/S1474-4422(14)70102-4

Ji RR, and Suter MR (2007). P38 MAPK, microglial signaling, and neuropathic pain. Molecular Pain, 3: 1-9. DOI: https://www.doi.org/10.1186/1744-8069-3-33

To cite this paper: Ghanbari A and Mohammadi M (2021). Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain. *World Vet. J.*, 11 (3): 327-332. DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

Kauppila T, Kontinen VK, and Pertovaara A (1998). Weight bearing of the limb as a confounding factor in assessment of mechanical allodynia in the rat. Pain, 74(1): 55-59. DOI: https://www.doi.org/10.1016/S0304-3959(97)00143-7

Kingery WS (1997). A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes. Pain, 73(2): 123-139. DOI: https://www.doi.org/10.1016/S0304-3959(97)00049-3

Masri R, Quiton RL, Lucas JM, Murray PD, Thompson SM, and Keller A (2009). Zona incerta: A role in central pain. Journal of Neurophysiology, 102(1): 181-191. DOI: https://www.doi.org/10.1152/jn.00152.2009

Minett MS, Quick K, and Wood JN (2011). Behavioral measures of pain thresholds. Current protocols in Mouse Biology, 1(3): 383-412. DOI: https://www.doi.org/10.1002/9780470942390.mo110116

Mizisin AP, Kalichman MW, Garrett RS, and Dines KC (1998). Tactile hyperesthesia, altered epidermal innervation and plantar nerve injury in the hindfeet of rats housed on wire grates. Brain Research, 788: 13-19. DOI: https://www.doi.org/10.1016/S0006-8993(97)01474-1

Mogil JS (2009). Animal models of pain: Progress and challenges. Nature Reviews. Neuroscience 10(4): 283-294. DOI: https://www.doi.org/10.1038/nrn2606

Pitcher GM, Ritchie J, and Henry JL (1999). Paw withdrawal threshold in the von frey hair test is influenced by the surface on which the rat stands. Journal of Neuroscience Methods, 87(2): 185-193. DOI: https://www.doi.org/10.1016/S0165-0270(99)00004-7

Pop NL, Nan A, Urda-Cimpean AE, Florea A, Toma VA, Moldovan R, Decea N, Mitrea DR, and Orasan R (2021). Chitosan functionalized magnetic nanoparticles to provide neural regeneration and recovery after experimental model induced peripheral nerve injury. Biomolecules, 11(5): 1-22. DOI: https://www. doi.org/10.3390/biom11050676

Popiolek-Barczyk K, and Mika J (2016). Targeting the microglial signaling pathways: New insights in the modulation of neuropathic pain. Current Medicinal Chemistry, 23(26): 2908-2928. DOI: https://www.doi.org/10.2174/0929867323666160607120124

Ren K (1999). An improved method for assessing mechanical allodynia in the rat. Physiology and Behavior, 67(5): 711-716. DOI: https://www.doi.org/10.1016/S0031-9384(99)00136-5

Ren K, Thomas DA, and Dubner R (1995). Nerve growth factor alleviates a painful peripheral neuropathy in rats. Brain Research, 699(2): 286-292. DOI: https://www.doi.org/10.1016/0006-8993(95)00920-L

Safakhah HA, Bazargani A, and Ghanbari A (2016). Effects of forced exercise on neuropathic pain induced by chronic constriction injury of sciatic nerve in male rat. Koomesh, 17(2): 411-418. Available at : http://koomeshjournal.semums.ac.ir/article-1-2969-en.html

Safakhah HA, Moradi Kor N, Bazargani A, Bandegi AR, Gholami Pourbadie H, Khoshkholgh-Sima B, and Ghanbari A (2017). Forced exercise attenuates neuropathic pain in chronic constriction injury of male rat: An investigation of oxidative stress and inflammation. Journal of Pain Research, 10: 1457-1466. DOI: https://www.doi.org/10.2147/JPR.S135081

Santos-Nogueira E, Redondo Castro E, Mancuso R, and Navarro X (2012). Randall-selitto test: A new approach for the detection of neuropathic pain after spinal cord injury. Journal of Neurotrauma, 29(5): 898-904. DOI: https://www.doi.org/10.1089/neu.2010.1700

Soignier RD, Taylor BK, Baiamonte BA, Lee FA, Paul D, and Gould HJ III (2011). Measurement of cfa-induced hyperalgesia and morphine-induced analgesia in rats: Dorsal vs plantar mechanical stimulation of the hindpaw. Pain Medicine (Malden, Mass.), 12(3): 451-458. DOI: https://www.doi.org/10.1111/j.1526-4637.2011.01066.x

Taiwo YO, Coderre TJ, and Levine JD (1989). The contribution of training to sensitivity in the nociceptive paw-withdrawal test. Brain Research, 487(1): 148-151. DOI: https://www.doi.org/10.1016/0006-8993(89)90950-5

Tal M, and Bennett GJ (1994). Extra-territorial pain in rats with a peripheral mononeuropathy: Mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve. Pain, 57(3): 375-382. DOI: https://www.doi.org/10.1016/0304-3959(94)90013-2

Vafaei AA, Safakhah HA, Jafari S, Tavasoli A, Rashidy-Pour A, Ghanbari A, Seyedinia SA, and Tarahomi P (2020). Role of cannabinoid receptors in crocin-induced hypoalgesia in neuropathic pain in rats. Journal of Experimental Pharmacology, 12: 97-106. DOI: https://www.doi.org/10.2147/JEP.S250738

Xiao WH, and Bennett GJ (1994). Magnesium suppresses neuropathic pain responses in rats via a spinal site of action. Brain Research, 666(2): 168-172. DOI: https://www.doi.org/10.1016/0006-8993(94)90768-4

Yam MF, Loh YC, Oo CW, and Basir R (2020). Overview of neurological mechanism of pain profile used for animal "pain-like" behavioral study with proposed analgesic pathways. International Journal of Molecular Sciences, 21(12): 1-26. DOI: https://www.doi.org/10.3390/ijms21124355

To cite this paper: Ghanbari A and Mohammadi M (2021). Comparison of Painful Response to Mechanical Stimulation of the Plantar and Dorsal Surface of Paw Following Chronic Constriction Injury-induced Neuropathic Pain. *World Vet. J.,* 11 (3): 327-332. DOI: https://dx.doi.org/10.54203/scil.2021.wvj45

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 333-342, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj46

Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt

Mostafa G. Al Naguib[1], Eman A. Morsy[1*], Mahmoud Samir[2], Marwa S. Khattab[3], Diaa G. Khelfa[1], and Gomaa A. Abdel-Alim[1]

[1]*Department of Poultry Diseases, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt*
[2]*Animal Health Institute, Agriculture Research Center, Giza 12211, Egypt*
[3]*Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt*

*Corresponding author's Email: emananter@cu.edu.eg ; ORCID: 0000-0002-1906-5416

ORIGINAL ARTICLE
pii: S232245682100046-11
Received: 22 June, 2021
Accepted: 14 Aug, 2021

ABSTRACT

Avian adenoviruses are an extremely diversified group of pathogens that recently triggering a variety of problems for poultry production. In particular, Inclusion Body Hepatitis-Hydropericardium Syndrome (IBH-HPS), which has been observed in broiler birds from 3 to 6 weeks of age and is associated with anemia, hemorrhagic disorders, hydropericardium, and high mortality. The disease has been reported worldwide, and recently it was reported in many Egyptian farms, causing severe economic losses. Therefore, the current study aimed to isolate, and genetically type the most common Adenovirus serotypes associated with this syndrome in Egyptian farms. A total of 50 broiler chicken farms (3-6 weeks old) located in different Egyptian governorates were examined. Macroscopically, the diseased flock revealed hydropericardium, enlarged friable livers with ecchymotic hemorrhages, and varying mortality rates (1 to 7.5%). Histopathologically, severe diffuse necrotizing enteritis, hepatitis, pericarditis, and diffuse lymphoid depletion of the spleen were the most prominent lesions. Liver tissues and cloacal swabs were collected from all examined flocks for FAdVs detection by conventional polymerase chain reaction (PCR) targeting the L1 loop in the hexon gene. The PCR products were sequenced for typing of the detected viruses. It was found that 10 out of 50 flocks examined were PCR positive for FAdVs (20%). Phylogenetic analysis of the sequenced genes revealed that the obtained viruses clustered with reference strains belonging to FAdV type D and E serotype 2, 11, and 8a respectively. The isolation of both FAdV type D and FAdV type E were carried out on a primary cell culture chicken embryo liver cell (CEL) and the presence of these viruses was confirmed by PCR after the appearance of cytopathic effect (CPE). From this study, it could be concluded that both FAdVs types D and E are the most common adenoviruses circulating in poultry farms suffering from hydropericardium and inclusion body hepatitis.

Keywords: Broiler chicken, Chicken embryo liver cell, Fowl adenovirus, Hexon gene, Histopathology, Inclusion body hepatitis–hydropericardium syndrome, PCR

INTRODUCTION

Adenoviruses (AdVs) belong to the family Adenoviridae which are non-enveloped double-stranded DNA viruses. This family is distributed worldwide and has been recently classified into five genera, including Aviadenovirus, Mastadenovirus, Atadenovirus, Siadenovirus, and Ichtadenovirus (Hess, 2000). Virological analyses have enabled the division of the aviadenoviruses into five species of 12 serotypes (FADV-1 to FADV-8a and FADV-8b to FADV-11) based on genomic restriction digest patterns (FAdV-A to FAdV-E) (Hess, 2000). It has been shown that hepatitis inclusion body (IBH), hydropericardium syndrome (HPS), and gizzard erosion (GE) are the most common diseases associated with FadV infection in chickens (Mittal et al., 2014) The FAdVs' 12 serotypes have been associated with IBH outbreaks (Li et al., 2018, Chen et al., 2018). However, FAdVs type D and E including serotypes; FAV-2, -3, -11 and FAV-6, -7, -8a, -8b respectively, are the most common relevant isolates (Schachner et al., 2018a).

Recently, outbreaks of IBH-HPS have hit several countries over the globe such as Japan (Nakamura et al., 2011), Malaysia (Mohamed Sohaimi et al., 2019), China (Li et al., 2018), India (Raj et al., 2018; Suohu et al., 2021) Spain (Oliver-Ferrando et al., 2017), Iran (Morshed et al., 2017), South Africa (Maartens et al., 2014) and Saudi Arabia (Hemida and Alhammadi 2017). The disease mortalities reach 5-10% and may surge to 30% if it is co-infected with other immunosuppressive agents, such as Chicken infectious anemia virus CIAV and Infectious bursal disease virus **(**IBDV) (McFerran and Smyth, 2000). Moreover, virulent HPS caused by FAdV serotype 4 (FAdV-4) leads to high mortality varying from 30-70% (Mansoor et al., 2011; Schachner et al., 2018; Khan et al., 2019).

Grossly, the infected broilers revealed lesions in the liver and heart in the form of the enlarged, pale, friable liver with ecchymotic hemorrhages in addition to the accumulation of straw yellow-colored fluids in the pericardium (Schachner et al., 2018). Histologically, the most common findings were hepatitis, pancreatitis, nephrosis, thymic and bursal atrophy, and lymphoid depletion in the bursa and spleen. Basophilic and eosinophilic inclusion bodies of variable

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.,* 11 (3): 333-342.

appearance were recorded in liver infected with IBH and rarely in the kidney. Moreover, HHS syndrome results in inflammatory and degenerative lesions mainly in the heart, kidneys, lungs, and intestine (Schachner et al., 2018).

Polymerase chain reaction (PCR) using primers specific to L1 loop as the most conserved region in hexon gene is used for characterization of FAdVs. Hexon is the main adenovirus protein and is typically used to detect FAdVs molecularly. Further FAdV typing was carried out using restriction enzyme length polymorphism (RFLP) and/or DNA sequencing (Hess, 2000) In Egypt, FAdVs outbreaks have been reported and confirmed by the sequence analysis of the viruses in collected samples (El-Tholoth and Abou El-Azm, 2019; Radwan et al., 2019; Elbestawy et al., 2020). Therefore**,** the current study was conducted for isolation, molecular characterization, typing, and histopathological picture of the most recent FAdVs circulating in broiler farms suffering from IBH/HPS in different Egyptian governorates.

MATERIALS AND METHODS

Ethical approval

The procedures for chicken handling were carried out in compliance with the applicable legislation of the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Cairo, Egypt (VetCU24112020261).

Sample collection

The examined farms were in Egypt at Menofya, Menia, Giza, Beheira, and Fayum Governorates during 2019-2020. Liver tissues and cloacal swabs were collected from 50 broiler chicken farms (5 birds from each farm with a total number of 250 birds as 200 freshly dead and 50 birds with severe clinical signs that were euthanized) with the age range of 3-6 weeks for the detection of FAdVs. In addition, the bursa of Fabricius was collected for the detection of IBDV. The pooled cloacal swabs from each farm were dissolved in 1 mL normal saline with a pH of 7.4 containing (1 mg/mL) streptomycin (50 µg/mL) gentamycin and (100,000 IE/mL) penicillin. The liver and bursa tissue samples were collected in phosphate-buffered saline (PBS) then transported to the laboratory in an icebox. All samples were stored at -20 °C for further processing and virus detection. In addition to the tissue specimens that were collected for histopathological examination.

Preparation of collected samples

The collected cloacal swabs samples were centrifuged at 700 × g for 10 minutes to clarify them. The collected supernatant was filtered by 0.2-µm pore size syringe filters. The pooled liver samples (5 from each farm) were ground by sterile mortar and pistol. A 20% (w/v) suspension was prepared using sterile saline solution. Centrifugation of the suspensions was carried out for 20 minutes at 2000 rounds per minute (r.p.m.) Collected supernatants were preserved at -20°C till use. (Radwan et al., 2019)

Histopathology

Tissue specimens from the intestine, liver, heart, and spleen of chickens in suspected farms were fixed in 10% neutral buffered formalin. The tissues were dehydrated, cleared, embedded in paraffin, and sectioned by microtome into 4 µm thick (Suvarna et al., 2018). Tissue sections were stained by hematoxylin and eosin stain, examined by a light microscope, and photographed by a digital camera (Olympus XC30, Tokyo, Japan).

Virus detection and Identification by PCR

Extraction of nucleic acid

The Genomic DNA isolation kit (Genedirex, Cat no. SN026-0100, Taiwan) was used to extract the total viral nucleic acid from 300 µL of the supernatant following the manufacturer's instructions.

Oligonucleotide primers

In this study, the forward (HexL1-s) and reverse (HexL1-as) primers corresponding to a conserved area in the hexon gene L1 region were used (Raue et al., 2005) and are listed in Table 1.

Table 1. Hexon gene L1 region primers sequence

Target gene/Amplicon size	Primer name	Nucleotide position	Sequence	Reference
Hexon/589 bp	Hex L1-s	301-323	5-ATGGGAGSACCTAYTTCGACAT-3	(Raue et al., 2005)
	Hex L1-as	890-868	5-AAATTGTCCCKRAANCCGATGTA-3	

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

Polymerase chain reaction

Amplification of the target gene was achieved in a reaction volume of 25 µL containing 5 µL DNA template (congruent to 20% of the total reaction volume), 1 µL of 10 µM forward (HexL1-s) and the same of reverse (HexL1-as) primers, 12.5 µL 2X One PCR^{TM} polymerase chain reaction (PCR) master mix (One PCRTM, Genedirex; Cat. No. MB203-0100), and 5.5 µL nuclease-free water. One initial denaturation cycle of 4 minutes at 94°C was required for the PCR cycling profile, followed by 35 amplification cycles of 45 seconds for denaturation at 94°C, 45 seconds for annealing at 51°C, and 1 minute for the extension at 72°C, and a final extension stage at 72°C for 10 minutes. In 1.5% agarose gel stained with ethidium bromide, the resulted amplified PCR products were electrophoresed and visualized under ultraviolet transillumination.

Sequence and phylogenetic tree analysis

The nucleotide sequence alignment was assembled and analyzed with representative reference to FAdVs strains' hexon gene sequences obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/) using the NCBI BLAST server to confirm the identity with FAdVs strains. In BioEdit version 7.0.1.4 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA), sequences of FAdV hexon genes were downloaded from the NCBI GenBank and aligned using the Clustal W program. The subsequent phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis version 6.0 constructed by the maximum likelihood approach based on the approximately 590-bp region corresponding to nucleotides 301-890 of the hexon gene and was used to demonstrate the clustering of the discrete species based on their hexon gene sequences. A similarity matrix has been created using the DNASTAR program (Lasergene, version 8.0).

Detection of concurrent infection with infectious bursal disease virus and chicken infectious anemia virus by conventional PCR

Detection of accompanied infections with infectious bursal disease virus (IBDV) and chicken infectious anemia virus (CIAV) was performed using the extracted viral DNA/RNA from the liver for CIAV and bursa of Fabricius tissues for IBDV from Adenovirus positive cases by conventional PCR assay as previously reported by Metwally et al. (2009) and Elbestawy et al. (2020).

Virus isolation on primary chicken embryo liver cells

Virus preparation

The supernatant of the collected FAdVs positive by PCR samples was passed through syringe filters with 0.2-µm pore size. Primary chicken embryo liver **(**CEL) cell was obtained from the liver of 15-day-old SPF embryonated chicken eggs (ECE) (Kumar et al., 2003).

Virus propagation

Flasks containing complete monolayer were washed two successive times with serum-free medium and inoculated with 0.1mL of the forementioned supernatant. For virus adsorption onto cells the inoculated flasks were incubated at 37˚C for one hour then a maintenance medium containing 2% fetal bovine serum (FBS) was added. Daily observation of the inoculated cells for CPE was done using an inverted microscope during the first three successive days post-inoculation (pi). After obvious CPEs development, the flasks were harvested three times by repeated freezing and thawing before centrifugation at 216 x g for 10 minutes. Viral fluid was collected and stored at -20˚C for further process. Complete monolayers were prepared for subsequent passage and inoculated with 0.1 mL of viral fluid. However, one monolayer was left uninoculated and was used as control cells (Mansoor et al., 2011)

Confirmation of fowl adenoviruses in cell culture by PCR

After the appearance of CPE in cell culture, the presence of FAdVs was confirmed by conventional PCR.

RESULTS

Clinical cases and gross lesions

During 2019-2020, 50 broiler flocks suffering from hydropericardium IBH syndrome in different governorates in Egypt were examined. The distribution and mortalities are summarized in Table 2 and Figure 1. Clear postmortem lesions in the form of a clear straw yellow serous fluid accumulation in the pericardium, swollen livers with necrotic foci, and petechial hemorrhages were observed (Figure 2).

Histopathological findings

Microscopy of the intestine showed severe diffuse necrotizing enteritis which was either acute or chronic according to the stage of infection. Intranuclear basophilic inclusion bodies were seen in infiltrating cells (Figure 3a, b, c). The hepatic lesions varied from vacuolation and necrobiotic changes to massive necrosis and dissociation of hepatocytes at the periphery of hepatic lobules with the presence of intranuclear eosinophilic inclusion bodies (Figure 3d, e, f). The heart had endothelial cell hypertrophy of blood vessels, pericarditis, degeneration of muscle bundles, necrotic foci with

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

mononuclear cells infiltration, edema, heterophils infiltration in between muscle bundles, and perivascular mononuclear cells infiltration in addition to the presence of intranuclear inclusion bodies in infiltrating leukocytes (Figure 3g, h, i). The spleen showed lymphocytolysis, lymphoid depletion, and reticular hyperplasia. Prominent intranuclear eosinophilic inclusion bodies were observed in lymphocytes and reticular cells (Figure 3j, k, l).

Virus detection by PCR

Ten out of 50 examined flocks were positive for FAdVs (20%). Positive samples with a DNA length fragment of 590 bp were detected from cloacal swabs and liver tissues (Figure 4). The percentage of positive flocks from different governorates is listed in Table 2. In addition, all FAdVs positive samples were negative for IBDV and CIAV by -PCR.

Table 2. Distribution of fowl adenovirus infections and mortality percentage in broiler flocks at different governorates in Egypt

Governorate	Number of investigated farms	Number of Positive flocks	Type of sample		Mortality (During the disease course, %)	Percentage of positive flocks/ Governorate
			Pooled livers	Pooled cloacal swabs		
Menofya	7	2	Positive	Negative	7.5	28.5
			Positive	Negative	6.6	
Fayoum	10	3	Negative	Positive	3	30
			Positive	Negative	5	
			Negative	Positive	2	
Menia	11	2	Negative	Positive	4	18.1
			Negative	Positive	1	
Giza	10	1	Negative	Positive	3.5	10
Dakhlya	6	1	Negative	Positive	4	16.6
Behera	6	1	Negative	Positive	2	16.6
Total	50	10	3	7	38.6	

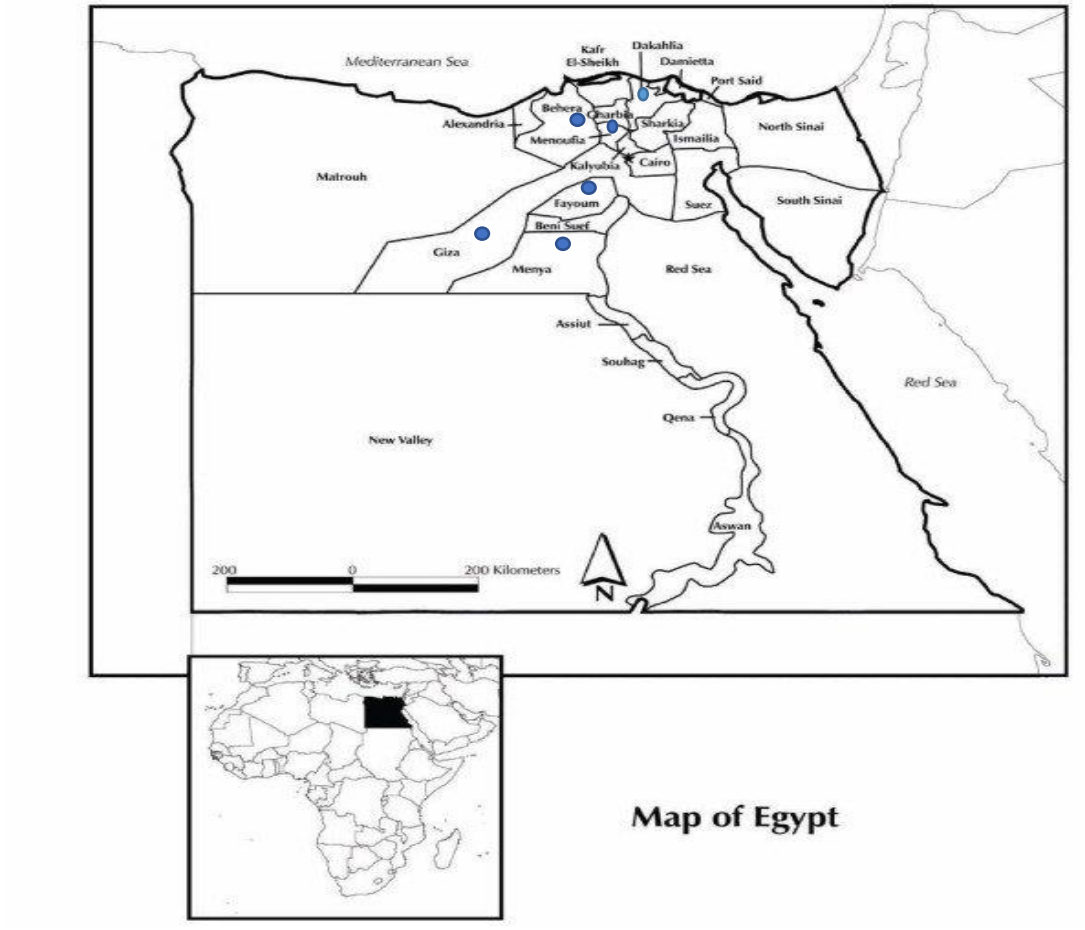


Figure 1. Fowl adenoviruses distribution in broiler chickens at different Egyptian governorates (Blue points)

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

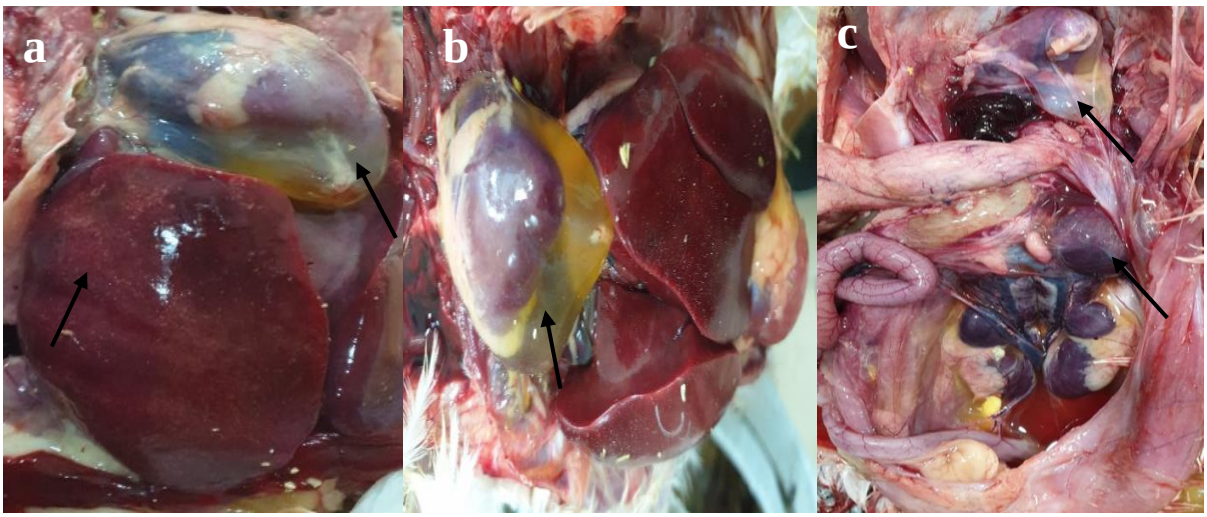


Figure 2. Gross pathology of naturally infected chickens with Inclusion Body Hepatitis-Hydropericardium Syndrome. **a** and **b:** Hydropericardium with necrosis and enlargement of the liver. **c:** hydropericardium and nephrosis.

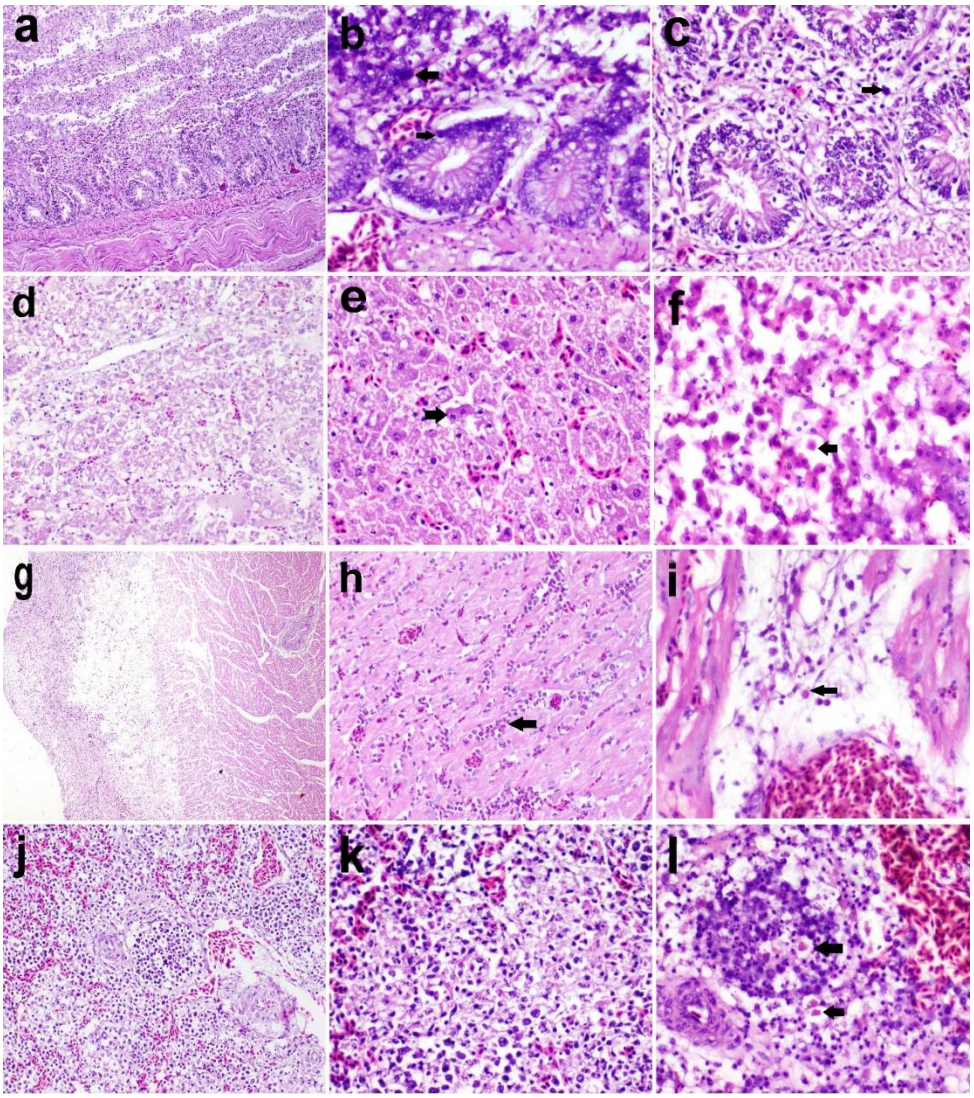


Figure 3. Histopathology of naturally infected chickens with Inclusion body hepatitis-hydropericardium syndrome. **a-c:** Chicken intestine, **a:** Chronic diffuse necrotizing enteritis (X100), **b:** Basophilic Intranuclear inclusion body (IN IB) (arrows) in the glandular epithelium**, c:** karyorrhexis in the glandular epithelium (X400). **d-f:** Chicken liver**, d: S**evere diffuse vacuolar degeneration of hepatocytes (X200), **e:** Necrobiotic changes and karyorrhexis of hepatocytes (arrow) (X400), **f:** Dissociation of hepatocytes with eosinophilic IN IB (arrow) (X400), **g:** Severe diffuse pericarditis (X100), **h:** Heterophils infiltration between muscle bundles (X200), **i:** Perivascular edema and perivascular mononuclear cells infiltration with eosinophilia in ib (arrow) (x400), **j-l:** Chicken spleen, **j:** Moderate diffuse depletion of white pulp (X200), **k:** Lymphocytolysis with small basophilic IN IB (arrow) (X400), **l**: Large eosinophilic IN IB in lymphocytes (arrow, X400, H & E stain).

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

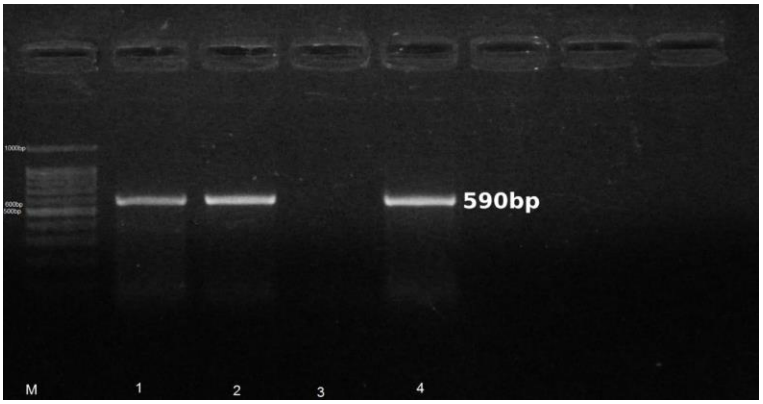


Figure 4. Visualization of 590-bp PCR product of hexon gene of FAdVs by agarose gel electrophoresis (1.5%) after staining with ethidium bromide. **Lane M:** 100bp DNA ladder, **Lanes 1, 2:** positive samples, **Lane 3:** Control negative, **Lane 4:** Control positive.

Phylogenetic analysis

A phylogenetic tree was created based on our sequences, and the reference strain's hexon gene sequences from different countries (Egypt, France, Brazil, Malaysia, China, Japan, and India) that were available on the GenBank (Table 3) to confirm the clustering of the various species. Phylogenetic analysis showed that the isolated FAdVs were classified as Fowl adenovirus type D serotype FAdV-2 and FAdV-11 in addition to Fowl adenovirus serotype FAdV-8a type E (Figure 5). Inter and intra-species analyses of genetic distance among the obtained sequences and other 14 aligned sequences available in the NCBI GenBank database of FAdV were performed (Figure 6). The genetic identity of FAdVs-type D had a high sequence homology (93.98% similarity) with the Indian FAdV type D, serotype 2 strains (MK816406.1 and MF770580.1) which previously has been isolated from liver tissues of broilers. On the other hand, the isolated FAdVs-type E viruses had a high sequence homology (99% similarity) with the Egyptian FAdV type E, serotype 8a (KT781516.1 and KT781517.1) that were isolated from cloacal swab samples of broiler chickens as illustrated in Figure 6. The partial hexon gene sequences of the two strains of FAdV-D and the FADV-E strain were submitted to the NCBI GenBank under accession numbers (MT386509.1, MT356630.1, MT893206.1, and MW847902), respectively (Table 3).

Virus isolation on cell culture

Three positive samples, including FAdV-D serotype 2, FAdV-D serotype 11, and FAdV-E (serotype-8a), were passaged on CEL. Different degrees of CPE on cell culture were observed in the inoculated cells as early as 24hr post inoculation after first passage with type E virus and after second passage from type D virus (Figure 7).

Table 3. Reference isolates of Fowl aviadenovirus serotypes on GenBank and isolates of the current study

Accession No.	Country	Host	Sample type	Strain	Collection date
MT708028.1	France	Numida meleagri guinea fowl	Pancreas	Fowl aviadenovirus A (fowl adenovirus type 1)	2018
KT781516.1	Egypt	Chicken	Cloacal swabs	Fowl adenovirus 8a	2015
KT781517.1	Egypt	Chicken	Cloacal swabs	Fowl adenovirus 8a	2015
MN453821.1	Brazil	Gallus gallus with RSS	Intestinal content	Fowl adenovirus 8a	2010
MT127100.1	Malaysia	SPF Embryonated Chicken Eggs	-	Fowl adenovirus 8b	2017
MT127101.1	Malaysia	SPF Embryonated Chicken Eggs	-	Fowl adenovirus 8b	2017
MT561444.1	Malaysia	SPF Embryonated Chicken Eggs	Chicken embryo liver cell	Fowl aviadenovirus E	2019
MT233532.2	Malaysia	Broiler	Chicken embryo liver cell	Fowl aviadenovirus E	2017
MK816406.1	India	Gallus gallus	-	Fowl aviadenovirus D	2017
MF770580.1	India	Poultry	Liver	Fowl aviadenovirus D	2016
MH492324.1	Japan	layer chickens	Oviducts	Fowl aviadenovirus 4	2014
MH159176.1	China	Chicken	-	Fowl aviadenovirus 4	2008
MH159177.1	China	Chicken	-	Fowl aviadenovirus 4	2017
MT386509.1	Egypt	Broiler chickens	Liver tissue	Fowl aviadenovirus D Serotype	2019 Current study
MT893206.1	Egypt	Broiler chickens	Liver tissue	Fowl aviadenovirus D Serotype	2019 Current study
MT356630.1	Egypt	Broiler chickens	Liver tissue	Fowl aviadenovirus D Serotype	2019 Current study
MW847902	Egypt	Broiler chickens	Cloacal swabs	Fowl aviadenovirus E 8a	2019 Current study

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

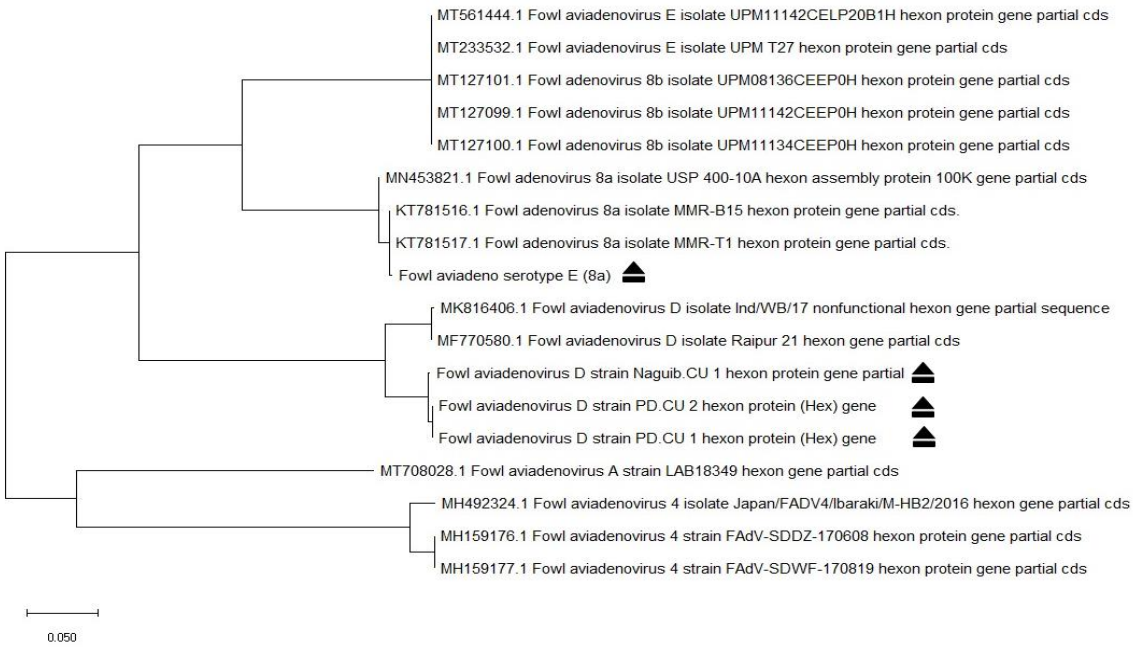


Figure 5. Phylogenetic tree of the partial hexon gene fragment nucleotide sequences of our isolates and reference strains from the GenBank (accession number of the reference strain's hexon gene sequences were mentioned beside each nucleotide sequence)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1		57.8	57.8	58.0	57.2	57.2	57.2	57.2	57.2	56.8	60.2	57.0	60.2	60.2	56.4	56.4	56.0	57.6	1
2	62.0		100.0	99.2	79.0	79.0	79.0	79.0	79.0	68.2	55.2	68.4	54.6	55.2	68.0	68.0	68.2	99.8	2
3	62.0	0.0		99.2	79.0	79.0	79.0	79.0	79.0	68.2	55.2	68.4	54.6	55.2	68.0	68.0	68.2	99.8	3
4	61.5	0.8	0.8		79.6	79.6	79.6	79.6	79.6	68.0	55.0	68.2	54.6	55.0	67.8	67.8	68.0	99.0	4
5	63.1	25.0	25.0	24.1		100.0	100.0	100.0	100.0	65.8	51.4	66.0	51.8	51.4	66.2	66.2	66.4	78.8	5
6	63.1	25.0	25.0	24.1	0.0		100.0	100.0	100.0	65.8	51.4	66.0	51.8	51.4	66.2	66.2	66.4	78.8	6
7	63.1	25.0	25.0	24.1	0.0	0.0		100.0	100.0	65.8	51.4	66.0	51.8	51.4	66.2	66.2	66.4	78.8	7
8	63.1	25.0	25.0	24.1	0.0	0.0	0.0		100.0	65.8	51.4	66.0	51.8	51.4	66.2	66.2	66.4	78.8	8
9	63.1	25.0	25.0	24.1	0.0	0.0	0.0	0.0		65.8	51.4	66.0	51.8	51.4	66.2	66.2	66.4	78.8	9
10	62.7	41.5	41.5	41.9	45.9	45.9	45.9	45.9	45.9		52.8	99.8	52.2	52.8	93.8	93.8	93.4	68.0	10
11	51.0	62.0	62.0	62.5	71.5	71.5	71.5	71.5	71.5	69.2		53.0	96.6	100.0	52.4	52.4	52.4	55.0	11
12	62.2	41.2	41.2	41.5	45.5	45.5	45.5	45.5	45.5	0.2	68.6		52.4	53.0	94.0	94.0	93.6	68.2	12
13	51.0	63.8	63.8	63.8	70.6	70.6	70.6	70.6	70.6	70.8	3.7	70.3		96.6	52.6	52.6	52.6	54.4	13
14	51.0	62.0	62.0	62.5	71.5	71.5	71.5	71.5	71.5	69.2	0.0	68.6	3.7		52.4	52.4	52.4	55.0	14
15	63.7	42.0	42.0	42.4	45.2	45.2	45.2	45.2	45.2	6.6	70.2	6.4	69.8	70.2		100.0	99.6	67.8	15
16	63.7	42.0	42.0	42.4	45.2	45.2	45.2	45.2	45.2	6.6	70.2	6.4	69.8	70.2	0.0		99.6	67.8	16
17	64.7	41.5	41.5	41.9	44.8	44.8	44.8	44.8	44.8	7.0	70.2	6.8	69.7	70.2	0.4	0.4		68.0	17
18	62.4	0.2	0.2	1.0	25.2	25.2	25.2	25.2	25.2	41.9	62.5	41.5	64.3	62.5	42.3	42.3	41.9		18
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

1 MT708028.1_Fowl_aviadenovirus_A_strain_
2 KT781516.1_Fowl_adenovirus_8a_isolate_M
3 KT781517.1_Fowl_adenovirus_8a_isolate_M
4 MN453821.1_Fowl_adenovirus_8a_isolate_U
5 MT127099.1_Fowl_adenovirus_8b_isolate_U
6 MT127100.1_Fowl_adenovirus_8b_isolate_U
7 MT127101.1_Fowl_adenovirus_8b_isolate_U
8 MT561444.1_Fowl_aviadenovirus_E_isolate
9 MT233532.1_Fowl_aviadenovirus_E_isolate
10 17_nonfunctional_hexon_gene_partial_seq
11 MH159177.1_Fowl_aviadenovirus_4_strain_
12 MF770580.1_Fowl_aviadenovirus_D_isolate
13 2016_hexon_gene_partial_cds
14 MH159176.1_Fowl_aviadenovirus_4_strain_
15 MT386509.1
16 MT356630.1
17 MT83206.1
18 MW847902

Figure 6. Similarity (percent identity) of L1 loop of the hexon gene sequences and their amino acids translate of FAV from broiler chickens in Egypt (representing numbers 15, 16, 17, and 18) as compared with the most similar reference sequences (GenBank).

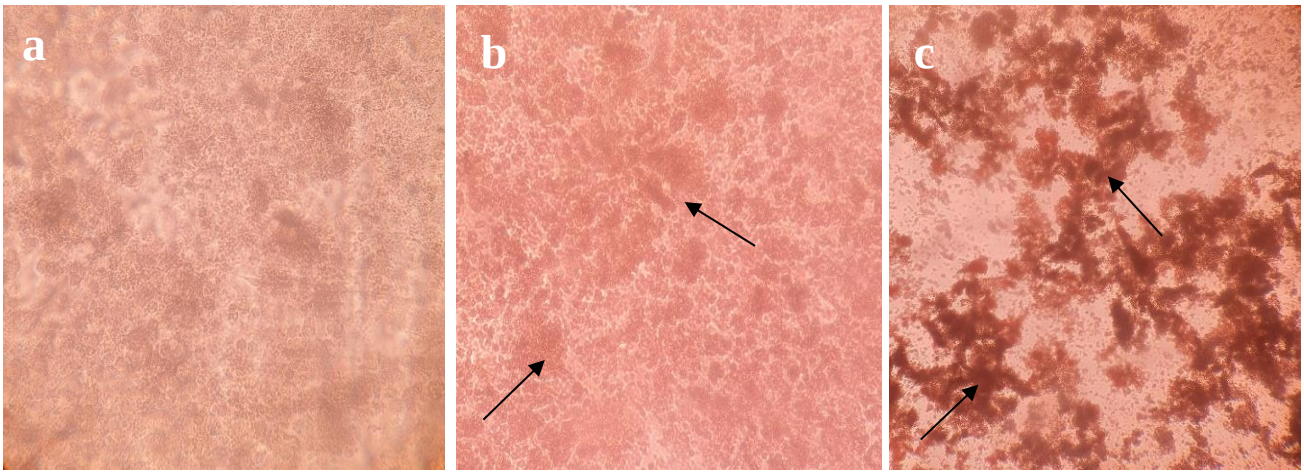


Figure 7. Different stages of growth of FAdV on chicken embryo liver. **a:** Spindle shape of the liver cells. **b:** Cytopathic effect after 24 hours appeared as (small areas of cell death). **c:** large areas of focal cell death with the beginning detachment of the cells (CPE after 2 dpi).

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

DISCUSSION

Over the past decade, inclusion body hepatitis–hydropericardium syndrome (IBH–HPS) has gained importance as one of the pivotal vertically transmitted diseases in broiler chickens that tackle the growing poultry industry all over the globe (Hafez, 2011; Shah et al., 2017; Schachner et al., 2018). In many countries, especially in Egypt, FAdV is considered to be the main pathogen within the first five weeks of broilers causing significant economic losses (Radwan et al., 2019).

In the current study, the examined flocks grossly revealed hepatic enlargement with pale pinpointed foci, liver ecchymotic hemorrhage, and straw-yellow fluid in pericardium up to 15 mL. In addition, nephrosis and enlargement of the spleen were noticed. These lesions resemble IBH-HPS findings previously described by (McFerran and Smyth, 2000; Hafez, 2011).

These gross lesions and mortality were attributed to the replication of FAVS in liver cells and extensive hepatic damage which leads to a decrease in the colloid osmotic pressure and increase of blood vessel permeability with a massive accumulation of fluid around the heart and finally cardiac tamponade and death (Niu et al., 2019). Histologically, degenerative and inflammatory lesions were recorded in the intestine, liver, pericardium, and heart with the presence of eosinophilic intranuclear inclusion bodies similar to previous studies (Steer et al., 2015; Niu et al., 2019; Radwan et al., 2019c).

Although it was assumed before that the heart was not the target organ of FAdV-4, severe pericarditis and heterophils infiltration were the most prominent lesions observed in the heart in the present study in addition to interstitial edema and widening of the gap between myocardial fibers (Niu et al., 2019).

Mortalities in the positive FAdVs tested flocks had a wide range from 1 to 7.5%. Variation in broilers mortalities due to IBH was recorded in many countries including Canada (Ojkic et al., 2008), Japan, Iran (Rahimi and Minoosh Siavosh Haghighi, 2015), China (Changjing et al., 2016), and Egypt (Hafez, 2011; Radwan et al., 2019).

IBDV and CIAV have been reported as common co-infection of FAdVs positive flocks which exacerbate the death rates as described by (Elbestawy et al., 2020). Despite this, there have been reports that IBH can cause mortality even when no other infective agent is present (Mohamed et al., 2018). Neither CIAV nor IBDV was detected in all FAdVs positive flocks in the current study. Therefore, FAdVs could be the primary pathogen responsible for mortality in the examined flocks.

Nevertheless, the clinical signs, post mortem lesions, and histopathology were highly indicative for FAdV infection, molecular detection by PCR assay was used to confirm the disease as previously reported earlier by (Raue et al., 2005; Günes et al., 2012; Mittal et al., 2014). By using the conventional PCR technique, only 10 out of 50 examined flocks were positive (20%) and this low percentage of virus detection by PCR may be interpreted by the usage of a conventional PCR in viral recognition, which is less sensitive in comparing with a real-time PCR (Günes et al., 2012). Only three farms were positive for liver tissue samples, and seven farms were positive for cloacal swabs. Consequently, sampling by cloacal swabs is considered better than liver tissues for detection of FAdv in broiler chicken, and this finding agrees with that recorded by (Radwan et al., 2019). In addition, FAdV virus isolation was carried out using primary CEL and cytopathic effects were noticed after 24 hours of virus inoculation in the form of cell rounding and small focal cells' areas detachment from the surface. Similar CPE in CEL of FAdVs was previously described by(Soumyalekshmi et al., 2014; Mohamed Sohaimi et al., 2019).

Further typing of the isolated viruses indicated that they are belonged to FAdV-type D serotypes 2 and 11, in addition to FAV- type E serotype 8a. The obtained sequences were clustered with fowl Adenoviruses strains isolated from Europe, Brazil, Malaysia, China, Japan, and India. Many countries all over the world reported detection of FAdV-D including Canada (Ojkic et al., 2008), South Africa (Maartens et al., 2014), China (Changjing et al., 2016), Poland (Niczyporuk, 2016), Lebanon (Shaib et al., 2017), Saudi Arabia (Mohamed et al., 2018) and Egypt (El-Tholoth and Abou El-Azm, 2019; Elbestawy et al., 2020). In addition, Radwan et al., 2019 previously detected two strains of serotype FAdV-8a (FAdV-E) in Egyptian chicken farms during 2015. These viruses had a high sequence homology with the viruses isolated during the current study that throws light on the nature, and resistance of adenoviruses over time.

Detection and characterization of FAdVs serotypes play a major role in epidemiologic studies of the disease spread, the development of preventative measures, and the application of vaccination programs. The current study spots light on the distribution and molecular characterization of FAdVs from cases of IBH-HPS that have been recently increased in broiler chicken flocks in Egypt and resulted in significant economic losses to the poultry industry.

CONCLUSION

In conclusion, the necessity to update the recent situation of Adenoviruses associated with hydropericardium syndrome in different broiler farms in many governorates in Egypt prompts us to conduct this study. Virus detection by PCR and sequence analysis indicated that both type D and type E adenovirus are the most common adenovirus associated with this syndrome in broiler farms in different governorates in Egypt. Further study will be conducted to study the pathogenicity of these isolated viruses in broiler chickens.

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

DECLARATIONS

Ethical considerations

Ethical issues including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by the authors.

Competing interests

The author has no conflict of interest.

Authors' contributions

DGK, MGA, EAM, and GAA contributed to the design of the study, sample collection, isolation, identification of the virus, sequencing, and sequence analysis. MS carried out virus isolation, identification, and titration on cell culture. MSK carried out the histopathological examination. All authors drafted the manuscript and participated in the subsequent discussions and revisions of the entire text. All authors read and approved the results and final manuscript.

Acknowledgments

The authors would like to thank the Poultry diseases department, Faculty of Veterinary Medicine, Cairo University, Egypt for its assistance and support. This work did not receive any specific grant from any funding agencies.

REFERENCES

Changjing L, Haiying L, Dongdong W, Jingjing W, Youming W, Shouchun W, Jida L, Ping L, Jianlin W, Shouzhen X et al. (2016). Characterization of fowl adenoviruses isolated between 2007 and 2014 in China. Veterinary microbiology, 197: 62-67. DOI: https://www.doi.org/10.1016/j.vetmic.2016.11.005

Chen Z, Shi S, Qi B, Lin S, Chen C, Zhu C, and Huang YJ (2018). Hydropericardium syndrome caused by fowl adenovirus serotype 4 in replacement pullets. Journal of Veterinary Medical Science, 81 (2): 245-251. DOI: https://www.doi.org/10.1292/jvms.18-0168

Elbestawy AR, Ibrahim M, Hammam H, Noreldin AE, Bahrawy AE, and Ellakany HF (2020). Molecular Characterization of Fowl Adenovirus D Species in Broiler Chickens with Inclusion Body Hepatitis in Egypt. Alexandria Journal of Veterinary Sciences, 64: 110-116. DOI: https://www.doi.org/10.5455/ajvs.74411

El-Tholoth M, and Abou El-Azm KI (2019). Molecular detection and characterization of fowl adenovirus associated with inclusion body hepatitis from broiler chickens in Egypt. Tropical Animal Health Production, 51: 1065-1071. DOI: https://www.doi.org/10.1007/s11250-018-01783-0

Günes A, Marek A, Grafl B, Berger E, and Hess M (2012). Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). Journal of Virological Methods, 183: 147-153. DOI: https://www.doi.org/10.1016/j.jviromet.2012.04.005

Hafez HM (2011). Avian adenoviruses infections with special attention to inclusion body hepatitis/hydropericardium syndrome and egg drop syndrome. Pakistan Veterinary Journal, 31: 85-92. DOI: http://www.dx.doi.org/10.17169/refubium-18486

Hemida M, and Alhammadi M (2017). Prevalence and molecular characteristics of fowl adenovirus serotype 4 in eastern Saudi Arabia. Journal of Veterinary and Science, 41: 506-513. DOI: https://www.doi:10.3906/vet-1612-19

Hess MJAP (2000). Detection and differentiation of avian adenoviruses: A review. Avian Pathology, 29: 195-206. DOI: https://www.doi.org/10.1080/03079450050045440

Khan AA, Sabri AN, Mansoor MK, and Hussain I (2019). Hydropericardium syndrome in Pakistan: A review. World's Poultry Science Journal, 61: 647-654. DOI: https://www.doi.org/10.1079/WPS200576

Kumar R, Chandra R, and Shukla S (2003). Isolation of etiological agent of hydropericardium syndrome in chicken embryo liver cell culture and its serological characterization. Indian Journal of Experimental Biology, 31: 821-826. Available at: http://hdl.handle.net/123456789/17161

Li L, Wang J, Chen P, Zhang S, Sun J, and Yuan W (2018). Pathogenicity and molecular characterization of a fowl adenovirus 4 isolated from chicken associated with IBH and HPS in China. BMC Veterinary Research, 14: 400. DOI: https://www.doi.org/10.1079/WPS200576

Maartens LH, Joubert HW, Aitchison H, and Venter EH (2014). Inclusion body hepatitis associated with an outbreak of fowl adenovirus type 2 and type 8b in broiler flocks in South Africa. Journal of the South African Veterinary Association, 85: 1-5. DOI: https://www.doi.org/10.4102/jsava.v85i1.1146

Mansoor MK, Hussain I, Arshad M, and Muhammad G (2011). Preparation and evaluation of chicken embryo-adapted fowl adenovirus serotype 4 vaccine in broiler chickens. Tropical Animal Health Production, 43: 331-338. DOI: https://www.doi.org/10.1007/s11250-010-9694-z

Mcferran J, and Smyth J (2000). Avian adenoviruses. Revue Scientifique et Technique (International Office of Epizootics), 19: 589-601. PMID: 10935281. Available at: https://www.oie.int/doc/ged/D9436.PDF

Metwally A, Yousif A, Shaheed I, Mohammed W, Samy A, and Reda I (2009). Re-emergence of very virulent IBDV in Egypt. International Journal of Virology, 5: 1-17. Available at: https://scialert.net/abstract/?doi=ijv.2009.1.17

Mittal D, Jindal N, Tiwari AK, and Khokhar RS (2014). Characterization of fowl adenoviruses associated with hydropericardium syndrome and inclusion body hepatitis in broiler chickens. Virus disease, 25: 114-119. DOI: https://www.doi.org/10.1007/s13337-013-0183-7

Mohamed MHA, El-Sabagh IM, Abdelaziz AM, Al-Ali AM, Alramadan M, Lebdah MA, Ibrahim AM, and Al-Ankari AS (2018). Molecular characterization of fowl aviadenoviruses species D and E associated with inclusion body hepatitis in chickens and falcons indicates possible cross-species transmission. Avian Pathology, 47: 384-390. DOI: https://www.doi.org/10.1080/03079457.2018.1457769

Mohamed Sohaimi N, Bejo MH, Omar AR, Ideris A, and Mat Isa N (2019). Molecular characterization of fowl adenovirus isolate of Malaysia attenuated in chicken embryo liver cells and its pathogenicity and immunogenicity in chickens. PLoS One, p. 14. DOI: https://doi.org/10.1371/journal.pone.0225863

Morshed R, Hosseini H, Langeroudi AG, Fard MHB, and Charkhkar S (2017). Fowl adenoviruses D and E cause inclusion body hepatitis outbreaks in broiler and broiler breeder pullet flocks. Avian Diseases, 61: 205-210. DOI: https://www.doi.org/10.1637/11551-120516-Reg.1

Nakamura K, Mase M, Yamamoto Y, Takizawa K, Kabeya M, Wakuda T, Matsuda M, Chikuba T, Yamamoto Y, Ohyama T et al. (2011). Inclusion body hepatitis caused by fowl adenovirus in broiler chickens in Japan, 2009-2010. Avian Diseases, 55: 719-723. DOI: https://www.doi.org/10.1637/9813-052511-Case.1

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

Niczyporuk JS (2016). Phylogenetic and geographic analysis of fowl adenovirus field strains isolated from poultry in Poland. Archives of Virology, 161: 33-42. DOI: https://www.doi.org/10.1007/s00705-015-2635-4

Niu Y, Sun Q, Liu X, and Liu S (2019). Mechanism of fowl adenovirus serotype 4-induced heart damage and formation of pericardial effusion. 98: 1134-1145. DOI: https://doi.org/10.3382/ps/pey485

Ojkic D, Martin E, Swinton J, Vaillancourt JP, Boulianne M, and Gomis S (2008). Genotyping of Canadian isolates of fowl adenoviruses. Avian Pathology, 37: 95-100. DOI: https://www.doi.org/10.1080/03079450701805324

Oliver-Ferrando S, Dolz R, Calderón C, Valle R, Rivas R, Pérez M, Biarnés M, Blanco A, Bertran K, and Ramis A (2017). Epidemiological and pathological investigation of fowl aviadenovirus serotypes 8b and 11 isolated from chickens with inclusion body hepatitis in Spain (2011–2013). Avian Pathology, 46: 157-165. DOI: https://www.doi.org/10.1080/03079457.2016.1232477

Radwan MM, El-Deeb AH, Mousa MR, El-Sanousi AA, and Shalaby MA (2019). First report of fowl adenovirus 8a from commercial broiler chickens in Egypt: Molecular characterization and pathogenicity. Poultry Science, 98: 97-104. DOI: https://www.doi.org/10.3382/ps/pey314

Rahimi M and Minoosh Siavosh Haghighi Z. (2015). Adenovirus-like inclusion body hepatitis in a flock of broiler chickens in Kermanshah province, Iran. Veterinary Research Forum. 6(1):95-98. Available at: https:// www.ncbi.nlm.nih.gov/pmc/articles/PMC4405693/pdf/vrf-6-095.pdf

Raj Narayan Trivedi, Rajesh Kumar, Mamta Metwal, Amir Showkat Khan, Akanksha Tiwari, Garima Panday and Avadhesh Kumar. (2018). Epidemiological Observations on Some Natural Outbreaks of Inclusion Body Hepatitis-Hydropericardium Syndrome (IBH-HPS) in Domestic Chicken. International Journal of Current Microbiology and Applied Sciences , 7(8): 3012-3022. DOI: https://www.doi.org/10.20546/ijcmas.2018.708.320

Raue R, Gerlach H, and Muller H (2005). Phylogenetic analysis of the hexon loop 1 region of an adenovirus from psittacine birds supports the existence of a new psittacine adenovirus (PsAdV). Archive of Virology, 150: 1933-1943. DOI: https://www.doi.org/10.1007/s00705-005-0578-x

Schachner A, Matos M, Grafl B, and Hess M (2018). Fowl adenovirus-induced diseases and strategies for their control–a review on the current global situation. Avian Pathology, 47: 111-126. DOI: https://www.doi.org/10.1080/03079457.2017.1385724

Shah MS, Ashraf A, Khan MI, Rahman M, Habib M, Chughtai MI, and Qureshi JA (2017). Fowl adenovirus: History, emergence, biology, and development of a vaccine against hydropericardium syndrome. Archive of Virology, 162: 1833-1843. DOI: https://www.doi.org/10.1007/s00705-017-3313-5

Shaib H, Ramadan N, Mahmoud G, Nassif G, and Chedid S (2017). Outbreak of inclusion body hepatitis causing Adenovirus in Lebanese Broiler flocks. EC Microbiology, 13: 92-101. Available at: https://www.ecronicon.com/ecmi/pdf/ECMI-13-00424.pdf

Soumyalekshmi S, Ajith M, Meshram C, and Technology (2014). Isolation of fowl adenovirus in chicken embryo liver cell culture and its detection by hexon gene-based PCR. Indian Journal of Scientific Research, 2: 33-36. Available at: http://www.indjsrt.com/admin/UploadFiles/6-31.pdf

Steer PA, Sandy JR, O'rourke D, Scott PC, Browning GF, and Noormohammadi AH (2015). Chronological analysis of gross and histological lesions induced by field strains of fowl adenovirus serotypes 1, 8b, and 11 in one-day-old chickens. Avian Pathology, 44: 106-113. DOI: https://www.doi.org/10.1080/03079457.2015.1007919

Suohu, Sedeneinuo; Rajkhowa, Tridib Kumar 2021 Prevalence and Molecular Diagnosis of Hydropericardium Hepatitis Syndrome in the Poultry Population of Mizoram, India. Indian Journal of Animal Research. 55 (1): 96-100. DOI: https://www.doi.org/10.18805/ijar.B-3923

Suvarna KS, Layton C, and Bancroft JD (2018). Bancroft's theory and practice of histological techniques E-Book, Elsevier Health Sciences. Available at: http://library.iautmu.ac.ir/file/download/page/1557923707-105-bancroft-s-theory-and-practice-of-histological-techniques-8th-edition-kim-s-suvarna-frcpath-christopher-9780702068645-elsevier-2019-574-209.pdf

To cite this paper: Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG, and Abdel-Alim GA (2021). Isolation and Molecular Characterization of Fowl Adenoviruses Associated with Inclusion Body Hepatitis-hydropericardium Syndrome in Broiler Chickens in Egypt. *World Vet. J.*, 11 (3): 333-342.

2021, Scienceline Publication
World's Veterinary Journal
World Vet J,11(3): 343-367, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes

Tewodros Fentahun*[1] and Jan Paeshuyse[2]

[1]*Department of Veterinary Biomedical Sciences, College of Veterinary Medicine and Animal Sciences, University of Gondar, P.O.Box 196, Gondar, Ethiopia*
[2]*KU Leuven, Faculty of Bioscience Engineering, Kasteelpark Arenberg 30 - bus 24723001, Leuven, Belgium*

*Corresponding author's Email: tedyvet@gmail.com ; ORCID: 0000-0002-2955-5638

ORIGINAL ARTICLE
pii: S232245682100047-11
Received: 21 June 2021
Accepted: 10 August 2021

ABSTRACT

Trypanosoma theileri (*T. theileri*) is a non-pathogenic, cosmopolitan, and commensal protozoa of cattle. The main objective of the current study was to investigate the biology and feasibility of *T. theileri* as a model candidate for the discovery of a novel drug. In the present study, the isolates of *T. theileri* obtained from the Institute of Tropical Medicine (ITM) in SDM 79 were cultivated at 26^{o}C. Eight experiments with different inoculum and different times were grown. The growth curve was plotted to check the growth trends. The doubling time in the logarithmic phase was determined to be 17.43 hours. In addition, an experimental infection was done on a 3-month-old Holstein Friesian calf to isolate the blood-streaming shape; however, it was not successful after the blood buffy coat smear and PBMC culture in RPMI 1640 and HMI 9. Furthermore, the viability was determined by quantitative colorimetric Resazurin assay in 96-well fluorescence Microplates containing 0.4 to 2.4 mM of Resazurin. On the other hand, the response to Pentamidine (1-100 ng/mL) showed a strong negative correlation between the fluorescence signal and the highest Pentamidine concentration. IC_{50} was 9.25 ng/mL. Genomic DNA was extracted using the phenol-chloroform method. The gradient PCR amplification using *T. theileri* specific PCR (Tth625-PCR) primers was detected at 465 base pair (bp). In addition, the full-length 18S rDNA sequence was detected at 730 bp. In the silico analysis using common anti-trypanosome drug targets, no significant similarity could be found on either the DNA or the protein level. Nevertheless, homologous sequences have been identified among the drug targets for Ornithine decarboxylase. Therefore, the analysis might show the possibility of using *T. theileri* as a model for the search of new drugs once they have entire genome sequences. Analysis of the whole genome and transcriptome indicated a phylogenetic relationship between *T. theileri* and other pathogenic trypanosomes which can be the basis for novel drug development.

Keywords: Drug model, Novel drug, PCR, Resazurin, SDM 79, *Trypanosoma theileri*

INTRODUCTION

The trypanosomatid parasites cause one of the most notorious human and animal trypanosomiasis in all parts of Africa and South America. Even if trypanosomes are the main cause of diseases in humans (sleeping sickness) and animals (Nagana), many other species are not pathogenic (Mott et al., 2011). Such pathogenic trypanosomatids occur globally and infect a large number of hosts. Among these, *Trypanosoma theileri* (*T. theileri*) is ubiquitous, 'truly cosmopolitan' cattle protozoan commensal found worldwide (Mott et al., 2011 and Lee et al., 2013).

Natural infections could be found in all age groups of cattle although they are rare in cattle younger than one year old. Neither its life cycle nor its host relationship is fully understood in the mammalian host. The main vector responsible for the transmission of the parasite is Tabanidae. However, ticks including *Hyalomma anatolicum* and *Boophilus microplus* were also later reported as vectors (Latif et al., 2004). Hence, *T. theileri* is typically characterized by a stercorarian type of transmission (Latif et al., 2004). After ingesting infected blood, trypanosomes develop in the vector's hindgut. The infection is then transmitted to new hosts through fecal contamination of the mucus membrane or abrasions of the skin (Lukes, 2009). In the newly infected host, the epimastigotes multiply in the bloodstream by binary fission. Besides epimastigotes and large trypomastigotes in the peripheral blood, flagellates have also been found in extra-vascular sites of lymph nodes, kidneys, spleen, and brain (Braun et al., 2002).

In order to isolate pathogenic African Trypanosomes, a kit called KIVI (Kit for In Vitro Isolation of trypanosome) was designed (Aerts et al., 1992). Similarly, Verloo et al. (2000) proved that this kit can be used as an excellent device for isolating *T. theileri* with much higher sensitivity than the Roswell Park Memorial Institute (RPMI) medium. On the other hand, there was evidence that the growth of *T. theileri* on the RPMI medium could be easily confirmed (Lee et al., 2013). Many trypanocidal drugs are available in the market. Among these drugs, pentamidine, diminazene aceturate (Berenil), isometamidium chloride (Samorin), and ethidium bromide are important anti-trypanosomal drugs (Shapiro and Englund, 1990).

High-Throughput Screening (HTS) and virtual screening are used as a standard means in drug discovery to identify novel lead compounds that target a biomolecule of interest. However, the latter is considered a cost-effective means

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.,* 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

(Ekins et al., 2007; Fatumo et al., 2013). Editing of trypanosomatid RNA could be used to identify the drug target for protozoal parasites that cause diseases, such as trypanosomiasis. Amaro et al. (2008) reported that RNA-Editing Lgase-1 (REL-1) could be used as drug-like inhibitors of a key enzyme in the editing machine. The identification of inhibitors was done through a strategy employing molecular dynamics to account for protein flexibility (Amaro et al., 2008). New parasitic inhibitors had been identified due to the availability of an automated approach to high content microscopy (Alonso-Padilla and Rodriguez, 2014).

For better pharmacology hypotheses and tests, the development of computational (In Silico) methods plays a significant role. This methodology comprises pharmacophores, databases, quantitative structure-activity relationships, homology models, and other molecular modeling approaches, machine learning, network analysis tools, and data analysis tools using a computer.

Although *T. theileri* was not naturally pathogenic, it can cause disease in stressed cattle. Moreover, little is known about *T. theileri*. Recently, however, it has become an area of interest and is viewed as a tool and a vector for treating pathogenic microorganisms, particularly protozoan parasites (Mott et al., 2011). Furthermore, the mixed infection of *T. theileri* cause pathogenic trypanosome on the same host (cattle), and the presence of homologous sequences with specific sequences of anti-trypanosomal drug targets from pathogenic trypanosomes could lead to the use of this parasite (*T. theileri*) as a model candidate for the development of new drugs for the treatment of pathogenic trypanosomes. To this end, the basics of the parasite should be studied to manipulate the parasite as a tool to combat pathogenic trypanosomes. Furthermore, little is known about the biology, cell growth pattern, doubling time, and viability of the parasite. There is little convincing data to determine whether *T. theileri* could be used as a model for discovering new drugs for the treatment of pathogenic trypanosomiasis.

The general aim of the present research was to provide fundamental insights into the biology of *T. theileri* to verify its feasibility as a model organism for the discovery of the new drug. The specific objectives were cultivation of *T. theileri* both *in vitro* and *in vivo*, comparing and analyzing the growth pattern with others to check viability with Resazurin assay analysis and response to the drug, and determining the presence of homologous sequences between its genome and the specific target (conserved) sequence of anti-trypanosome drugs.

MATERIALS AND METHODS

Ethical approval

During the entire experimental period, the care and maintenance of the calf in its pen was performed based on the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. Furthermore, the experimental protocols were used after the approval by the Animal Research and Ethical Review Committee at the KU Leuven University (Permit No: P024/2017)

Parasites and *in vitro* culture

The sample used in the current study was *T. theileri*, kindly donated by the Diagnostic Parasitology Department of the Institute of Tropical Medicine, Belgium. These cryogenically preserved trypanosomes in culture were isolated from a small farm nearby Antwerp, Belgium by Verloo et al. (2000). Different growth media and conditions, under which other trypanosomes were grown in the institute, were assessed to determine the optimal conditions and favorable growth media for culturing the parasite. After identifying the appropriate medium (SDM 79) and the growth condition (at 26^{o}C, without CO_2), the cryostablate was seeded (1: 10 ratio) and propagated three times with SDM 79 medium (BioConcept AMIMED company, Switzerland) in a cell culture flask (25cm^2, Thermo Scientific™ Nunc™ Cell Culture Flasks). It was counted daily with a hemocytometer and recorded in the logarithmic table. A growth curve was then plotted to determine the growth pattern. In addition, the minimum and the maximum number of cells in the eight-cell culture flasks (eight experiments) that could serve as a potential indicator of the logarithmic phase and considered for both the experimental infection and Resazurin assay development were stored. Moreover, *T. theileri* was sub-passaged every three days. Epimastigotes were harvested in the exponential growth phase. Epimastigoteswas were centrifuged (1500 rpm for 10 minutes to sediment, washed, and re-suspended with phosphate buffer saline (PBS, pH = 7.2) before inoculation to the experimental calf while it was being used for DNA extraction.

Experimental animal and infection

A Holstein Friesian calf (aged three months) was randomly selected from the zootechnical Centre, KU Leuven, Belgium. It was confirmed to be trypanosome-negative by taking the blood sample and smear and culture in HMI-9 medium. After confirmation, it was inoculated with the sub-cultured epimastigotes of *T. theileri* (4.5 ×10^6/mL to 7.3 ×10^6/mL), intravenously (IV) through the jugular vein in a volume of 5-9 mL. In addition to parenteral inoculation, the calf was orally drenched with the same amount of inoculation. The calf was monitored and examined for parasitemia for three consecutive weeks. Blood samples (10 mL) were collected using Ethylenediaminetetraacetic Acid (EDTA) coated

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

vacutainer tubes. The samples were collected two times after experimental infection (every week after experimental infection). After the blood samples were transported to the Host-Pathogen Interaction Laboratory of KU Leuven, Belgium, the samples were examined for the presence of trypanosomes using the standard parasitological methods of the wet and thin blood smear (stained with Giemsa), PBMC, and buffy coat technique within two to three hours of sampling per day (Murray et al., 1977).

The buffy coat/PBMCculture

After extracting the buffy coat (Murray et al., 1977) and PBMC (Ficoll-Paque method), the buffy coat samples were transferred to two cell culture flasks (25cm^2, Thermo Scientific™ Nunc™ Cell Culture Flasks) containing HMI-9 medium and RPMI 1640 supplemented with 10% fetal calf serum (FCS, Sigma) and 200 IU/mL of penicillin and 100µg/Ml streptomycin (Invitrogen, Carlsbad, CA) according to Hirumi and Hirumi (1989). A ratio of 1:10 (1 ml of buffy coat sample to 10 ml of the HMI-9 medium) was used. The inoculated cell culture flasks containing HMI-9 medium were then incubated at 37^{o}C and exposed to 5% CO_2. The tests were checked daily under an inverted microscope at 40 × magnification to monitor the progress of growth in both cell culture flasks inoculated for a maximum of two weeks.

***In vitro* sensitivity assays (Resazurin assay)**

A reagent, Resazurine obtained from Sigma-Aldrich, was used as a quantitative colorimetric assay based on the oxidation (blue) and reduction (pink) indicators to measure *T. theileri* viability and its response to pentamidine. Resazurin stock solution (0.4 to 2.4 mM) was prepared in PBS withpH 7and filter-sterilized (Miriam et al., 2006).

Optimization of Resazurin to Trypanosoma theileri viability

To reduce the background signal for a better sensitivity assay and also to avoid light piping between wells, the Black Microtiter® 96-Well Fluorescence microplates were used. Cells ranging from 5.2 ×10^5/mL to 8.5 ×10^6/mL with a logarithmic phase were obtained after incubation in cell culture flasks at 26°C for 48 hours. Then, 120 µL cells of *T. theileri* were removed from this cell culture flask into wells of Microtiter® 96-Well Fluorescence Microplates (Thermo Scientific) and incubated again at 26^{o}C for 48 hours. After 48 hours of incubation, 20 µl of different concentrations of Resazurin solution (0.4 to 2.4 mM) was added to each inoculum while an equivalent amount of SDM 79 medium was added for blank wells. The plates were returned to the incubator for 24 hours for optimal oxidation and reduction. The Fluorescence signal was read 1, 3, 5, 7, 18, and 24 hours after the addition of Resazurin by dual-wavelength using a GFP protocol-VICTOR™ X Series Multi-label Plate Reader (Perkin Elmer Instruments Inc.) at λ_{exc}485, and λ_{em} 535 nm. A single micro-titer plate was used for three different concentrations of Resazurin per experiment. The background was subtracted from each reading. The experiments were performed three times and an average was taken.

Standard curves

Following incubating epimastigotes in the range of 1.3 ×10^6 to 5.3 ×10^6 epimastigotes/mL for 48 hours, 120 µL of epimastigotes /mL per well were seeded to a 96 well microtiter plate for further 48 hours of incubation at 26^{o}C. Then, 20 µL of 2.4 mM Resazurin was added followed by fluorescence signal reading after 7 hours of incubation. This procedure was performed twice in all three times.

Drugs sensitivity assay

Pentamidine was dissolved in concentrations from1.0 to 80ng/ml. In each wellofCostarTM 96-well microtitre plates, 120µl of epimastigotes of *T. theileri* in the logarithmic phase (1.4-5.3 ×10^6/mL) was seeded with 20 µL of different concentrations of pentamidine (1.0 to 80ng/ml). Three drug concentrations were tested per plate (column 1-3 with 1.0ng/ml, column 4-6 with 40 ng/ml, column 7-9 with 80 ng/ml pentamidine, column 10 without the drug, and column 11SDM 79 medium used as control medium). The plates were then incubated with pentamidine for 48 hours. The trypanosome density was counted with a hemocytometer up to 48 hours of incubation before the addition of Resazurin. Afterwards, the plates were incubated by adding a 20 µL Resazurin to each well and incubated at 26°C for an additional seven hours. IC_{50} values were also calculated at concentrations 1-100 ng/mL using this assay and microscopic counting. Columns 1-10 were tested for drugs, column 11 was without the drug, and column 12 included only SDM 79 as the control medium.

Data Evaluation and analysis

The plate was read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm in a fluorescence/microplate reader (GFP protocol-VICTOR™ X Series Multi-label Plate Reader (PerkinElmer Instruments Inc.). The data were transferred into a graphics program (Excel) and analyzed using the GraphPad Prism 7.0. Descriptive

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

statistics and Pearson r^2(r) correlation coefficient were also calculated. To measure the anti-epimastigotes activity (%AE), the following formula according to Miriam et al. (2006) was used.

$$\mathbf{AE}\ (\%) = \frac{(\mathbf{Gc-Gp})}{\boldsymbol{Gc}} \times \mathbf{100} \quad \text{(Equation 1)}$$

Where, Gc represents the mean number of parasites per milliliter in the control, and Gp shows the mean number of parasites per milliliter according to the different doses of drugs.

Furthermore, the doubling time was calculated based on the following equation used for *T.b.brucei* by Sykes and Avery (2009) and Melissa et al. (2009).

$$\mathrm{TD} = (\mathrm{t2} - \mathrm{t1})\frac{\log(2)}{\log(\mathrm{q2/q1})} \text{(Equation 2)}$$

Where, Td refers to doubling time (q1), t1 is the first quantity for the first time, and (q2) at the time (t2) denotes the second quantity at the second time.

Genomic DNA extraction and PCR amplification

A *T. theileri* culture in a high density of the logarithmic phase ranging from 3.6 $\times 10^6$ cells/ml to 7.8 $\times$ 10^6 epimastigotes/mL was utilized to extract genomic DNA. Both the phenol-chloroform method and DNeasy®Blood and Tissue Kit (Qiagen, Hilden, Germany) were used. The concentration and purity of the DNA were determined with NanoDrop ™Spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis (1%).

DNeasy®Blood and tissue kit

The genomic DNA extraction was conducted according to the protocol recommended by the manufacture Purification of Total DNA from Animal Blood or Cells (DNeasy 96 Protocol), DNeasy®Blood and tissue kit (Qiagen, Hilden, Germany).

Phenol-chloroform method

Cultivated *T. theileri* cells (3.6$\times$ 10^6 cells/ml to 7.8 $\times$ 10^6 cells/ml) in 2ml Eppendorftubeswere lysed by centrifugation at 13000 rpm for a minute. The supernatants were removed and 500 µL of T10N150E10wereadded and mixed thoroughly by pipetting several times. The content was then centrifuged at 13000 rpm for 10 minutes and the supernatant was removed, followed by the addition of500 µL of $T_{10}N_{150}E_{10.}$ This step was repeated twice.

Eight hundred microliter of freshly prepared Glouton-Buffer (10mM Tris HCl, 10 mM EDTA, 100mM NaCl, 10% SDS, 3.9Mm DTT) were added and mixed well by pipetting up and down. It was then incubated at 65^{o}C for one hour. Afterwards, 20µl of 10 µg/mL proteinase K was added and incubated at 56^{o}C overnight.

An equal volume(1000 µL) of phenol/chloroform/isoamyl alcohol in the ratio of 25:24:1 was added and mixed gently. After centrifuging at 13000 rpm for 10minutes, the supernatant was transferred to a new tube. The same amount of phenol/chloroform was added to a new tube, mixed, and centrifuged as above-mentioned. The supernatants were transferred to a new tube again and the same amount of chloroform was added, mixed, and centrifuged as indicated above. The aqueous layer (500 µl) was transferred to a new tube and mixed gently with 1166 µL 100 % Ethanol. The supernatants were removed after centrifugation at 13000 rpm for 1 minute. The tubes were left open to dry DNA for 3 hours, then it was resuspended with 30 µL of Mili-Q water.

PCR amplification and gel electrophoresis

Species-specific PCR identification was performed using the following primers in Table 1 as described by Rodrigues et al. (2003) and Lee et al. (2013). Amplification was conducted using 25 µL reaction mixture with 200 ng template DNA (genomic DNA), 2.5 IU (International Unit) TagDNA polymerase (Promega, U.S.A), 0.2 mM dNTP, PCR buffer, 1.5 mM $MgCl_2$, (Promega, U.S.A), and 0.3µM of primer, according to the manufacturer's instructions. For gradient PCR Amplification, the procedure involved 95°C for 5 minutes, followed by 20 cycles of 94°C for 10 seconds, 53°C for 15 seconds, and 67°C for 1 minute, with a final extension at 72°C for 3 minutes.

Finally, PCR products were run on 1% agarose gel (Sigma) electrophoresis using 1× TBE buffer and stained with 7µL ethidium bromide in 50 mL agarose (0.5 ng/ mL) and made visible using UV transillumination (Vilmar Lourmat).

Table 1. Primers used to detect *Trypanosoma theileri*

	Primer (Forward and Reverse)	Purpose
1	Tth625a (5'-CCG CTG GAG CTA AGA ATA GA-3') and Tth625b (5'-AAT TGC ATA AAC ACA GCT CCC-3')	For species-specific PCR amplification (Tth625-PCR)
2	Forward primer 18STnF2 (5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3') and Reverse primer 18STnR3 (5'-TGC GCG ACC AAT AAT TGC AAT AC-3')	The full length 18S ribosomal DNA sequence Analysis
3	Kin1 reverse (5'- GCG TTC AAA GAT TGG GCA AT-3') and Kin2 forward (5'-CGC CCG AAA GTT CAC C-3')	For single PCR amplification to anneal internal transcribed spacer of ribosomal genes (ITS) sequence

Source: Rodrigues et al. (2003) and Lee et al.(2013)

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

***In silico analysis by* multiple sequence alignments**

Multiple Sequence Alignments (MSA) were conducted with commonly used anti-trypanosomal drug target sequences (Table 1) from pathogenic *Trypanosoma* species and *T. Theileri* genome/proteome using FFT in ExPASy tool, Switzerland (Rodrigues et al., 2003). In addition, phylogenetic trees were created to study the relationships between the different drug targets and the *T. theileri* genome/proteome, as well as between the drug target sequences.

Risk analysis

The *T. theileri* is a non-pathogenic parasite and does not cause disease in either livestock or humans. There is no biological hazard to the calf from parasite inoculation. Therefore, there are no biochemical hazards for the farm and environment when carrying out such an experiment.

RESULTS

Growth medium and conditions

Different types of cell culture media commonly used to culture other types of *Trypanosoma* in ITM were screened to assess the optimal and conducive conditions to culture *T. theileri* epimastigotes. Cryogenically preserved 2.5× 10^6 epimastigotes/mL were seeded into a cell culture flask (25cm^2, Thermo Scientific™ Nunc™ Cell Culture Flasks) in four different media and growth conditions (Figures 1 and 2). It was seeded at a 1:10 ratio and sub-passaged every three days for two consecutive weeks. Finally, the optimal conditions and favorable growth media for *T. theileri* epimastigotes were identified. As a result, significant growth of the *T. theileri* could be observed in SDM 79 at 26°C without CO_2, and RPM'3I 1640 at 37°C with 5 % CO_2 with 10 % Fetal Calf Serum (FCS), as shown in Figures 1 and 2, respectively. However, relatively slower growth was observed for the later third day. On the other hand, there was a prominent growth in RPMI 1640 with 10 % FCS than other serum types used (Figure 2).

Growth pattern and doubling time

After inoculation (2.5 × 10^6 epimastigotes/mL) of *T. theileri* in eight flasks containing SDM 79 with10 % FCS, the growth pattern was determined by counting 10 µL from each flask daily with a Haemocytometer. As indicated in Figure 3, maximum growth was observed on the sixth day in the entire eight-cell culture flask, except for the seventh experiment, which took place on the seventh day. The maximum number of epimastigotes that could be grown among the eight flasks was estimated to be 1.7 × 10^7cells/mL (Experiment 2). The doubling time was calculated per day assuming they were in the logarithmic phase from the third to sixth day as indicated in Figure 3. Since there was a significant growth of the parasite in this period, from 3.2× 10^4 cells/mL, which was the lowest on the third day from the second experiment to the highest 1.3×10^7 cells/mL from the same flask on the sixth day. The calculation was based on the manipulation of the doubling time equation used for *T.b.brucei* from Sykes and Avery(2009) and Melissa et al. (2009) as described in materials and methods. Therefore, the doubling time calculated in the logarithmic growth phase averaged 17.43 hours (0.73 day) for eight experiments.

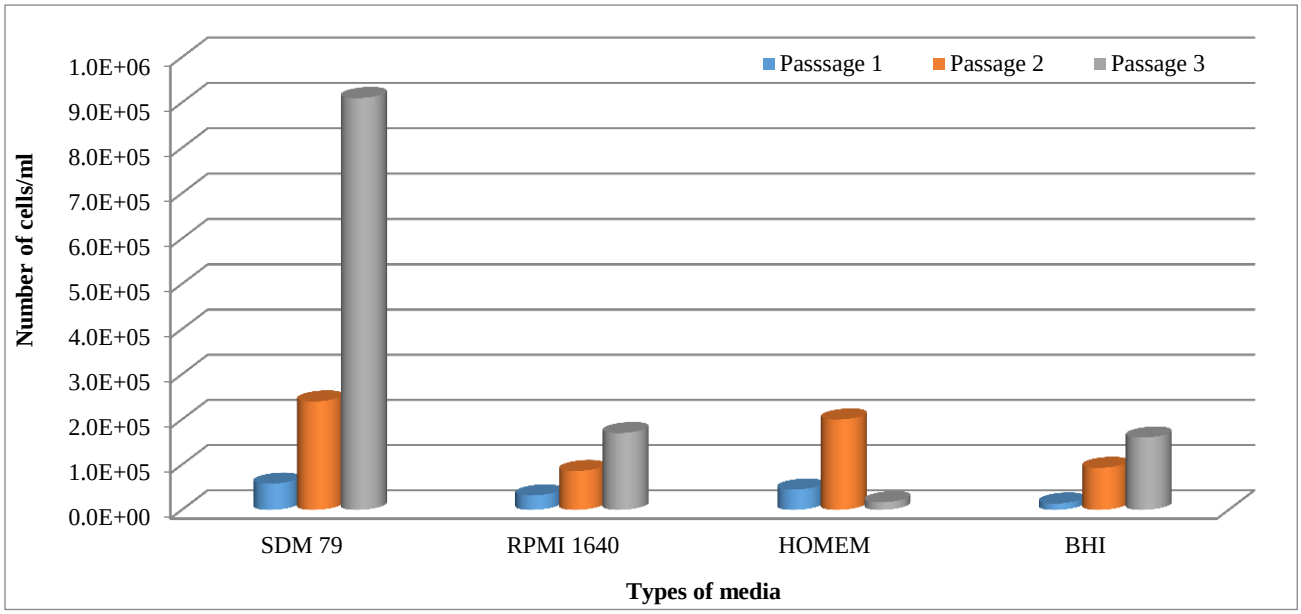


Figure 1. Growth of *Trypanosoma theileri* with various media at 26°C without CO_2 in cell culture flasks. It was performed at the Institute of Tropical Medicine Antwerp, Belgium for two consecutive weeks

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

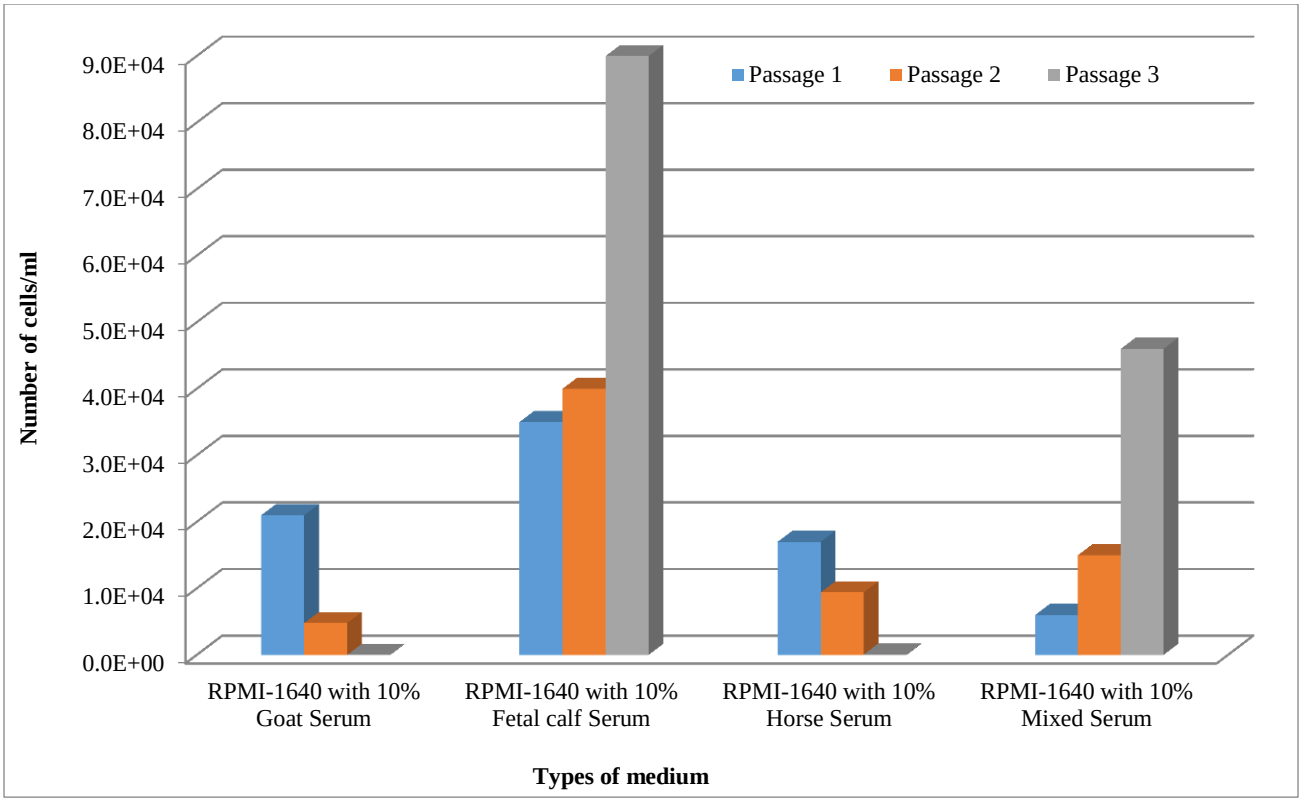


Figure 2. Growth of *Trypanosoma theileri* with RPMI 1640 in different serum types at 37°C with 5 %CO_2 in cell culture flask at ITMor two weeks

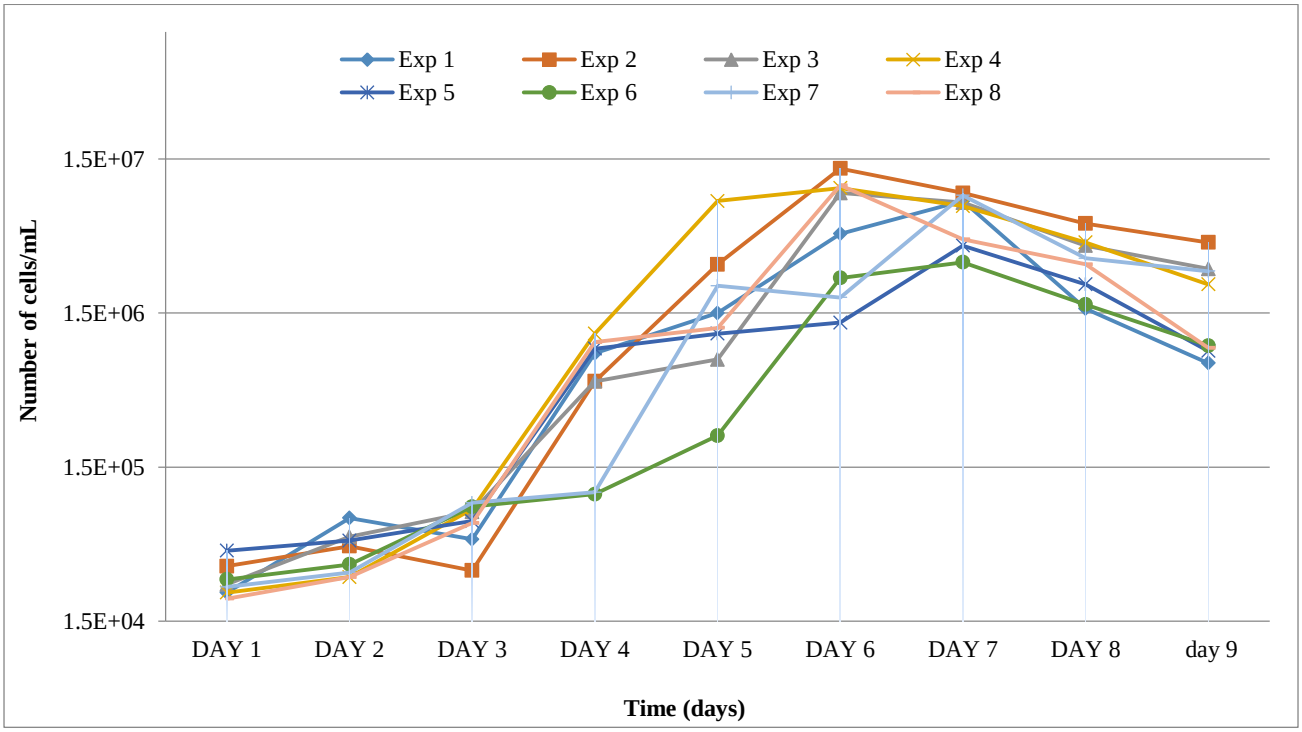


Figure 3. The growth pattern of *Trypanosoma theileri* in eight tissue culture flasks considered as experiments for nine days incubated in SDM 79at 26°C without CO_2. Exp: Experiment

To assess the morphology and the relative difference between other stages, Giemsa and DAPIstains (4′,6-diamidino-2-phenylindole) were performed on slides. Some of the slides were stained at ITM and some others in the Host Interaction lab, KU Leuven, Belgium (Figure 4).

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

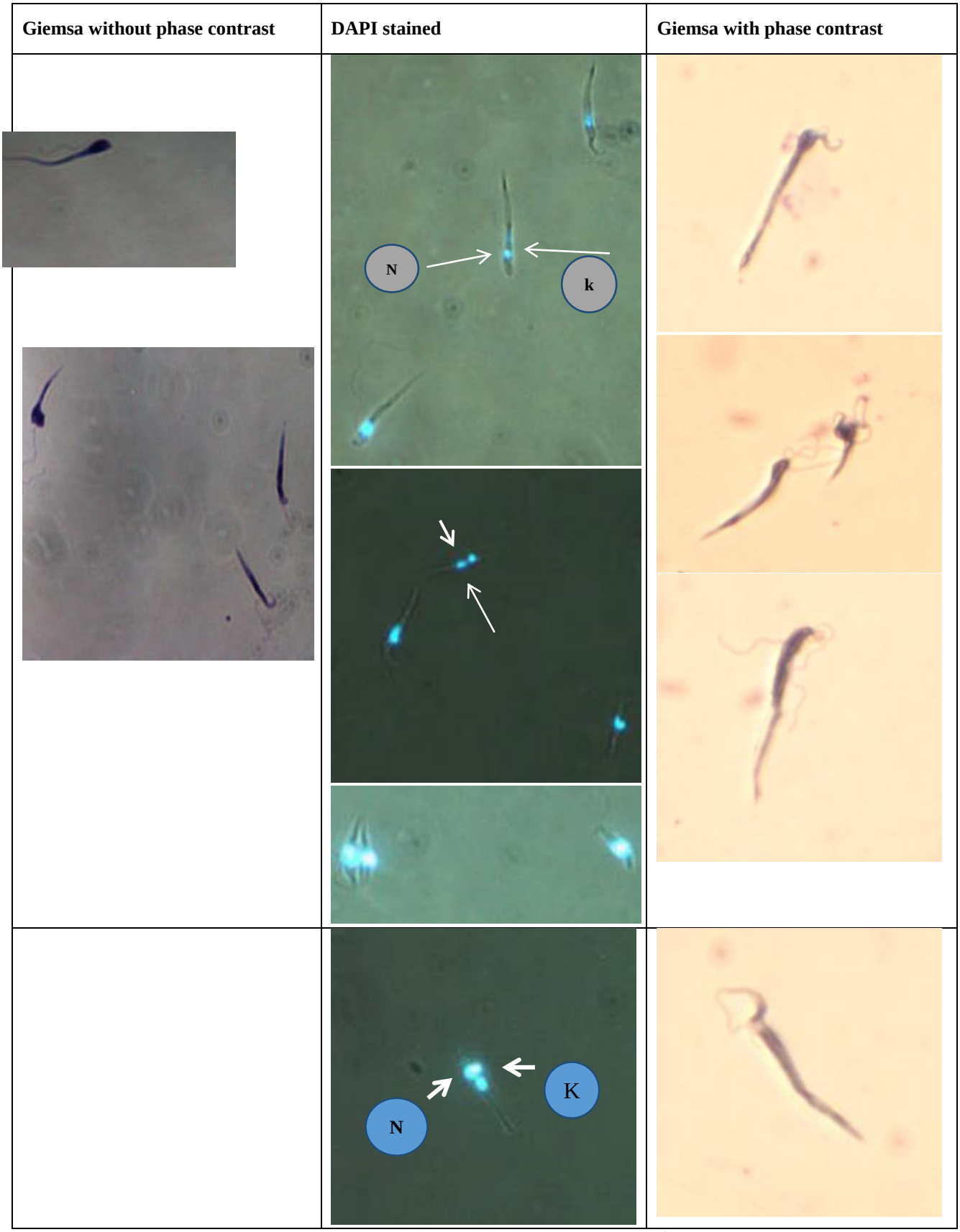


Figure 4. Light microscopy of *Trypanosoma theileri* epimastigotes cultured at 26°C (Giemsa and DAPI stained, 400 × magnifications). A kinetoplast is anterior to the nucleus, unlike a trypomastigote which has a kinetoplast posterior to the nucleus. N: Nucleus, K: Kinetoplast.

Resazurin assay

After optimization, *T. Theileri* was grown in microtiter plates and reached the level to produce a detectable fluorescence signal by incubation with 20 µL of each Resazurin solution. The magnitude of fluorescence increased remarkably up to7 hours, after which the fluorescence saturation occurred, particularly at 3.5×10^6 epimastigotes /mL, which had the highest density and then gradually decreased (Figure 5).

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

A statistically significant difference in the fluorescence of Resazurin solution at three concentrations (0.4 mM to 2.4 mM) was observed ($p < 0.05$) with an increase in the number of parasites and the incubation time (7 hours) during the assay (Figures 5 and 6). There was a positive correlation (r=0.75 to 0.925) between the magnitude of fluorescence of various cell densities and the three Resazurin concentrations (Table 6). However, the growth declined from 3.5×10^6 to 7.9×10^6 epimastigotes/mL, which indicated that the epimastigote was reaching the stationary phase. Hence, the upper limit of the assay was a plating density of 3.5×10^6 epimastigotes/ml (Figure5).

Trypanosoma theileri were seeded with 1.6×10^6 epimastigotes/ml, which resulted in a low level of fluorescence on the first reading (in the first hour after the addition of Resazurin) at the beginning of the assay process. Then, it gradually increased and a maximum signal was received seventh hours after adding Resazurin, and then significantly declined until the last reading (24 hours after adding Resazurin, Figure 6). Overall, the fluorescence signal of Resazurin solutions had a positive correlation (r = 0.75 to 0.925) with cell density and incubation time. There was a statistically significant difference between Resazurin fluorescence and the number of epimastigotes and also with reading time after incubation ($p < 0.05$, Table 6).

There was a positive, and linear correlation between the fluorescence and density of epimastigote at 1.6×10^6 cells/mL for a 2.4 mM Resazurin concentration at7hours of incubation (r = 0.8297), compared to the other two concentrations of Resazurin (Table 3). After incubation of 2.4 mM Resazurin at 26°C for 7 hours, a very high and linear association (r = 0.9876) in the range of 1.3×10^6 to 5.3×10^6 was observed in the fluorescence signal of the parasite (Table 3). For a comparative demonstration of the Resazurin based colorimetric assay, 1.8×10^6 epimastigotes/Ml (300 µL/well) with 2.4 mM (25 µL) of Resazurin was seeded in a Corning® 24-well culture plate (Sigma Aldrich) containing to appreciate the color changes from blue to pink. The test showed a gradual colorimetric change in the three concentrations of Resazurin and one control (Figure 8).

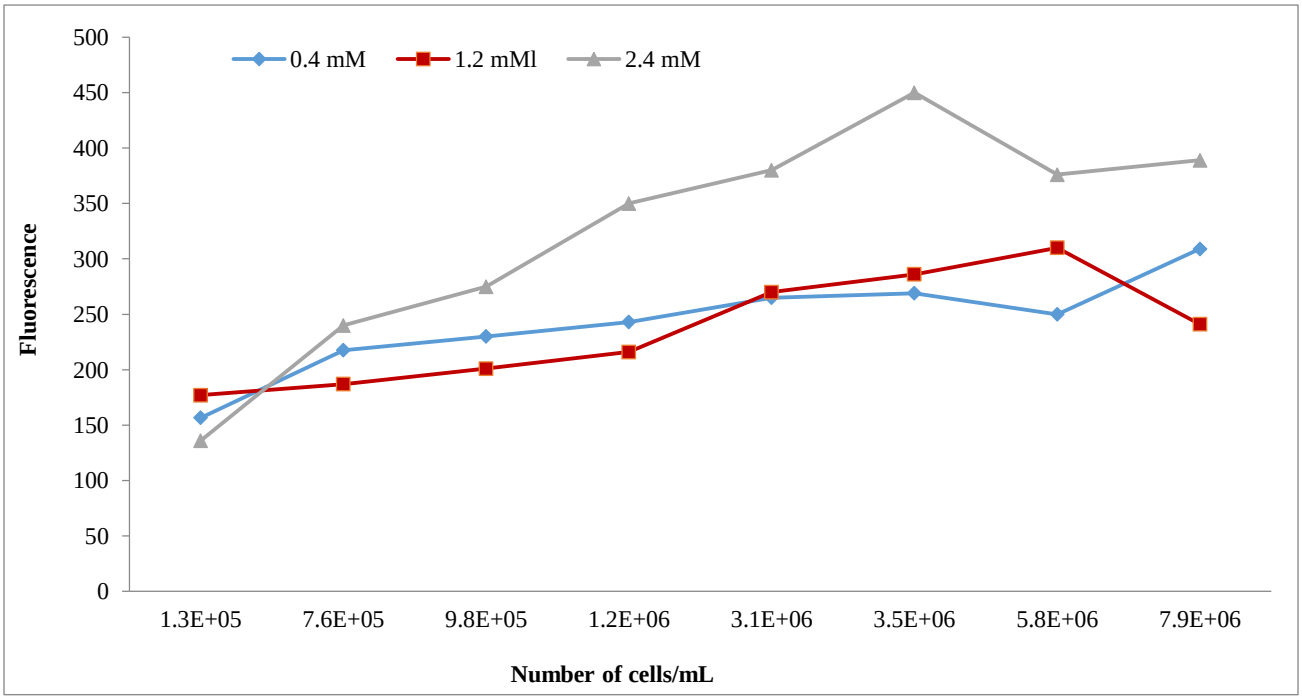


Figure 5. The relationship between the different fluorescence concentrations of Resazurin and the growth of epimastigote cultures after 7 hours of incubation time

Table 2. Statistical values of fluorescence for different cell concentrations concerning Resazurin concentrations

	Cells/ml and their respective fluorescence								r-value	P-value
Resazurin	$1.3X10^5$	$7.6X10^5$	$9.8X10^5$	$1.2X10^6$	$3.1X10^6$	$3.5X10^6$	$5.8X10^6$	$7.9X10^6$		
0.4 mM	156.63	217.6	230	243	280	292	268	290	0.752	0.0315
1.2 mM	145	187	201	216	255	310	298	341	0.925	0.0010
2.4 mM	184	240	275	341	380	489	450	490	0.865	0.0056

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

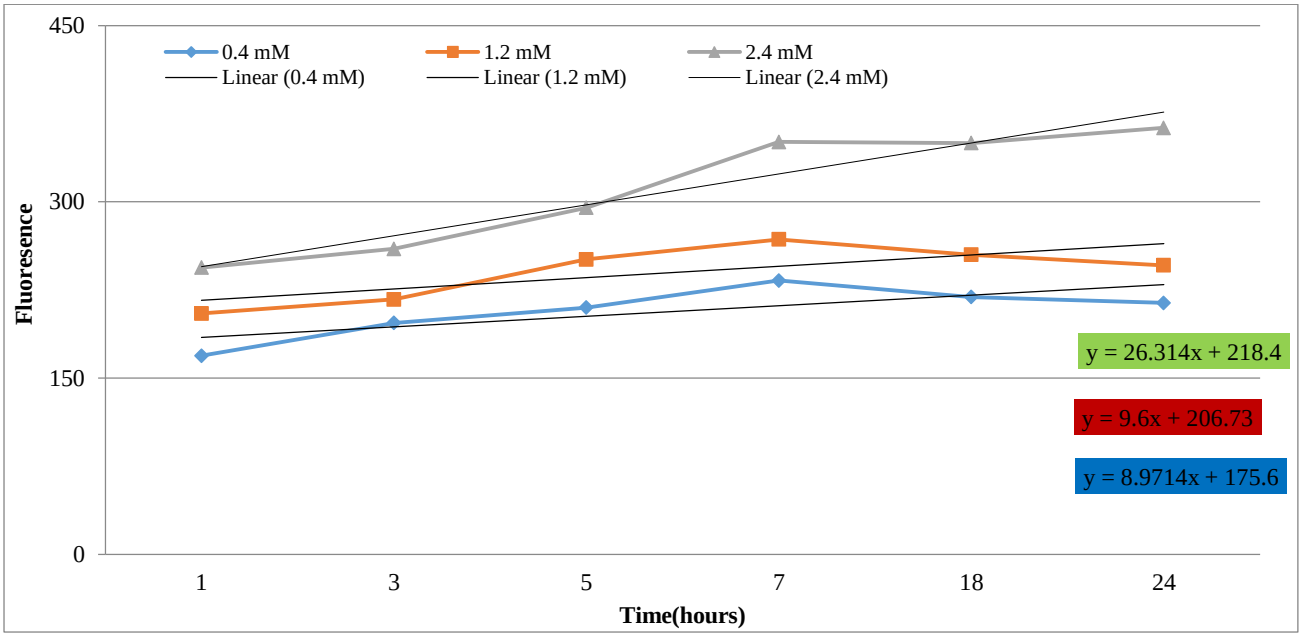


Figure 6. Fluorescence of Resazurin concerning incubation time and cell density of 1.6×10^6 epimastigotes/mL of *Trypanosoma theileri*. All experiments were performed two times each in three duplicates, and average values were taken.

Table 3. Correlation between fluorescence signal and an incubation time of *Trypanosoma theileri* epimastigote

	Time (Hours)						r -value	P -value
Resazurin	1	3	5	7	18	24		
0.4 mM	169	197	210	233	219	214	0.5066	0.3051
1.2 mM	205	217	251	268	257	246	0.4851	0.3295
2.4 mM	244	260	295	351	350	363	0.8297	0.0410

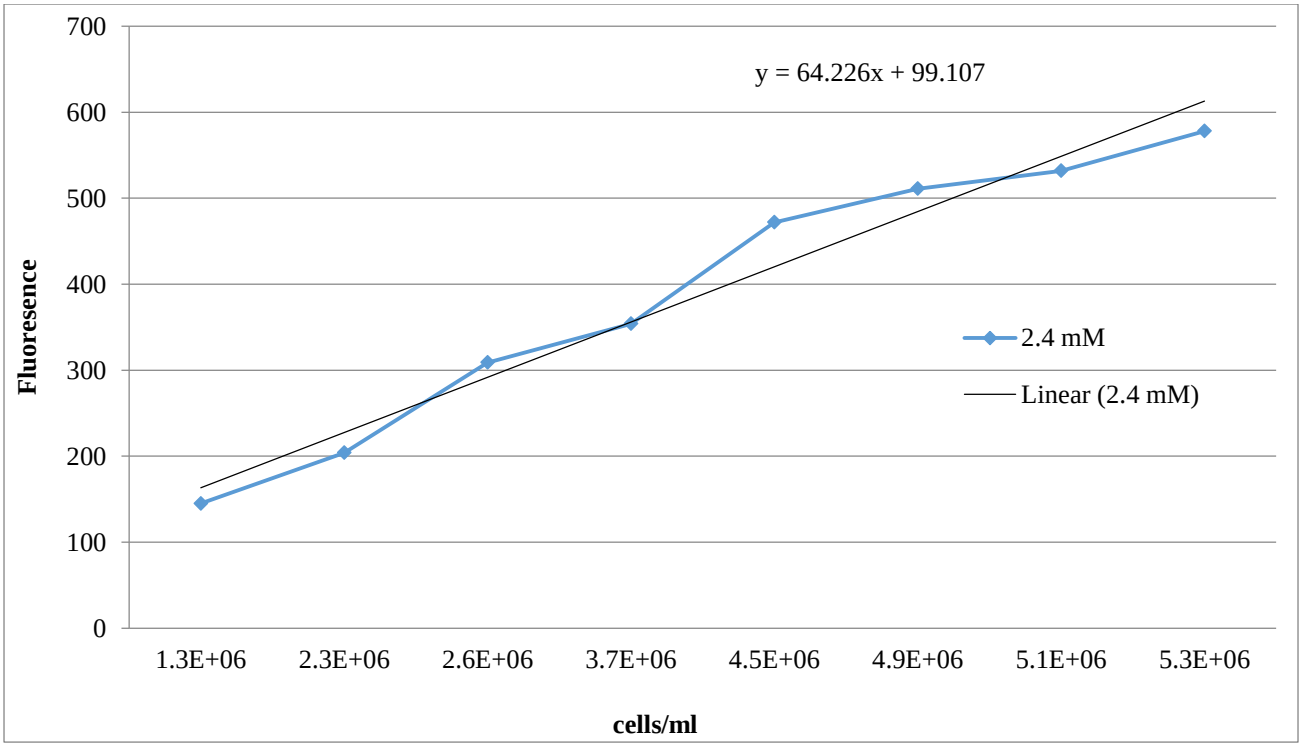


Figure 7. Standard curves for Resazurin fluorescence and the number of epimastigotes in the logarithmic growth phase, r = 0.9876, $p < 0.05$

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

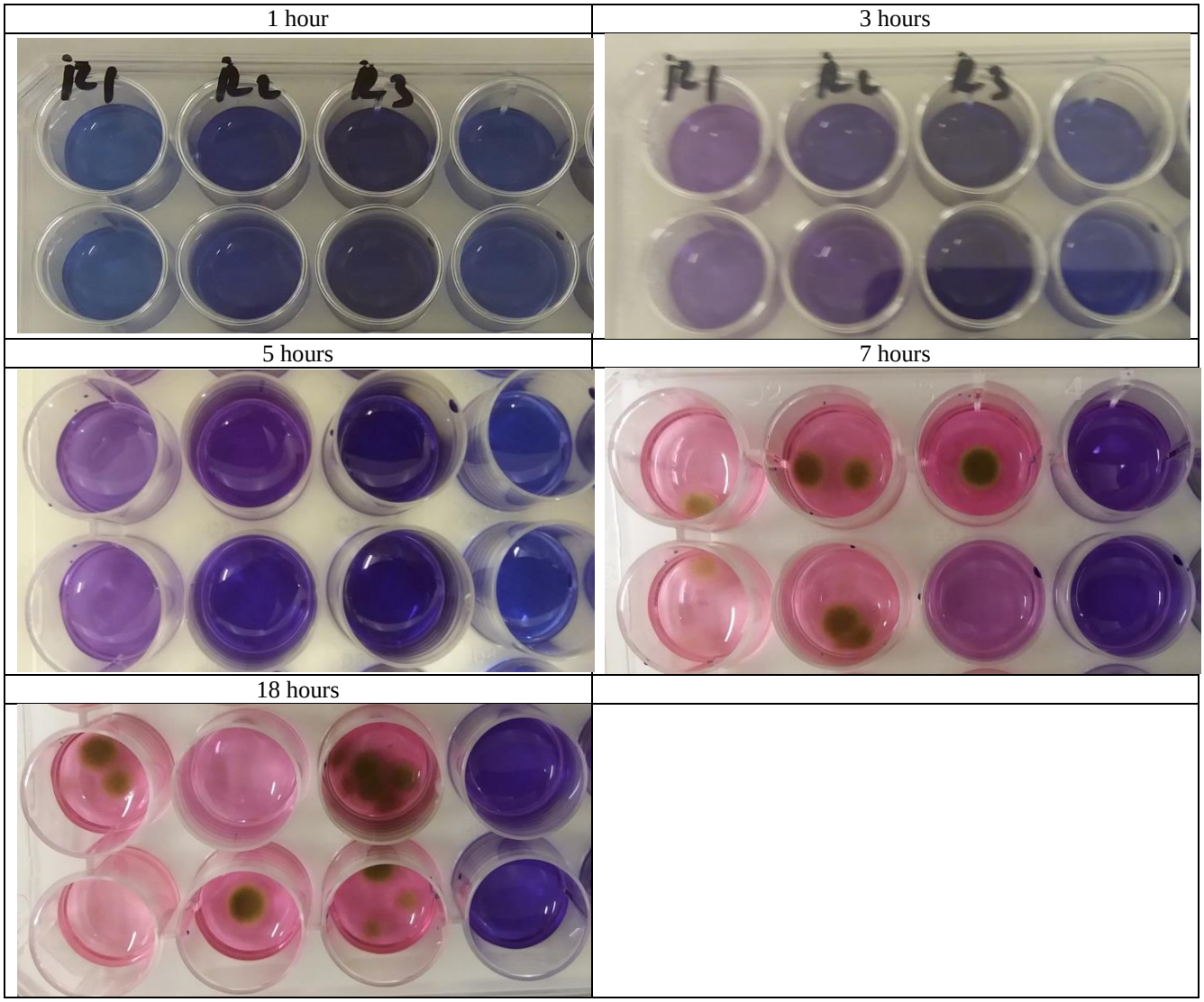


Figure 8. A plate to see the colorimetric changes of Resazurin (2.4 mM) in each reading time after incubation with pentamidine (80 ng/mL)

Drug sensitivity assays

Based on the optimized Resazurin assay for cell density, Resazurin concentration, and incubation period (Figure 5), Resazurin was deployed for the sensitivity of *T. theileri* epimastigote to pentamidine. Accordingly, Resazurin solution at a concentration of 2.4 mM was used to assess the response of *T. theileri* epimastigotes to different concentrations (1-80 ng/mL) of pentamidine (Figure 9). Therefore, there was a negative correlation (-0.8826) between the reduction in fluorescence signal and a significant increase in drug concentration ($p < 0.05$), as described in Figure 9and Table 4.

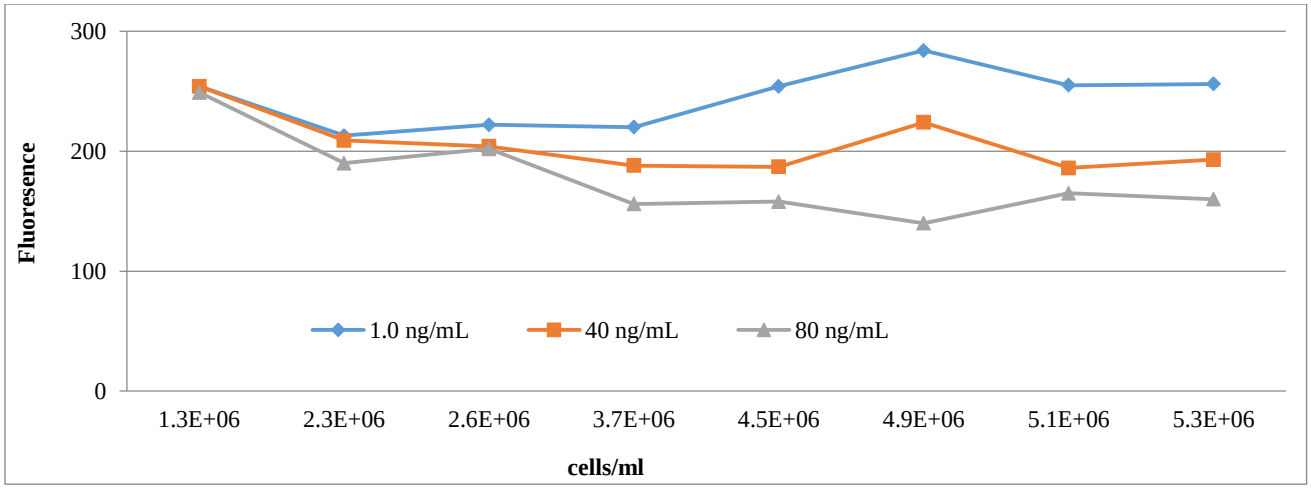


Figure 9. The reduction in fluorescence of epimastigotes of *Trypanosoma theileri* due to the pentamidine at different concentrations using 2.4 mM Resazurin solution

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

Table 4. Signals from Resazurin (2.4 mM) after addition of three different concentrations of pentamidine

	Cells/mL with its respective fluorescence								r value	P value
Pentamidine	1.3×10^6	2.3×10^6	2.6×10^6	3.7×10^6	4.5×10^6	4.9×10^6	5.1×10^6	5.3×10^6		
1ng/ml	254	213	222	220	254	284	255	256	0.533	0.1740
40ng/ml	254	209	204	188	187	224	186	193	-0.649	0.0817
80ng/ml	249	190	202	156	158	140	165	160	-0.8826	0.0037

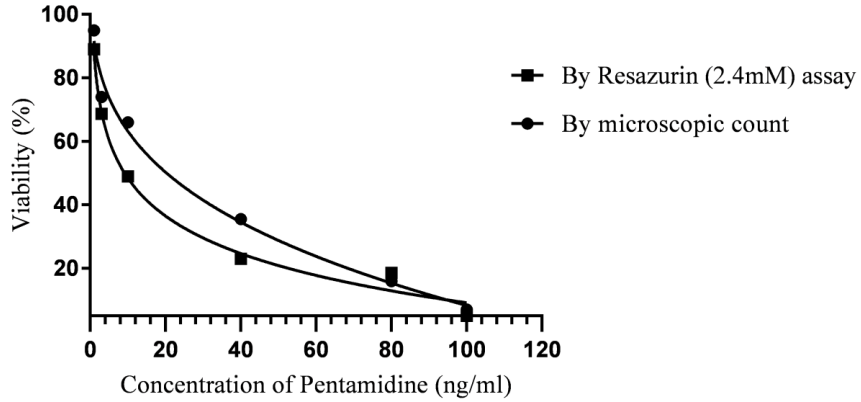


Figure 10. Viability of epimastigote after exposure to Pentamidine (1-100ng/mL) using Resazurin and microscopic counting

The role of Resazurin colorimetric assay was compared by calculating percentages of anti-epimastigotes (% AE) activity of pentamidine(1 to 100 ng/mL). For this purpose, a microscopic count was done and calculated based on Equation 1as described in materials and methods. As indicated in Figure 10, the Resazurin assay and microscopic count were compared. As a result, there was a significant reduction in the percentage of viability of the epimastigotes as the pentamidine concentration increased after 48 hours of contact time. IC_{50} values of 9.25ng/mL and 16.29 ng/mL were determined using Resazurin ($r = -0.957$; $p < 0.05$) and manual counting ($r = 0.90$, $p < 0.05$), respectively.

Experimental infection of *Trypanosoma theileri*

Before inoculating a calf with *T. theileri*, blood samples were taken to check whether there was a natural infection. No *T. theileri* was confirmed either in Giemsa stained slides or by culturing PBMC and buffy coat in RPMI 1640 and HMI 9 medium. The calf was inoculated intravenously through a jugular vein with a density of 4.5×10^6/mL to 7.3×10^6/mL in 5-8ml. Moreover, the same amount and concentration were administered orally. Experimental infections were performed three times.

PCR confirmation of *Trypanosoma theileri*

WithDNeasy®Blood and Tissue Kit (Qiagen, Hilden, Germany) a very low concentration of DNA could be achieved. As a result, a higher DNA concentration (16.5-274 µg/m) could be extracted using the phenol-chloroform method. Gradient PCR amplification using *T. theileri* specific PCR (Tth625-PCR) revealed the 465 bp amplification product (Figure 11). In addition, the full-length 18S ribosomal DNA sequence of *T. theileri* DNA was detected at 730 bp (Figure 12). They were stained with ethidium bromide in 1% agarose gel. A DNA ladder (100 bp) was used on the left side of the gel. The PCR amplification for the third primer (for annealing the Internal Transcribed Spacer (ITS) of the sequence of the ribosomal gene) was not included due to the poor image quality.

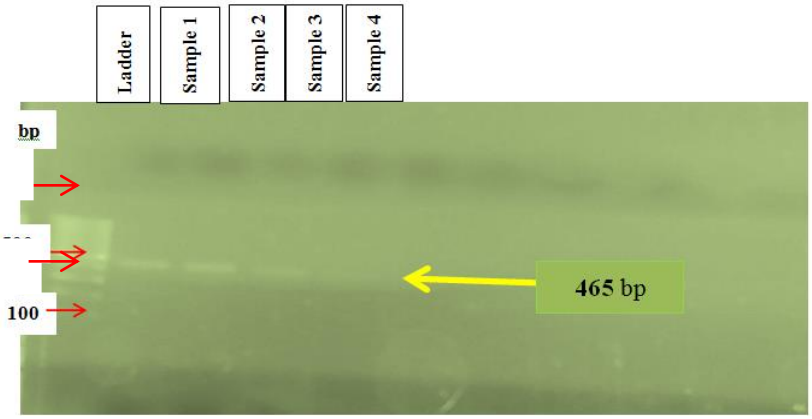


Figure 11. Detection of PCR of *Trypanosoma theileri* DNA from the cultured epimastigote using species-specific Tth625-PCR primers. The products were separated on a 1% agarose mini-gel

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

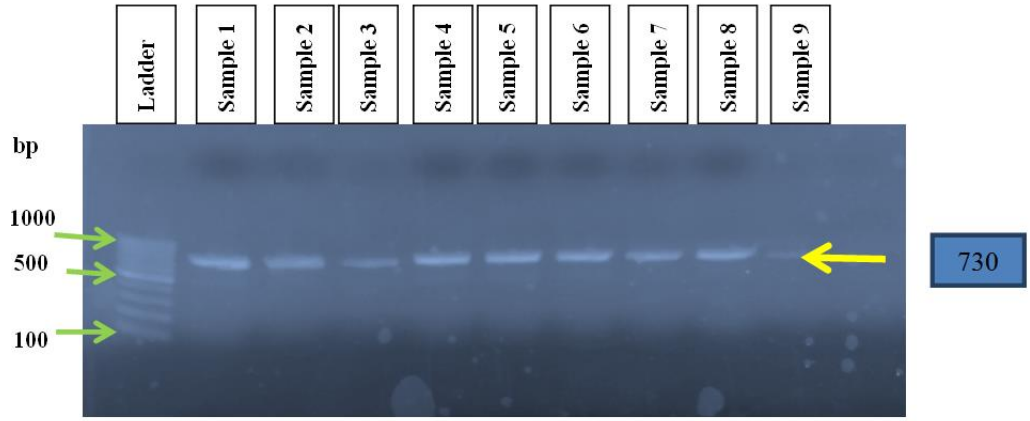


Figure 12. Detection of PCR of *T. theileri* of 18S ribosomal DNA from the cultured epimastigote using 18STnF2 and 18STnR3. The products were separated on a 1% agarose mini-gel

In silico analysis

Multiple Sequence Alignment of the sequences of commonly used trypanocidal drug targets (Figure 13) with *T. theileri* nucleotide found in the NCBI database was run. Multiple Sequence Alignment was performed at both the genomic (DNA) and protein levels. The Basic Local Alignment Search Tool (BLAST) and Clustal W program (from ExPASy bioinformatics resource portal) was run to demonstrate homology between *T. theileri* genome/ proteome and /or drug target sequences. Based on the determined protein/nucleotide sequence, phylogenetic trees were constructed to find out the possibility of evolutionary relationships. At the genomic (DNA) level, no significant similarity was found. However, to a lesser extent, a similarity of 294/392 (75%) was observed between *T.grayi* cathepsin L-like protein (CATL) gene (NCBI Gene bank accession No: XM_009318006.1) and HQ664735.1 of *T. theileri* isolate Tthb19 clone 3 cathepsin L-like protein (CATL) gene. The Cluster W alignment of these two similar genes from both species of *Trypanosome* was is described in Figure 14.

On the other hand, MSA among anti-trypanosome drug targets excluding *T. theileri* showed that there was above 99.85% identity among accession number of XM_824336.1(100%), J02771.1(99%), and AF042286 (99%). These listed accession numbers all indicated the Ornithine decarboxylase gene in *T.brucei*. In addition, the phylogenetic tree showed that these were similarities with accession Numbers: DQ887563.1 and XM_009318006.1, which both described the topoisomerase gene from *T.congolense* and the CATL gene from *T. grayi*, respectively (Figure 15).

CLUSTAL format alignment by MAFFT FFT-NS-i (v7.215)

```
XM_009318006.1  AACGGCGGCTTGATGGACGACGCCTTCACATGGATCATCCAGGACCACAACGGCACGGTG
HQ664735.1      AATGGCGGCTTGATGGACGACGCCTTCCAGTGGCTCGTGGATTCGAACAAGGGCAAGGTG
                **.************************  .*** **.*  *     **** **** ****

XM_009318006.1  GACACAGAGGCCAGCTACCCCTACGTCTCGGGCGCGGGCTACTCCCCGAAGTGCAGGACA
HQ664735.1      TACACGGAGAACAGCTATCCCTACGTCTCTGGCTCCGGTCAAACGCCGGCGTGCTCGACA
                 ****.***. ******.*********** *** * **..*  * ***. ****  ****

XM_009318006.1  GCTAGCCACGAGTTCGGCGCAGCCATCAGCGGCTACAATGACCTGCCGAATGATGAGGAC
HQ664735.1      AGTGAACATGAGGTTGGTGCGACAATCACCGGCTTTGTGGACTTGCCAAAAGATGAGGAC
                . *.. **.*** *.**.**..* **** ***** ..  ***.****.** *********

XM_009318006.1  AAGATGGCCGCGTGGCTGGCTGTCCACGGCCCCATTGCCATCGCCGTCGACGCCACCAGC
HQ664735.1      AAGATGGCGGCATGGCTTGCTACCAATGGCCCCATTGCTATCGCTGTCGACGCCAACAGC
                ******** **.***** ***..* *.**********.*****.********** ****

XM_009318006.1  TTCCAGTTCTACATGGGTGGCGTCCTGACGAACTGCATCTCTGAGCAGCTCGACCACGGG
HQ664735.1      TTTCTGTCGTACGTAAGTGGTGTTTTGACGAACTGTGAATCGGACCAGTTGAACCACGGT
                **.* **. ***.*..****.**..**********..  ** ** ***.* .*******

XM_009318006.1  GTGCTTCTTGTGGGCTACGACGACAGCAACAGCCCGCCGTACTGGATCATCAAGAACTCG
HQ664735.1      GTGCTTCTTGTCGGCTACGACGACAGCAGCAATCCACCGTACTGGATCATCAAGAAC---
                ********** ***************.**..**.********************
```

Figure 13. Cluster W alignment of *Trypanosoma theileri* (HQ664735.1) and *T.grayi* (XM_009318006.1) for the cathepsin L-like protein gene

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

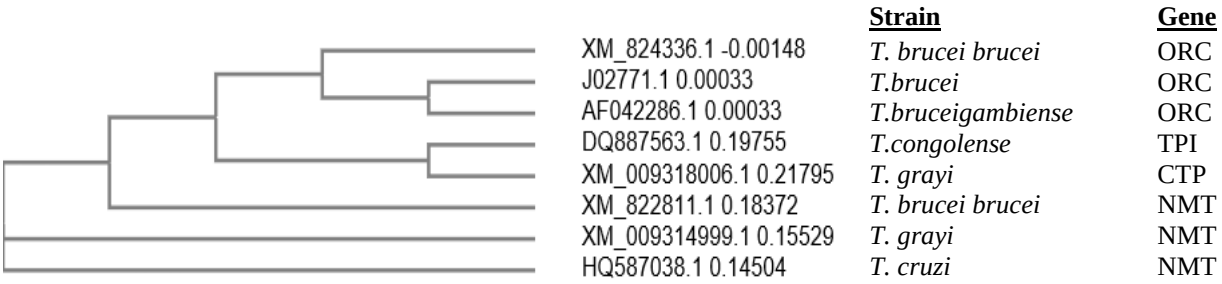


Figure 14. Multiple sequence alignments phylogenetic tree of the genome (DNA) of trypanosome showing relationships between common drug targets. ORC: Ornithine decarboxylase, TPI: Topoisomerase, CTP: Cathepsin L-like protein partial mRNA, NMT: N-myristoyl transferase

Table 5. Percentage of identity and divergence between common anti-trypanosomal drug targets identity

Divergence		1	2	3	4	5	6	7	8	
	1: XM_824336.1	100	99.93	99.85	44.01	41.48	40.87	44.4	42.34	1
	2: J02771.1	99.93	100	99.93	42.89	41.22	41.2	43.46	42.2	2
	3: AF042286.1	99.85	99.93	100	43.07	41.22	41.25	43.42	42.08	3
	4: DQ887563.1	44.01	42.89	43.07	100	58.45	43.5	44.17	43.38	4
	5: XM_009318006.1	41.48	41.22	41.22	58.45	100	41.52	43.44	40	5
	6: XM_822811.1	40.87	41.2	41.25	43.5	41.52	100	63.21	65.15	6
	7: XM_009314999.1	44.4	43.46	43.42	44.17	43.44	63.21	100	68.7	7
	8**:** HQ587038.1	42.34	42.2	42.08	43.38	40	65.15	68.7	100	8

```
XM_824336.1     TCTGTGAATTGTCTTGTAGCACAAACGGAGAAATCTATGGACATTGTCGTGAACGATGAC
J02771.1        TCTGTGAATTGTCTTGTAGCACAAACGGAGAAATCTATGGACATTGTCGTGAACGATGAC
AF042286.1      TCTGTGAATTGTCTTGTAGCACAAACGGAGAAATCTATGGACATTGTCGTGAACGATGAC
                ************************************************************

XM_824336.1     TTGAGTTGTCGCTTTCTTGAAGGGTTTAATACGAGGGATGCCCTCTGTAAAAAGATCAGT
J02771.1        TTGAGTTGTCGCTTTCTTGAAGGGTTTAATACGAGGGATGCCCTCTGTAAAAAGATCAGT
AF042286.1      TTGAGTTGTCGCTTTCTTGAAGGGTTTAATACGAGGGATGCCCTCTGTAAAAAGATCAGT
                ************************************************************

XM_824336.1     ATGAATACGTGTGACGAAGGTGATCCGTTTTTTGTTGCCGATCTCGGGGACATTGTAAGG
J02771.1        ATGAATACGTGTGACGAAGGTGATCCGTTTTTTGTTGCCGATCTCGGGGACATTGTAAGG
AF042286.1      ATGAATACGTGTGACGAAGGTGATCCGTTTTTTGTTGCCGATCTCGGGGACATTGTAAGG
                ************************************************************

XM_824336.1     AAGCACGAAACATGGAAAAAATGCCTTCCCCGCGTCACGCCGTTTTACGCGGTCAAATGC
J02771.1        AAGCACGAAACATGGAAAAAATGCCTTCCCCGCGTCACGCCGTTTTACGCGGTCAAATGC
AF042286.1      AAGCACGAAACATGGAAAAAATGCCTTCCCCGCGTCACGCCGTTTTACGCGGTCAAATGC
                ************************************************************

XM_824336.1     AACGATGACTGGCGCGTACTTGGAACGCTGGCGGCTCTCGGCACGGGATTTGATTGTGCT
J02771.1        AACGATGACTGGCGCGTACTTGGAACGCTGGCGGCTCTCGGCACGGGATTTGATTGTGCT
AF042286.1      AACGATGACTGGCGCGTACTTGGAACGCTGGCGGCTCTCGGCACGGGATTTGATTGTGCT
                ************************************************************

XM_824336.1     AGCAACACTGAGATACAACGTGTGAGAGGCATTGGTGTGCCACCGGAAAAAATAATATAT
J02771.1        AGCAACACTGAGATACAACGTGTGAGAGGCATTGGTGTGCCACCGGAAAAAATAATATAT
AF042286.1      AGCAACACTGAGATACAACGTGTGAGAGGCATTGGTGTGCCACCGGAAAAAATAATATAT
                ************************************************************

XM_824336.1     GCGAACCCTTGTAAACAAAATTCACACATACGGTACGCGCGTGATAGCGGCGTTGATGTC
J02771.1        GCGAACCCTTGTAAACAAATTTCACACATACGGTACGCGCGTGATAGCGGCGTTGATGTC
AF042286.1      GCGAACCCTTGTAAACAAATTTCACACATACGGTACGCGCGTGATAGCGGCGTTGATGTC
                ******************* ****************************************

XM_824336.1     ATGACATTTGATTGCGTGGATGAACTGGAAAAGGTCGCTAAAACGCATCCAAAGGCAAAG
J02771.1        ATGACATTTGATTGCGTGGATGAACTGGAAAAGGTCGCTAAAACGCATCCAAAGGCAAAG
AF042286.1      ATGACATTTGATTGCGTGGATGAACTGGAAAAGGTCGCTAAAACGCATCCAAAGGCAAAG
                ************************************************************

XM_824336.1     ATGGTATTAAGAATTTCTACGGATGATTCGTTGGCTCGATGCCGTCTCAGTGTGAAGTTT
J02771.1        ATGGTATTAAGAATTTCTACGGATGATTCGTTGGCTCGATGCCGTCTCAGTGTGAAGTTT
AF042286.1      ATGGTATTAAGAATTTCTACGGATGATTCGTTGGCTCGATGCCGTCTCAGTGTAAAGTTT
                ***********************************************.******
```


To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

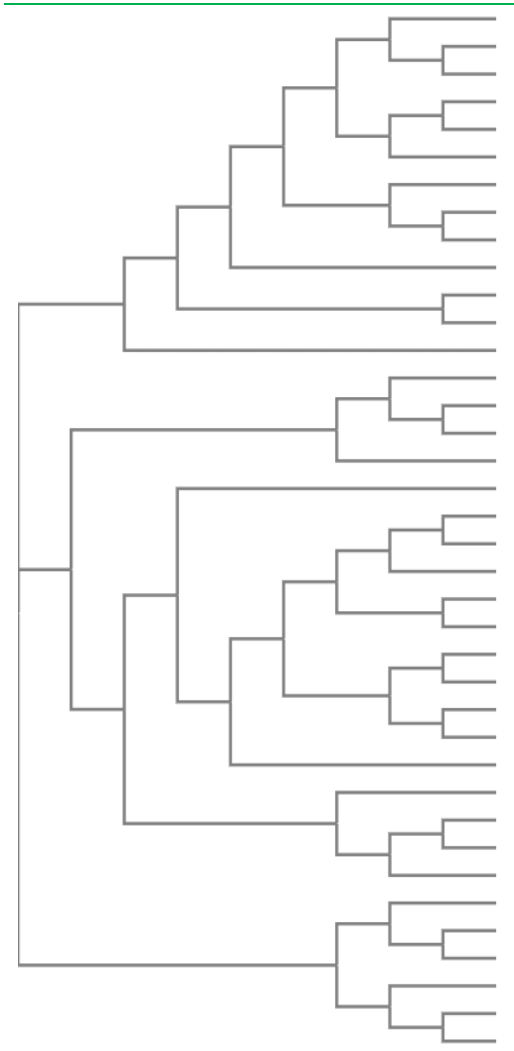
```
XM_824336.1     GGTGCAAAGGTGGAAGACTGTAGGTTTATCTTGGAGCAGGCAAAGAAACTGAATATCGAC
J02771.1        GGTGCAAAGGTGGAAGACTGTAGGTTTATCTTGGAGCAGGCAAAGAAACTGAATATCGAC
AF042286.1      GGTGCAAAGGTGGAAGACTGTAGGTTTATCTTGGAGCAGGCAAAGAAACTGAATATCGAC
                ************************************************************

XM_824336.1     GTCACTGGTGTGAGTTTTCACGTGGGAAGCGGATCTACAGATGCCTCTACCTTCGCTCAA
J02771.1        GTCACTGGTGTGAGTTTTCACGTGGGAAGCGGATCTACAGATGCCTCTACCTTCGCTCAA
AF042286.1      GTCACTGGTGTGAGTTTTCACGTGGGAAGCGGATCTACAGATGCCTCTACCTTCGCTCAA
                ************************************************************

XM_824336.1     GCCATATCTGACTCCCGTTTCGTTTTCGACATGGGTACTGAGCTTGGGTTCAATATGCAC
J02771.1        GCCATATCTGACTCCCGTTTCGTTTTCGACATGGGTACTGAGCTTGGGTTCAATATGCAC
AF042286.1      GCCATATCTGACTCCCGTTTCGTTTTCGACATGGGTACTGAGCTTGGGTTCAATATGCAC
                ************************************************************

XM_824336.1     ATTCTTGATATCGGTGGTGGGTTTCCAGGGACGAGGGATGCACCACTTAAATTTGAAGAG
J02771.1        ATTCTTGATATCGGTGGTGGGTTTCCAGGGACGAGGGATGCACCACTTAAATTTGAAGAG
AF042286.1      ATTCTTGATATCGGTGGTGGGTTTCCAGGGACGAGGGATGCACCACTTAAATTTGAAGAG
                ************************************************************

XM_824336.1     ATTGCTGGTGTCATCAACAATGCGCTGGAAAAACATTTTCCACCTGACCTCAAGCTTACC
J02771.1        ATTGCTGGTGTCATCAACAATGCGCTGGAAAAACATTTTCCACCTGACCTCAAGCTTACC
AF042286.1      ATTGCTGGTGTCATCAACAATGCGCTGGAAAAACATTTTCCACCTGACCTCAAGCTTACC
                ************************************************************

XM_824336.1     ATTGTTGCCGAGCCGGGAAGGTACTACGTTGCTTCAGCTTTCACACTTGCCGTAAATGTT
J02771.1        ATTGTTGCCGAGCCGGGAAGGTACTACGTTGCTTCAGCTTTCACACTTGCCGTAAATGTT
AF042286.1      ATTGTTGCCGAGCCGGGAAGGTACTACGTTGCTTCAGCTTTCACACTTGCCGTAAATGTT
                ************************************************************

XM_824336.1     ATTGCCAAGAAGGTGACACCAGGGGTTCAGACCGACGTCGGTGCCCATGCTGAATCAAAC
J02771.1        ATTGCCAAGAAGGTGACACCAGGGGTTCAGACCGACGTCGGTGCCCATGCTGAATCAAAC
AF042286.1      ATTGCCAAGAAGGTGACACCAGGGGTTCAGACCGACGTCGGTGCCCATGCTGAATCAAAC
                ************************************************************

XM_824336.1     GCACAGAGTTTTATGTATTATGTGAATGATGGCGTGTATGGTTCATTTAATTGCATCCTG
J02771.1        GCACAGAGTTTTATGTATTATGTGAATGATGGCGTGTATGGTTCATTTAATTGCATCCTG
AF042286.1      GCACAGAGTTTTATGTATTATGTGAATGATGGCGTGTATGGTTCATTTAATTGCATCCTG
                ************************************************************

XM_824336.1     TATGACCACGCAGTCGTCAGGCCTTTGCCCCAGAGGGAGCCAATCCCCAATGAAAAGCTC
J02771.1        TATGACCACGCAGTCGTCAGGCCTTTGCCCCAGAGGGAGCCAATCCCCAATGAAAAGCTC
AF042286.1      TATGACCACGCAGTCGTCAGGCCTTTGCCCCAGAGGGAGCCAATCCCCAATGAAAAGCTC
                ************************************************************

XM_824336.1     TATCCCTCAAGTGTATGGGGTCCCACATGTGATGGTCTTGATCAGATAGTTGAACGATAC
J02771.1        TATCCCTCAAGTGTATGGGGTCCCACATGTGATGGTCTTGATCAGATAGTTGAACGATAC
AF042286.1      TATCCCTCAAGTGTATGGGGTCCCACATGTGATGGTCTTGATCAGATAGTTGAACGATAC
                ************************************************************

XM_824336.1     TATCTTCCCGAGATGCAAGTGGGGGGAATGGCTGCTCTTTGAGGATATGGGTGCCTACACG
J02771.1        TATCTTCCCGAGATGCAAGTGGGGGGAATGGCTGCTCTTTGAGGATATGGGTGCCTACACG
AF042286.1      TATCTTCCCGAGATGCAAGTGGGGGGAATGGCTGCTCTTTGAGGATATGGGTGCCTACACG
                ************************************************************

XM_824336.1     GTCGTAGGAACTTCTTCCTTTAATGGATTCCAGAGTCCGACTATTTACTATGTAGTCTCC
J02771.1        GTCGTAGGAACTTCTTCCTTTAATGGATTCCAGAGTCCGACTATTTACTATGTAGTCTCC
AF042286.1      GTCGTAGGAACTTCTTCCTTTAATGGATTCCAGAGTCCGACTATTTACTATGTAGTCTCC
                ************************************************************

XM_824336.1     GGGCTACCAGACCATGTTGTCCGGGAGTTGAAAAGTCAAAAATCATAA------------
J02771.1        GGGCTACCAGACCATGTTGTCCGGGAGTTGAAAAGTCAAAAATCATAAATGGAAGCGAAG
AF042286.1      GGGCTACCAGACCATGTTGTCCGGGAGTTGAAAAGTCAAAAATCATAAATGGAAGCGAAG
                ********************************************
```

Figure 15. Cluster W alignment between the three homologous sequences of anti-trypanosome drug targets

Furthermore, apart from the genomic level (DNA), MSA was conducted at the protein level. After the BLAST and Cluster W analysis of protein sequences of drug targets with *T. theileri* proteome, which was found in NCBI databases, these drug targets showed a 12/17 (71%) homology with hypothetical *T. theileri* proteins (TM35) and pyruvate kinase (Figures 17 and 18).

On the other hand, among the drug targets themselves, the highest identity was detected in accession number ofXP_829429.1 (445/445(100%)), which indicates ornithine decarboxylase protein (Figure 19). Furthermore, there was homology with AAD02222.1 and AAA30219.1, which encode the same protein from *T.grayi* and *T.brucei*, respectively. The MSA and the phylogenetic trees witnessed the homology of these sequences.

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47



	Target protein	Species/strain
XP_829429.1 0.00049	Ornithine decarboxylase	TB
AAD02222.1 -0.00118	Ornithine decarboxylase	TG
AAA30219.1 0.00118	Ornithine decarboxylase	TB
ORC93851.1 0.37181	**Dihydroxy-3-ketomethyldioxygenase**	**TT**
ORC93850.1 0.37127	Hypothetical protein TM35_000017270	TT
ORC93848.1 0.37177	**Hypothetical protein TM35_000017250**	**TT**
XP_827904.1 0.18659	N-myristoyl transferase	TB
XP_009313274.1 0.1557	N-myristoyl transferase	TG
AEP82716.1 0.14652	N-myristoyl transferase partial	TC
ORC93856.1 0.40966	Hypothetical Protein TM35_000017330	TT
ORC93859.1 0.41854	Hot spot (RHS) protein, partial	TT
ORC93840.1 0.42146	Hypothetical protein TM35_000017170	TT
ORC93853.1 0.41573	Hypothetical protein TM35_000017310	TT
ABI96917.1 0.39109	**Topoisomerase**	**TC**
ORC93854.1 0.36738	**Hypothetical protein TM35_000017310**	**TT**
ORC93843.1 0.31262	Hypothetical protein TM35_000017200	TT
ORC93842.1 0.41766	Coatomer alpha subunit	TT
ORC93849.1 0.3919	Hypothetical protein TM35_000017260	TT
XP_009309990.1 0	Pyruvate kinase 2	TG
KEG11772.1 0	Pyruvate kinase 2	TG
ORC92589.1 0.05295	**Pyruvate kinase 2**	**TT**
ESL09806.1 0.05353	Pyruvate kinase 2	TR
3QV9_B 0.05068	Pyruvate Kinase(Tcpyk)	TC
CCC94421.1 0.05016	Pyruvate kinase 1	TC
CCC52316.1 0.03772	Pyruvate kinase 1, partial	TV
EAN78782.1 0	Pyruvate kinase 1	TB
XP_827894.1 0	Pyruvate kinase 1	TB
ORC93858.1 0.38152	Hot spot (RHS) protein, partial	TT
ORC93855.1 0.4241	Hypothetical protein TM35_000017320	TT
XP_009316281.1 0.3720	Cathepsin L-like protein	TG
ORC93844.1 0.37795	Proteasome beta 7 subunit	TT
ORC93841.1 0.40854	Alkylated DNA repair protein	TT
CAA44870.1 0.37945	Trypanothione reductase	TB
ORC93857.1 0.2975	**Hypothetical Protein TM35_000017340**	**TT**
ORC93847.1 0.31333	RNA-binding protein	TT
ORC93852.1 0.40894	Hypothetical protein TM35_000017300	TT
ORC93845.1 0.36971	Phosphatidylinositol 3-kinase tor	TT
ORC93846.1 0.38247	Cell differentiation protein	TT

Figure 16. Phylogenetic tree following MSA of the drug targets and *Trypanosoma theileri.* TB: *T. brucei*, TG: *T. grayi*, TT: *T. theileri* , TC: *T. cruzi*, TR: *T. rangeli*, TV: *T. vivax*, TC: *T.congolense*

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

```
XP_009309990.1  GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQATINNVRTAAAELGLHIGIALDTK
3720
KEG117721       GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQATINNVRTAAAELGLHIGIALDTK
3720
ORC925891       GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQTTINNVRTAAAELGMHIGIALDTK
3720
CCC944211       GPSTQSVEALKGLMKSGMSVARMNFSHGSYE—YHQTTINNVRAAAAELGLHIGIALDTK
3720
CCC523161       GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQTTIKNVRQAAAELGLHIGIALDTK
3720
ESL098061       GPSTQSIEALRSLIKSGMSVARMNFSHGSHE—YHQTTINNVRAASAELGVHIGIALDTK
3720
3QV9_B          GPSTQSVEALKGLIRSGMSVARMNFSHGSHE—YHQTTINNLRAAATELGAHIGLALDTK
3720

XP_0093099901   GPEIRTGLFVGGEAVL-NPGDTVFVTTDPAFE----------KKGTKEKFYVDYPRLAT
3780
KEG117721       GPEIRTGLFVGGEAVL-NPGDTVFVTTDPAFE----------KKGTKEKFYVDYPRLAT
3780
ORC925891       GPEIRTGLFVGGEAIL-MTGDTVLVTTDPAFE----------KTGTKEKFYIDYPRLAT
3780

XP_0093099901   ----FHRLTDRKGCNLPGCDVDLPAVSAKDREDLKFGVEQGVDIIFASFIRTAEQVQEVR
3850
KEG117721       ----FHRLTDRKGCNLPGCDVDLPAVSAKDREDLKFGVEQGVDIIFASFIRTAEQVQEVR
3850
ORC925891       ----GHRLTDRKGCNLPGCEVDLPAVSAKDREDLKFGVEQGVDMIFASFIRTAEQVREVR
3850
CCC944211       ----HHRLTDRKGINLPGCEVDLPAVSEKDRKDLQFGVEQGVDMIFASFIRTADQVREVR
3850
CCC523161       ----HHRLTDRKGCNLPGCDVELPAVSEKDRKDLIFGVEQGVDMIFASFIRTAEQVREVR
3850
ESL098061       ----AHYLTDRKGCNLPGCEVDLPAVSEKDREDLKFGVEQGVDMIFASFIRTAEQVREVR
3850
3QV9_B          ----AHFLTDRKGCNLPGCEVDLPAVSEKDREDLKFGVEQGIDMVFASFIRTAEQVQEVR
3850
EAN787821       ----HHRLTDRRGINLPGCEVDLPAVSEKDRKDLEFGVAQGVDMIFASFIRTAEQVREVR
3850
XP_8278941      ----HHRLTDRRGINLPGCEVDLPAVSEKDRKDLEFGVAQGVDMIFASFIRTAEQVREVR
3850

KEG117721       AQMMLISKCNVAGK------------------PVICATQMLESMTANPRP---TRAEVSD
3970
ORC925891       AQMTLISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
CCC944211       AQMCIISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVTD
3970
CCC523161       AQMCIISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
ESL098061       AQMILISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
3QV9_B          AQMILISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
EAN787821       AQMCIISKCNVVGK------------------PVICATQMLESMTSNPRP---TRAEVSD
3970
XP_8278941      AQMCIISKCNVVGK------------------PVICATQMLESMTSNPRP---TRAEVSD
3970
```


To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.,* 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

```
XP_0093099901   VANAVFNG-----------ADCVMLSGETAKGKYPNEVVRYMARICVEAQSATNQ-----
4010
KEG117721       VANAVFNG-----------ADCVMLSGETAKGKYPNEVVRYMARICVEAQSATNQ-----
4010
ORC925891       VANAVFNG-----------ADCVMLSGETAKGHYPNEVVQYMARICVVAQSATNQ-----
4010
CCC944211       VANAVFNG-----------ADCVMLSGETAKGKYPNEVVQYMVRICIEAQSATHD-----
4010

ESL098061       VANAVFNG-----------ADCVMLSGETAKGKYPSEVVQYMARICVEAQSATNQ-----
4030
3QV9_B          VANAVFNG-----------ADCVMLSGETAKGKYPNEVVQYMARICLEAQSATNQ-----
4030
EAN787821       VANAVLNG-----------ADCVMLSGETAKGKYPNEVVQYMARICVEAQSATHD-----
4030
XP_8278941      VANAVLNG-----------ADCVMLSGETAKGKYPNEVVQYMARICVEAQSATHD-----
4030

XP_0093099901   SNSGRSARLTSKYRPDCPIICVTTRMRTCRQ---------------------LNVTRSVE
4150
KEG117721       SNSGRSARLTSKYRPDCPIICVTTRMRTCRQ---------------------LNVTRSVE
4150
ORC925891       SNSGRSARLASKYRPNCPIICATTRMRTCRQ---------------------LNITQSVE
4150
CCC944211       SNTGRSARLISKYRPNCPIICATTRLLTCRQ---------------------LNVTRSVE
4150

AAD022221       GPTCDGLDQIVERYYLPEMQVGEWLLFEDMGAYTVVGTS---SFNGFQSPTIYYVVSGLP
4210
AAA302191       GPTCDGLDQIVERYYLPEMQVGEWLLFEDMGAYTVVGTS---SFNGFQSPTIYYVVSGLP
4210
XP_8294291      GPTCDGLDQIVERYYLPEMQVGEWLLFEDMGAYTVVGTS---SFNGFQSPTIYYVVSGLP
4210

XP_0093099901   SVFYDAERCGADEDKENRVQLG--VESAKKKGYVVPG-------DIVVAVHADHKVKGYP
4210
KEG117721       SVFYDAERCGADEDKENRVQLG--VESAKKKGYVVPG-------DIVVAVHADHKVKGYP
4210
ORC925891       SVFYDAERYGPDDDKENRVQLG--VEFAKKKGYVVPG-------DVMVVVHADHKVKGYP
4210
CCC944211       SVYYDVDAHGEDNDREKRVQLG--VDWAKTKGYVSAG-------DVMVIVHADHSVKGYP
4210
```

Figure 17. Alignment of drug targets with *Trypanosoma theileri* (XP_0093099901) protein found in the NCBI database

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.,* 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

```
AAD022221       -------EIQRVRGIGVP-PEKIIYANPCKQISHIR---------------------- 550
AAA302191       -------EIQRVRGIGVP-PEKIIYANPCKQISHIR----------------------
XP_8294291      -------EIQRVRGIGVP-PEKIIYANPCKQNSHIR----------------------
ESL098061       -------YLTDRKGCNLPGCEVDLPAVSEKDREDLK-----------------------
3QV9_B          -------FLTDRKGCNLPGCEVDLPAVSEKDREDLK-----------------------
XP_0093099901   -------RLTDRKGCNLPGCDVDLPAVSAKDREDLK-----------------------
KEG117721       -------RLTDRKGCNLPGCDVDLPAVSAKDREDLK-----------------------
CCC523161       -------RLTDRKGCNLPGCDVELPAVSEKDRKDLI-----------------------
EAN787821       -------RLTDRRGINLPGCEVDLPAVSEKDRKDLE-----------------------
XP_8278941      -------RLTDRRGINLPGCEVDLPAVSEKDRKDLE-----------------------

AAD022221       --YARDSGVDVMTFDCVDELEKVAKTHPKAKMVLR-ISTDDSLARCRLSVKFGAKVEDCR 610
AAA302191       --YARDSGVDVMTFDCVDELEKVAKTHPKAKMVLR-ISTDDSLARCRLSVKFGAKVEDCR
XP_8294291      --YARDSGVDVMTFDCVDELEKVAKTHPKAKMVLR-ISTDDSLARCRLSVKFGAKVEDCR
ESL098061       --FGVEQGVDMIFASFIRTAEQVREVRAALGEKGKDILVISKIENHQGVQNIDAIIEASD
3QV9_B          --FGVEQGIDMVFASFIRTAEQVQEVREALGEKGKDILIISKIENHQGVQNIDGIIEASD
XP_0093099901   --FGVEQGVDIIFASFIRTAEQVQEVRAALGEKGKDTLVISKIENHQGVQNIDGIIAVSD
KEG117721       --FGVEQGVDIIFASFIRTAEQVQEVRAALGEKGKDTLVISKIENHQGVQNIDGIIAVSD
CCC523161       --FGVEQGVDMIFASFIRTAEQVREVRAVLGEKGKDTMIISKIENHQGVQNIDAIIEASD
EAN787821       --FGVAQGVDMIFASFIRTAEQVREVRAALGEKGKDILIISKIENHQGVQNIDSIIEASN
XP_8278941      --FGVAQGVDMIFASFIRTAEQVREVRAALGEKGKDILIISKIENHQGVQNIDSIIEASN
CCC944211       --FGVEQGVDMIFASFIRTADQVREVRAALGEKGKDTLIISKIENHQGVQNIDAIIEASD
XP_0093132741   --ENHLEPRIICEINFLCVHKQLREKRMAPILIQEVTRRVNLLNIWQAIYTAGALLPTP-
AEP827161       --EXYLEPRKICEINFLCVHKLLRAKRLAPILIKEVTRRVHLMNIWQAVYTAGRLLPTP-

AAD022221       F--ILEQAKKLNIDVTGVSFHVGSGSTDASTFAQ--------------AISDSRFVFDMG 670
AAA302191       F--ILEQAKKLNIDVTGVSFHVGSGSTDASTFAQ--------------AISDSRFVFDMG
XP_8294291      F--ILEQAKKLNIDVTGVSFHVGSGSTDASTFAQ--------------AISDSRFVFDMG
ESL098061       G--IMVARGDLGVEIAAEKVVVAQMILISKCNVAG-----------KPVICATQMLESMT
3QV9_B          G--IMVARGDLGVEIPAEKVVVAQMILISKCNVAG-----------KPVICATQMLESMT
XP_0093099901   G--IMVARGDLGVEIPAEKVVVAQMMLISKCNVAG-----------KPVICATQMLESMT
KEG117721       G--IMVARGDLGVEIPAEKVVVAQMMLISKCNVAG-----------KPVICATQMLESMT
CCC523161       G--IMVARGDLGVEIPAEKVVVAQMCIISKCNVAG-----------KPVICATQMLESMT
EAN787821       G--IMVARGDLGVEIPAEKVCVAQMCIISKCNVVG-----------KPVICATQMLESMT
XP_8278941      G--IMVARGDLGVEIPAEKVCVAQMCIISKCNVVG-----------KPVICATQMLESMT
CCC944211       G--IMVARGDLGVEIPAEKVVVAQMCIISKCNVAG-----------KPVICATQMLESMT
XP_0093132741   ----FTSGRYFHRSLNPEKLVAIAFSRIPPQYQKF-----------QNPMSMLKRFYQVP
AEP827161       ----FATADYYHRSLNPEKLVAVGFSXIPQQYQKF-----------QNPLSMIKRFYELP
XP_8279041      ----FAKGHYFHRSLNSQKLVDVKFSGIPPHYKRF-----------QNPVAVMERLYRLP

AAD022221       KKVTPGVQTDVGAHAESNAQSFMYYVNDGVYGSFNCILYDHAVVRPLPQ----------910
AAA302191       KKVTPGVQTDVGAHAESNAQSFMYYVNDGVYGSFNCILYDHAVVRPLPQ----------
XP_8294291      KKVTPGVQTDVGAHAESNAQSFMYYVNDGVYGSFNCILYDHAVVRPLPQ----------
ESL098061       SPEDAVCSSAVNSVYEVRAKVLLVLSNSGRSARLASKYRPNCPIVCATT-----------
3QV9_B          SPEEAVCSSAVNSVYEVRAKALLVLSNSGRSARLASKYRPDCPIICATT-----------
XP_0093099901   SPEEAVCCSAVNSVYEVRAKVLLVLSNSGRSARLTSKYRPDCPIICVTT-----------
KEG117721       SPEEAVCCSAVNSVYEVRAKVLLVLSNSGRSARLTSKYRPDCPIICVTT-----------

EAN787821       CPEEAVCSSAVASAFEVQAKAMLVLSNTGRSARLISKYRPNCPIICVTT----------- 910
XP_8278941      CPEEAVCSSAVASAFEVQAKAMLVLSNTGRSARLISKYRPNCPIICVTT-----------

AAD022221       REPIPNEKLYPSSVWGPTCDGLDQIVERYYLPEMQVGE-------------WLLFEDMGA
AAA302191       REPIPNEKLYPSSVWGPTCDGLDQIVERYYLPEMQVGE-------------WLLFEDMGA 970
XP_8294291      REPIPNEKLYPSSVWGPTCDGLDQIVERYYLPEMQVGE-------------WLLFEDMGA
ESL098061       RMRTCRQLTITRSVEPVFYDAERYGDDESKEKRVQLG---------------VEWAKKRG
3QV9_B          RMRTCRQLTITRSVDAVFYDAERYGEDENKEKRVQLG---------------VDCAKKKG
XP_0093099901   RMRTCRQLNVTRSVESVFYDAERCGADEDKENRVQLG---------------VESAKKKG
KEG117721       RMRTCRQLNVTRSVESVFYDAERCGADEDKENRVQLG---------------VESAKKKG
```

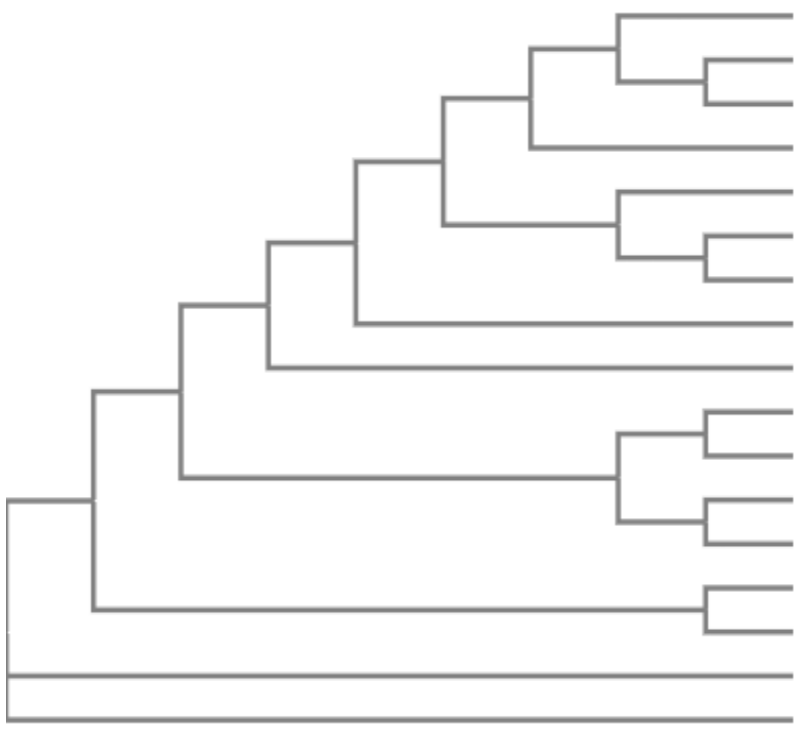
Figure 18. Multiple sequence alignmentsamong the drug targets excluding *Trypanosoma theileri* protein

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.,* 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

Table 6. Percent identity and divergence of Cluster W alignment of drug targets

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1: XP_829429.1	100	99.78	99.76	18.72	17.82	17.82	17.52	15.81	17.22	16.92	17.03	17.03	14.58	14.5	13.33	10.98	18.22	1
2: AAD02222.1	99.78	100	100	18.72	17.82	17.82	17.52	15.81	17.22	16.92	17.03	17.03	14.88	14.5	13.33	10.98	18.22	2
3: AAA30219.1	99.76	100	100	19.23	16.5	16.5	16.18	13.47	16.18	15.53	16.79	16.79	15.19	14.41	13.53	10.98	18.6	3
4: ABI96917.1	18.72	18.72	19.23	100	24.4	24.4	25.81	23.55	24.8	24.19	23.36	23.36	20.66	15.37	14.78	10.68	18.86	4
5: XP_009309990.1	17.82	17.82	16.5	24.4	100	100	82.97	88.18	86.37	86.17	80.34	80.34	22.2	16.77	18.81	16.81	18.37	5
6: KEG11772.1	17.82	17.82	16.5	24.4	100	100	82.97	88.18	86.37	86.17	80.34	80.34	22.2	16.77	18.81	16.81	18.37	6
7: CCC94421.1	17.52	17.52	16.18	25.81	82.97	82.97	100	91.21	82.16	82.36	86.89	86.89	23.17	17.07	18.51	16.38	19.59	7
8: CCC52316.1	15.81	15.81	13.47	23.55	88.18	88.18	91.21	100	84.55	85.15	87.91	87.91	23.59	17.14	19.25	16.74	17.2	8
9: ESL09806.1	17.22	17.22	16.18	24.8	86.37	86.37	82.16	84.55	100	89.58	80.34	80.34	22.44	17.38	20	17.67	15.92	9
10: 3QV9_B	16.92	16.92	15.53	24.19	86.17	86.17	82.36	85.15	89.58	100	80.63	80.63	21.71	17.07	18.81	17.24	16.33	10
11: EAN78782.1	17.03	17.03	16.79	23.36	80.34	80.34	86.89	87.91	80.34	80.63	100	100	23.16	17.86	17.77	14.97	18.27	11
12: XP_827894.1	17.03	17.03	16.79	23.36	80.34	80.34	86.89	87.91	80.34	80.63	100	100	23.16	17.86	17.77	14.97	18.27	12
13: CAA44870.1	14.58	14.88	15.19	20.66	22.20	22.20	23.17	23.59	22.44	21.71	23.16	23.16	100	14.33	13.65	14.07	19.83	13
14: XP_827904.1	14.5	14.5	14.41	15.37	16.77	16.77	17.07	17.14	17.38	17.07	17.86	17.86	14.33	100	59.64	63.52	13.58	14
15: XP_009313274.1	13.33	13.33	13.53	14.78	18.81	18.81	18.51	19.25	20	18.81	17.77	17.77	13.65	59.64	100	69.45	11.93	15
16: AEP82716.1	10.98	10.98	10.98	10.68	16.81	16.81	16.38	16.74	17.67	17.24	14.97	14.97	14.07	63.52	69.45	100	12.28	`6
17: XP_009316281.1	18.22	18.22	18.6	18.86	18.37	18.37	19.59	17.2	15.92	16.33	18.27	18.27	19.83	13.58	11.93	12.28	100	17

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47



	Target protein	Species/strain	
XP_829429.1 0	Ornithine decarboxylase		T B
AAD02222.1 -0.00239	Ornithine decarboxylase		TG
AAA30219.1 0.00239	Ornithine decarboxylase		TB
XP_009316281.1 0.40926	Cathepsin L-like protein		TG
XP_827904.1 0.19547	N-myristoyl transferase		TB
XP_009313274.1 0.14334	N-myristoyl transferase		TG
AEP82716.1 0.16212	N-myristoyl transferase partial		TC
ABI96917.1 0.38256	Topoisomerase		TC
CAA44870.1 0.39386	Trypanothione reductase		TB
XP_009309990.1 0	Pyruvate kinase 2		TG
KEG11772.1 0	Pyruvate kinase 2		TG
ESL09806.1 0.05239	Pyruvate kinase 2		TR
3QV9_B 0.05182	Pyruvate Kinase(Tcpyk)		TC
EAN78782.1 0	Pyruvate kinase 1		TB
XP_827894.1 0	Pyruvate kinase 1		TB
CCC94421.1 0.05135	Pyruvate kinase 1		TC
CCC52316.1 0.03653	Pyruvate kinase 1, partial		TV

TB: *T.brucei*, TG: T. grayi, TT: *T. theileri* , TC: *T.cruzi*, TR: *T. rangeli*, TV: *T.vivax*, TC: *T.congolense*

Figure 18. Phylogenetic tree following multiple sequence alignments showing relationships among common trypanocidal drug targets

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

DISCUSSION

Growth media and condition

Trypanosoma theileri is one of the least studied trypanosomes although it is prevalent worldwide. This distribution may be due to the pathogenic nature of the parasite, which, compared to pathogenic ones, cannot cause a remarkable loss of production and productivity in livestock. However, nowadays researchers are focusing on *T. theileri* since it has been used as a setup and new tool for trypanosomatid-based delivery (Mott et al., 2011) for the treatment of pathogenic trypanosome and other hemoparasites affecting livestock and humans. Nevertheless, due to its low parasitemic nature, it is rarely detected by direct smear microscopy. In view of this, Verloo et al. (2000) developed a kit called KIVI as an excellent tool for isolating *T. Theileri* which they found successful.

There are a number of media that must be cultured for both epimastigote and trypanosome's blood flow stages. For the blood flow stages of *T. theileri* (Trypomastigotes), RPMI medium 1640 with 10% FCS, supplemented with murine spleen cells as a feeder layer, was used (Verloo et al., 2000). In addition, it could be cultured in tissue culture fluid NCTC-109 (Splitter and Soulsby, 1967), 50% HMI-9 medium (Hirumi and Hirumi, 1989) supplemented with 20% FCS, 10% Serum, and 50% MDBK-conditioned media (Mott et al., 2011).

In the present study, it was possible to grow *T. theileri* epimastigotes in SDM 79 with 10 % FCS at 26^{o}C without CO_2betweenfour media tested (Figure 2). Under similar growth conditions, Wink (1979) cultured *T. Theileri* epimastigotes at 25°C with 10 % FCS, but with different growth media, Glossina Cell culture Medium (GCM). Moreover, in the second growth condition (at 37°C with 5% CO_2) there was slight growth of *T. theileri* epimastigotes in RPMI 1640 (Figure 3), which was confirmed by Verloo et al. (2000), who could culture *T. theileri* with RPMI 1640. However, the growth of *T. theileri* epimastigote in RPMI 1640 at 26^{o}C with CO_2 was less than the time cultivated at 26^{o}C without CO_2 (Figure 2) in the present study. This could be due to the reluctance to use PMI conditioning since they used a feeder layer of murine spleen cells. In addition, Verloo et al. (2000) cultured the blood flow stage that was directly isolated from the blood.

Growth pattern and doubling time

The culture of *T. theileri* in eight different tissue culture flasks showed maximum growth on the sixth day during the eight experiments, except for the seventh experiment on the seventh day. On the other hand, as can be seen in Figure 3, the log phase starts clearly from the third day. This indicated that the logarithmic phase of growth was started from the third to the sixth day and from the third to the seventh day for the seventh experiment. The maximum number of cells that could be grown among the eight flasks was estimated to be 1.7×10^7 cells/mL (Experiment 2). Hence, the logarithmic phase extended from 3.2×10^4cells/mL, which was on the third day of the experiment. Second, to the highest 1.3×10^7cells/mL from the same flask on the sixth day except for Experiment seven. The doubling time in the logarithmic growth phase was estimated to range from 13.43 to 19.0 hours with an average estimate of 17.43 hours (0.73 day) in eight experiments. The doubling time of the present study is slightly higher than the doubling time of *T. theileri* reported by Wink (1979) with a doubling time of 10-14 hours. However, the growth pattern of *T. theileri* is2.5 times higher than the doubling time of *T.b.brucei*as described by Sykes and Avery (2009) and Melissa et al. (2009).

The doubling time could indicate that *T. theileri* grows more slowly than other similar reports. The slow growth rate could be due to different media types and growing conditions. It is in a sense that, if the parasite has got a favorable growth environment, they may have the opportunity to divide within a short period. Furthermore, the slower growth of *T. theileri* compared to the growth of other pathogenic Trypanosomesmay be linked to the factors that could make the parasite a pathogen. This means that the longer replication time and the lower parasitemic features can naturally cause the least amount of anemia. The serious losses from pathogenic trypanosomes are principally due to anemia. Furthermore, the lower detection level in the blood can limit the distribution of the parasite to different visceral organs and haemopoietic tissues, such as the spleen. Moreover, virulent trypanosomes have a shorter incubation period than pathogen ones (Magona et al., 2008). The longer the doubling time which was needed in the present study, can be related to this point. The same applies to the study by Böse et al. (1987), who reported that after identifying the stages of infection of *T. theileri* in the gut and feces of tabanids, it could be determined that the minimum prepatent period of around four days in infected cattle despite no apparent signs of disease were detected.

Resazurin assay

The epimastigote (5.2×10^5/mL to 8.5×10^6/mL) was grown in a microtiter plate for three different concentrations of Resazurin. Initially, there was a lower fluorescence signal throughout the three Resazurin concentrations. However, it increased significantly from 1.2×10^6 cells/ml and reached the highest fluorescence signal detection at 3.5×10^6 cells/mL following the seventh hour of incubation with Resazurin. It indicated that there was a positively correlated ($r = 0.7517$-0.9252; $p < 0.05$) fluorescence signal with an increase in cell density and Resazurin concentrations.

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

On the other hand, there was a positive correlation ($r = 0.8297$; $p < 0.05$) among fluorescence signals as a result of 2.4 mM Resazurin than the other two concentrations with an increase in incubation time (Figure 8) until 7 hours using an optimal 1.2 $\times 10^6$ cells/mL of cell density. The fluorescence decreased dramatically after the seventh hour of incubation with Resazurin (Figure 7). It is in agreement with a study by Tana et al. (2012) who reported that Resazurin was reduced linearly after three to four hours of incubation with 25 mg/mL (20 µL/well) Resazurin though it was done on *T. brucei*. They also reported that the maximum fluorescence was recorded in the range of 2-5 $\times 10^6$ cell/mL which was almost similar to the optimal cell density found for higher fluorescence (1.2-3.5$\times$ 10^6 cells/mL) with 2.4Mm Resazurin. There was a very high and linear correlation ($r = 0.9876$)observed in the fluorescence signal of the parasite in the range of 1.3 $\times$ 10^6 to 5.3 $\times$ 10^6 cells/ml upon incubating with 2.4 mM Resazurin for 7 hours at 26°C. It is slightly higher than the incubation time used for fluorescence assay for *T. cruzi* which was 5 hours as reported by Miriam et al. (2006). Similarly, according to Tana et al. (2012), there was a linear reduction of Resazurin after a 3-4 hours period of contact time since there might be a saturation of the fluorescence at such high parasite densities. A report by Miriam et al. (2006) also showed the highest concentrations of Resazurin (3 mM) among the Resazurin concentrations they tested (0.5 to 3 mM), showed the highest Resazurin reduction. The same is true from our experiment since the highest Resazurin concentration (2.4 mM) showed a similar pattern. It may be due to the fact that whenever we use the lowest concentration of Resazurin, the Resazurin (blue) can be converted to a more fluorescent resorufin(pink) immediately within a short time. Then, if it was allowed for a long incubation time, the resorufin which is the fluorescent one will be converted to a non-fluorescent stage finally. It shows an imbalance between the highest numbers of cells with the lowest concentration of Resazurin used. Due to this, maybe the highest concentration showed a significant correlation in the magnitude of fluorescent signal with an increase in cell density and incubation time.

Drug sensitivity

The Resazurin assay enables the measurement of parasite viability as an indicator of the ability to recover from compound effects (Nare et al., 2010; Tana et al., 2012). By using the optimization conditions we established for cell density and Resazurin concentration, the Resazurin-lead assay was applied to assess the susceptibility of *T. theileri* epimastigotes to Pentamidine (Sigma, 1-100 ng/mL). As a consequence, a negatively correlated ($r = -0.8826$) and statistically significant ($p < 0.05$) difference were observed between the reduction in fluorescence signal and an increase in pentamidine concentration predominantly in the highest drug concentration. A study was done on *T.b.brucei* by Tana et al. (2012) also indicated a similar response of the parasite following 72 hours of incubation with Pentamidine which result in a dramatic reduction of Resazurin signal. The viability percentage was determined by comparing both microscopic counting which is anti-epimastigotes (%AE) and the resazurin assay with Pentamidine (1-100 ng/mL). IC_{50} values of 9.25 ng/mL and 16.29 ng/mL were found by using Resazurin ($r = -0.957$, $p < 0.05$) and microscopic counting ($r = 0.90$, $p < 0.05$) respectively. These two tests showed roughly similar outcomes though Resazurin assay was more preferable since it has relatively lower IC_{50} (Figure 11). Furthermore, it allowed screening a large sample size as far as microscopic counting is time-consuming. It was in agreement with the report by Miriam et al. (2006) and Sykes and Avery (2009) mentioning that Resazurin was preferable to test the viability of *T.b. brucei* for Pentamidine and Suramin. The IC_{50} value was higher than the reports by Sykes and Avery (2009) and Tana et al. (2012), who reported 5ng/mL and 40 nM, respectively for *T.brueci.*

Experimental infection of calves

During the experimental infection of the present study, the identification of parasites both in Giemsa stained slides and through culturing the PBMC and the buffy coat in RPMI 1640 and HMI 9 medium was unsuccessful. However, a similar method of experimental infection was followed by Mott et al. (2011). The problems could be, first, the parasite could die in transit prior to inoculation since the farm was a few kilometers away from the laboratory where the parasite was cultured. Secondly, it might be due to the time it took for replication to happen. According to the doubling time calculated during the in vitro culture, they needed 13.43 to 19 hours. Third, based on the amount of the parasite, the calf was inoculated between five and nine ml. Finally, maybe another co-founding factor has not yet been realized. Nevertheless, a similar study was done by Mott et al. (2011) after experimental infection. However, they extracted DNA directly from whole blood samples than growing the parasite after identification from buffy coat and/or PBMC since it's rarely detected due to the low level of parasitemia. However, the focus of the present study was on the culturing of the blood-streaming form in mass after isolating from the blood for further proteomic studies; to compare the epimastigote and trypomastigotes proteomics.

PCR confirmation of *Trypanosoma theileri*

Trypanosoma theileri could be differentiated from other trypanosomes through a species-specific primer (Tth625) as described by Rodrigues et al. (2003), PCR-amplified spliced-leader transcript, 18S ribosomal DNA, and internal transcribed spacer of ribosomal genes (ITS gene, Geysen et al., 2003). In the present study, the results obtained by PCR

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

and agarose gel electrophoresis based on Species-specific primers confirmed a 465 bp amplification product which was comparable with 450 bp DNA fragment (Tth625 fragment) by Rodrigues et al. (2003)and 472 bp by Lee et al. (2013). For the second amplification for the 18S rDNA sequence of *T. theileri*, a band with a size of730 bp was detected, which was almost expected to be 722 bp, as reported by Geysen et al. (2003).

In silico analysis

The BLAST and Clustal W alignment were done both at nucleotide (DNA) and protein level with target sequences of common anti-trypanosome drugs. Following a BLAST and Cluster W alignment using these common drug targets, there was only one slight homogeneity (75%) gene (CATL gene) which was found in both *T.grayi* (XM_009318006.1) and HQ664735.1 of *T. theileri* isolate in common. On the other hand, among the drug targets only, excluding *T. theileri* genome; they had a very high identity especially among XM_824336.1 (100%), J02771.1 (99%), and AF042286 (99%) which entirely indicated Ornithine decarboxylase gene in*T.brucei.* The phylogenetic tree and ClusterW alignment also showed these relationships. Lee et al. (2013)have got the homology of their isolate (TWTth1) from Taiwan with isolates by Rodrigues et al. (2003), Gene Bank Accession No: AF537201 (99.5%) and AF537202 (98.8%) from Brazil. They did the comparison based on *T. theileri* DNA. In addition, based on the full-length 18S rDNA sequence amplicon of their isolate, they found 100% identity with AB007814.1. Based on ITS sequences, Lee et al.(2013)reported that there was a similarity amongAB007814 (100%), AY773707 (97.3%), and AY773708 (98.0%) which are Japanese and Brazilian isolates.

At the protein level, there was almost no similarity of these drug targets with the proteins found in the NCBI database except a slight similarity with hypothetical *T. theileri* proteins (TM35). However, among the drug targets, a prominent homology was detected among XP_829429.1(100%), AAD02222.1, and AAA30219.1 which indicated ornithine decarboxylase protein as was obtained after analyzing at DNA level in the current study. The MSA and the phylogenetic trees witnessed the homology of these sequences.

Accordingly, BLAST was used in the current study and aligned only with partial and hypothetical sequences of the *T. theileri.* However, there were homologies of sequences from anti-trypanosome drug targets specifically for Ornithine decarboxylase from *T.brucei*. Similarly, Rodrigues et al. (2003) reported that *T. theileri* and '*T. brucei* clade' trypanosomes shared artiodactyl host species with overlapping distributions and commonly bovids carrying mixed trypanosome infection in the field.

CONCLUSIONS

In the present study, *T. theileri* was successfully cultured *in vitro* in SDM 79 at 26^{o}C. The growth pattern, viability, and response to pentamidine were assessed by Resazurin assay. *T. theileri* parasite took a longer time to double the population, compared to other trypanosomes. Moreover, the Resazurin assay using pentamidine was deployed as the reference drug to confirm the effectiveness of this assay technique. Consequently, it could be possible to use such a sensitive and inexpensive assay for high-throughput screening of anti-trypanosome compounds. On the other hand, after extraction of the DNA by the PhenolChloroform protocol from cultured *T. theileri*, the parasite was confirmed using PCR amplification by species-specific primers.

Furthermore, the BLAST and MSA were performed with common anti-trypanosome target sequences. Subsequently, in contrast to the anti-trypanosome targets of pathogenic trypanosomes, significant similarity at both the DNA and protein level with *T. theileri* was detected. However, some similarities to hypothetical. *theileri* proteins (TM35) were observed. Both at DNA and protein level, significant homologyamongXP_829429.1, AAD02222.1, and AAA30219.1 were detected which all referred to ornithine decarboxylase protein in *T.brucei*. The lack of homology in *T. theileri* might be due to the lack of a complete genome sequence. Finally, whole-genome and transcriptome analyses using *T. theileri* can reveal the phylogenetic relationship between *T. theileri* and other pathogenic trypanosomes, which can be used as a tool in the development of new therapeutic drugs for the treatment of the pathogenic trypanosome.

Based on the above conclusions, the following recommendations are put forward. The present study provides baseline data for the next research on the parasite. In the case of experimental infection, it is better to infect the test animal as soon as possible, while the parasite could soon die if transported with PBS. Long and continuous blood sampling should be performed after experimental infection. In the present study, only two consecutive weeks were checked after each infection. There is a chance that the parasite may not be immediately naturally isolated due to a low level of parasitemia. Hence, most of the isolation of *T. theileri* from natural infection was not purposively confirmed. Rather, it was found in an unexpected time. It can be isolated while researchers pursue other goals. For example, the total leukocyte and differential count, and PCV are examined during the macrophage culture while the Bovine Leukemia Virus(BLV) is examined from lymphocyte cultures of cows infected with it. It is better to use a reader with higher fluorescence to get a higher magnitude of the fluorescence signal. Better to use more than two drugs for treatment so that the drug sensitivity assay is representative and comparable.

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

DECLARATIONS

Authors' contribution

Tewodros Fentahun contributed to data collection, lab activities, and a write-up of the manuscript. Jan Paeshuyse was involved in data analysis. Both authors confirmed the final revised manuscript.

Competing interests

The authors have no conflicts of interest.

Acknowledgments

The authors would like to thank VLIR OUS, Katholic University of Leuven, and Institute of Tropical Medicine, Belgium, for their collaboration in the preparation of this research.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Aerts D, Truc P,PenchenierL, Claes Y, and Ray Le(1992). A kit for in vitro isolation of trypanosomes in the field: first trial with sleeping sickness patients in the Congo Republic.Transaction of the Royal Society of Tropical Medicine and Hygiene, 86: 394-395. Available at:http://www.sleeping-sickness.ird.fr/pdf/35977.pdf.

Alonso-Padilla Jand Rodríguez A (2014). High Throughput Screening for Anti*Trypanosoma cruz*i Drug Discovery. PLoS Neglected Tropical Diseases, 8(12): e3259. DOI: https://doi.org/10.1371/journal.pntd.0003259

Amaro RE, Schnaufer A, Interthal H, Hol W, Stuart KD, and McCammon JA (2008). Discovery of drug-like inhibitors of an essential RNA-editing ligase in Trypanosoma brucei. Proceedings of the National Academy of Sciences of the United States of America, 105(45): 17278-83. DOI: https://doi.org/10.1073/pnas.0805820105.

Böse R, Friedhoff KT, and Olbrich S (1987). Transmission of Megatrypanum trypanosomes to Cervus dama by Tabanidae. Journal of Protozoology, 34: 110-113. DOI: https://doi.org/10.1111/j.1550-7408.1987.tb03143.x

Braun U, Rogg E, Walser M, Nehrbass D, Guscetti F, Mathis A, Deplazes P(2002) *Trypanosoma theileri* in the cerebrospinal fluid and brain of a heifer with suppurative meningoencephalitis. VeterinaryRecords, 150: 18-19. Available at:https://europepmc.org/article/med/11817859

Ekins S, Mestres J, and Testa B (2007). In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling. British Journal of Pharmacology, 152:9-20. DOI: https://doi.org/10.1038/sj.bjp.0707305.

Fatumo S, Adebiyi M A, anddebiyi E (2013). In silico models for drug discovery. (M. Lill, Edition), InSilico Models for Drug Discovery. DOI: https://doi.org/10.1007/978-1-62703-342-8.

Geysen D, Delespaux V, and Geerts S (2003). PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of Trypanosoma species in cattle. Veterinary Parasitology, 110: 171-180. DOI: https://doi.org/10.1016/S0304-4017(02)00313-8.

Hirumi H and Hirumi K (1989). Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. Journal of Parasitology, 75: 985-989. DOI:https://doi.org/10.2307/3282883

Latif AA, Bakheit M a, Mohamed AE, and Zweygarth E (2004). High infection rates of the tick Hyalomma anatolicum anatolicum with *Trypanosoma theileri*. The Onderstepoort Journal of Veterinary Research, 71(4): 251-256. Available at:https://journals.co.za/content/opvet/71/4/EJC86189

Lee YF, Cheng CC, Chen JS, Lin NN, Hung YW, Wang JM, and Chiu YT (2013). Evidence of intracellular stages in *Trypanosoma* (Megatrypanum) *theileri* in non-phagocytic mammalian cells. Veterinary Parasitology, 191(3–4): 228-239. DOI: https://doi.org/10.1016/j.vetpar.2012.08.027.

Lukes J (2009). Trypanosoma Gruby 1843. Version 02 January 2009 (under construction). Available at: http://tolweb.org/Trypanosoma/98034/2009.01.02%20in%20The%20Tree%20of%20Life%20Web

Magona JW, Walubengo J, and Odimin JT(2008). Acute haemorrhagic syndrome of bovine trypanosomosis in Uganda. Acta Tropica, 107: 186-191. DOI: https://doi.org/10.1016/j.actatropica.2008.05.019.

Melissa S, Avery T, and Vicky C(2009). A luciferase based viability assay for ATP detection in 384-well format for high throughput whole cell screening of Trypanosoma brucei brucei bloodstream form strain 427. Parasites and Vectors, 2(1): 54. DOI: https://doi:10.1186/1756-3305-2-54.

Miriam R, Celeste V, Jose Antonio E, and Alicia B(2006). Development of resazurin microtiter assay for drug testing of *Trypanosoma cruzi* epimastigotes. Parasitology Research, 99(2): 103-107. DOI: https://:doi:10.1007/s00436-006-0126-y.

Mott GA, Wilson R, Fernando A, Robinson A, MacGregor P, Kennedy D, and Matthews KR (2011). Targeting cattle-borne zoonoses and cattle pathogens using a novel trypanosomatid-based delivery system. PLoS Pathogens, 7(10): 1-9. DOI:https://doi.org/10.1371/journal.ppat.1002340.

Murray M, Murray P K, and McIntyre W I(1977). An improved parasitological technique for the diagnosis of African trypanosomiasis.Transactions of The Royal Society of Tropical Medicine and Hygiene, 71(4): 325-326. DOI:

https://doi.org/10.1016/0035-9203(77)90110-9.

Nare B, Wring S, Bacchi C, Beaudet B, Bowling T, Brun R, Chen D, Ding C, Freund Y, Gaukel E et al. (2010). Discovery of novel orally bioavailable oxaborole 6-carboxamides that demonstrate cure in a murine model of late-stage central nervous system African trypanosomiasis. Antimicrobial Agents and Chemotherapy, 54: 4379-4388. DOI: https://DOI: 10.1128/AAC.00498-10.

Rodrigues AC, Campaner M, Takata CSA, Dell' Porto A, Milder RV, Takeda GF, and Teixeira MMG (2003). Brazilian isolates of *Trypanosoma* (Megatrypanum) *theileri*: Diagnosis and differentiation of isolates from cattle and water buffalo based on biological characteristics and randomly amplified DNA sequences. Veterinary Parasitology, 116(3): 185-207. DOI: https://doi.org/10.1016/S0304-4017(03)00236-X.

Shapiro Ta, and Englund PT (1990). Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. Proceedings of the National Academy of Sciences of the United States of America, 87(3): 950-954. DOI: https://doi.org/10.1073/pnas.87.3.950.

Shastri UV, and Deshpande PD (1981). Hyalomma anatolicum anatolicum (Koch, 1844) as a possible vector for transmission of *Trypanosoma theileri*, Laveran, 1902 in cattle. Veterinary Parasitology, 9(2): 151-155. DOI: https://doi.org/10.1016/0304-4017(81)90034-0.

Splitter EJ, and Soulsby EJ (1967). Isolation and continuous cultivation of *Trypanosoma theileri* inmedia containing tissue culture fluids. Experimental Parasitology, 21(2): 137-148. Available at:http://www.ncbi.nlm.nih.gov/pubmed/4169719.

Sykes M, and Avery V (2009). Development of an alamar Blue™ viability assay in 384-well format for high throughput whole cell screening of *Trypanosoma brucei brucei*bloodstream form strain 427.The American Journal of Tropical Medicine and Hygiene, 81(4): 665-674. DOI: https://doi.org/10.4269/ajtmh.2009.09-0015

Tana Bowling, Luke Mercer,Robert Don,Robert Jacobs, and Bakel Narea (2012). Application of a resazurin-based high-throughput screening assay for the identification and progression of new treatments for human African trypanosomiasis. International Journal of Parasitology and Drugs Drug Resistance, 2: 262-270. DOI: https://doi.org/10.1016/j.ijpddr.2012.02.002.

Verloo D, Brandt J, Van Meirvenne N, and Büscher P (2000). Comparative in vitro isolation of *Trypanosoma theileri* from cattle in Belgium. Veterinary Parasitology, 89(1–2): 129-132. DOI: https://doi.org/10.1016/S0304-4017(00)00191-6.

Wink M (1979). *Trypanosoma theileri:* In vitro cultivation in tsetse fly and vertebrate cell culture systems.International Journal for Parasitology, 9(6): 585-589. DOI:https://doi.org/10.1016/0020-7519(79)90016-X

To cite this paper: Fentahun T and Paeshuyse J (2021). Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes. *World Vet. J.*, 11 (3): 343-367. DOI: https://dx.doi.org/10.54203/scil.2021.wvj47

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 368-376, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium

Linda Miftakhul Khasanah[1], Teguh Budipitojo[2], and Yuda Heru Fibrianto [3*]

[1]*Doctoral Student of Veterinary Science, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[2]*Department of Anatomy, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[3]*Departement of Physiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

*Corresponding author's Email: yudaf@ugm.ac.id; ORCID: 0000-0002-5886-6697

ORIGINAL ARTICLE
pii: S232245682100048-11
Received: 18 June 2021
Accepted: 29 July 2021

ABSTRACT

About 60-80 million couples in the world are suffering from infertility disease. Infertility is a major problem in patients coping with chemotherapy. The chemotherapy process can degenerate non-target organs, especially in testes. Infertility in male or testicular dysfunction is caused by the failure of proliferation and differentiation of the spermatogenic cells. Many studies reported that mesenchymal stem cells-conditioned medium promoted regenerative processes. The present study aimed to investigate the effect of mesenchymal stem cells-conditioned medium on the cisplatin-induced testicular dysfunction by examining the immunolocalization of steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc which are considered as markers of steroid production. All experimental animals were divided into three groups, namely the control group, mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.2 ml/kg body weight (BW, P1), and mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.5 ml/kg BW (P2). Cisplatin was injected into both treated groups to induce testicular dysfunction. The testicular tissues were processed by the paraffin method, then cut to a thickness of 5 µm, followed by immunohistochemical staining. The HSD3B1 immunoreactivities were found only in Leydig cells, and the intensity increased every week after the injection of mesenchymal stem cells-conditioned medium. The variety of weeks and groups was significantly different in the number of immunoreactive cells of HSD3B1. The results indicated a significant difference between one week after the first injection and the one week after the third and fourth injection. The findings showed a significant difference between the treated group with an injection dose of 0.2 ml/kg BW and the control group. The number of immunoreactive cells of HSD3B1 with an injection dose of 0.5 ml/kg BW was greater compared to the group that received an injection dose of 0.2 ml/kg BW. The intensity of HSD3B1 and HSD17B1 increased every week. The p450scc immunoreactive cells were only found in Leydig cells. The intensity of positive cells of p450scc in the treated group with an injection dose of 0.5 ml/kg BW was more intense, compared to the treated group with an injection dose of 0.2 ml/kg BW. The results of the current study showed that the injection of mesenchymal stem cells-conditioned medium can improve the regeneration of spermatogenic cells, and recover spermatogenesis proved by positive cells of HSD3B1, HSD17B1, and p450scc as markers of steroid production.

Keywords: Cisplatin, HSD17B1, HSD3B1, Mesenchymal stem cells-conditioned medium, P450scc, Testicular dysfunction

INTRODUCTION

About 60-80 million couples in the world are suffering from an infertility disease, and about 40-50% of the infertility is caused by male factors. Infertility is an extremely common physical disorder in adults, which occurs due to trauma, infections, tumor growth, and radiation therapy in patients coping with chemotherapy (Zhang et al., 2017). The chemotherapy process can degenerate non-target organs, especially in testes, that decrease the quality and quantity of sperm. Infertility in male or testicular dysfunction is caused by the failure of proliferation and differentiation of the spermatogenic cells. Testicular dysfunction in an animal model can be induced by anticancer drugs, such as busulfan, cyclophosphamide, doxorubicin, procarbazine, or cisplatin (Atessahin et al., 2006). Cisplatin is an anticancer drug potential; however, a high dose of cisplatin is extremely toxic for testicles. Some researchers reported that the administration of antioxidants, such as lycopene, royal jelly, curcumin, melatonin, Vitamin C, montelukast, resveratrol, selenium, and amifostine, can improve the degeneration of testis (Beytur et al., 2012; Reddy et al., 2016; Simsek et al., 2016).

Mesenchymal Stem Cells (MSCs) have been used widely in the treatment of organ dysfunctions, such as testicular dysfunction. Many studies have reported the improvement of androgen deficiency after stem cell therapy. Human Umbilical Mesenchymal Stem Cells (HUMSCs) can increase testosterone levels, and Leydig-like cells are functioning

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.,* 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

well in hypogonadism models (Zhang et al., 2017). Transplanted rat adipose-derived MCSs into D-galactose-treated aging rats can improve testicular function by being differentiated into 3-β-Hydroxysteroid Dehydrogenase (HSD3B1) positive Leydig-like cells (Yang et al., 2015). Tamadon et al. (2015) reported that transplanted Bone Marrow-Mesenchymal Stem Cells (BM-MSCs) could successfully induce spermatogenesis in seminiferous tubules of azoospermia hamsters. Mesenchymal stem cells-conditioned medium or secretome is a factor secreted by stem cells containing growth factors and cytokines. Many studies reported that mesenchymal stem cells-conditioned medium promotes regenerative processes, such as healing diabetes mellitus type 1 by improving the pancreatic beta cells (Nugroho et al., 2016), and regenerating the healing burns (Padeta et al., 2017). It also regenerates the incise wound healing (Kusindarta et al., 2016). The present study aimed to investigate the effect of mesenchymal stem cells-conditioned medium in cisplatin-induced testicular dysfunction by examining the immunolocalization of HSD3B1, 17β-Hydroxysteroid Dehydrogenase (HSD17B1), and P450 Side-Chain Cleavage enzyme (P450scc).

MATERIALS AND METHODS

Ethical approval

The present study was approved by Ethical Clearance from Universitas Gadjah Mada, Yogyakarta, Indonesia with number 00035/04/LPPT/V/2017

Experimental animals

A total number of 36 healthy male Wistar rats (Rattus norvegicus) aged three-month-old, with an average weight of about 300 grams, were randomly divided into three groups. The groups included the control group, and the mesenchymal stem cells-conditioned medium treated groups with an injection dose of 0.2 ml/kg BW (P1), and the mesenchymal stem cells-conditioned medium treated groups with an injection dose of 0.5 ml/kg BW (P2). The control group was not injected with cisplatin, but injected with 0.9% physiological NaCl. The P1 and P2 groups were injected with cisplatin to induce testicular dysfunction, and continued with an injection dose of 0.2 ml/kg BW and 0.5 ml/kg BW of mesenchymal stem cells-conditioned medium. The rats in the treated groups were injected with Cisplatin (PT Dankos Farma, Kalbe Company, Indonesia) at a dose of three mg/kg BW three times at three-day intervals (Reddy et al., 2016; Prihatno et al., 2018). The mesenchymal stem cells-conditioned medium was injected one time a week for four times after all doses of cisplatin were injected.

Sample collection

Testicular tissues were collected one week after the first, second, third, and fourth injection of mesenchymal stem cells-conditioned medium. Testicular tissues were fixed in Bouin's solution for 24 hours, embedded in paraffin, sectioned to a thickness of 5 µm, and stained with the immunological method (Prihatno et al., 2020) to visualize the immunoreactivity of HSD3B1, HSD17B1, and P450scc.

Immunohistochemical staining

The first step of immunohistochemical staining was deparaffinization with xylene. Then, the slides were rehydrated with ethanol, and rinsed in running water for 5 minutes. Distillate water was placed in the microwave for 20 minutes for pre-heating the antigen retrieval, and then the slides were immersed in pre-heating distillate water for 10 minutes for antigen retrieval. Endogenous peroxide activity was blocked by incubating the slides in H_2O_2 3% in absolute methanol (1 mL H_2O_2 30%, 9 mL absolute methanol) for 30 minutes at room temperature. The slides were washed in Phosphate Buffer Saline (PBS) for five minutes, then repeated three times. Immunoreactivity was visualized with rabbit Diaminobenzidine (DAB) Detection IHC Kit (Fine Test, Wuhan Fine Biotech Co., Ltd., China) (Prihatno et al., 2020). After that, the slides were incubated overnight at 4^0C with HSD3B1 polyclonal antibody (1: 200, MBS2530192, Mybiosource, USA), HSD17B1 polyclonal antibody (1:400, A10839, ABclonal, USA), and Cytochrome P450scc Enzyme polyclonal antibody (1:500, AB1244, MilliporeSigma, USA). After that, the slides were washed again in PBS for five minutes, then it was repeated three times, and they were incubated with poly-HRP Goat Anti-Rabbit IgG for 60 minutes, and then for the last time, the slides were washed again in PBS three times for five minutes. Sites of immunoreactivity were visualized with DAB Chromogen. Harris hematoxylin was used for counter-staining the slides, followed by rinsing the slides with running water for 10 minutes. Lastly, the slides were dehydrated, cleared, and mounted. A light microscope was used to examine the stained slides, and Optilab® camera was used to take a photomicrograph.

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.*, 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

Statistical analysis

The number of immunoreactive cells of HSD3B1 was analyzed statistically using the Analysis of Variance (ANOVA) test followed by Duncan's post hoc test with software IBM SPSS Statistic 22. In this study, $p < 0.05$ was considered significant.

RESULTS

The immunoreactivity of HSD3B1 was detected in Leydig cells, and the intensity increased every week (Figures 1 and 2). The intensity of both treated groups, P1 and P2, on one week after the first injection, the immunoreactivity showed weak intensity (Figures 1-A and 2-A). One week after the second injection, the immunoreactivity of treated groups, both P1 and P2, showed no difference compared to the control group. One week after the third and the fourth injections, the intensity of both treated groups was stronger compared to the control group.

The obtained results of different time periods (one week after the first, second, third, and fourth injections) indicated a significantly different number of immunoreactive cells of HSD3B1 ($p < 0.05$). The investigated groups (P1, P2, and the control group) were significantly different regarding the number of immunoreactive cells of HSD3B1 ($p < 0.05$, Table 1). The results revealed a significant difference between the obtained results of one week after the first injection and one week after the third and fourth injection. However, one week after the first injection was not significantly different compared to one week after the second injection (Table 2). The statistical analysis of groups showed a significant difference between the P1 and control groups ($p < 0.05$). However, the P1 group was not significant in the number of immunoreactive cells of HSD3B1 compared to the P2 group. The control group was not significantly different in the number of immunoreactive cells of HSD3B1 compared to the P2 group (Table 3). The number of immunoreactive cells of HSD3B1 in the P2 group was greater compared to the P1 group (Table 3).

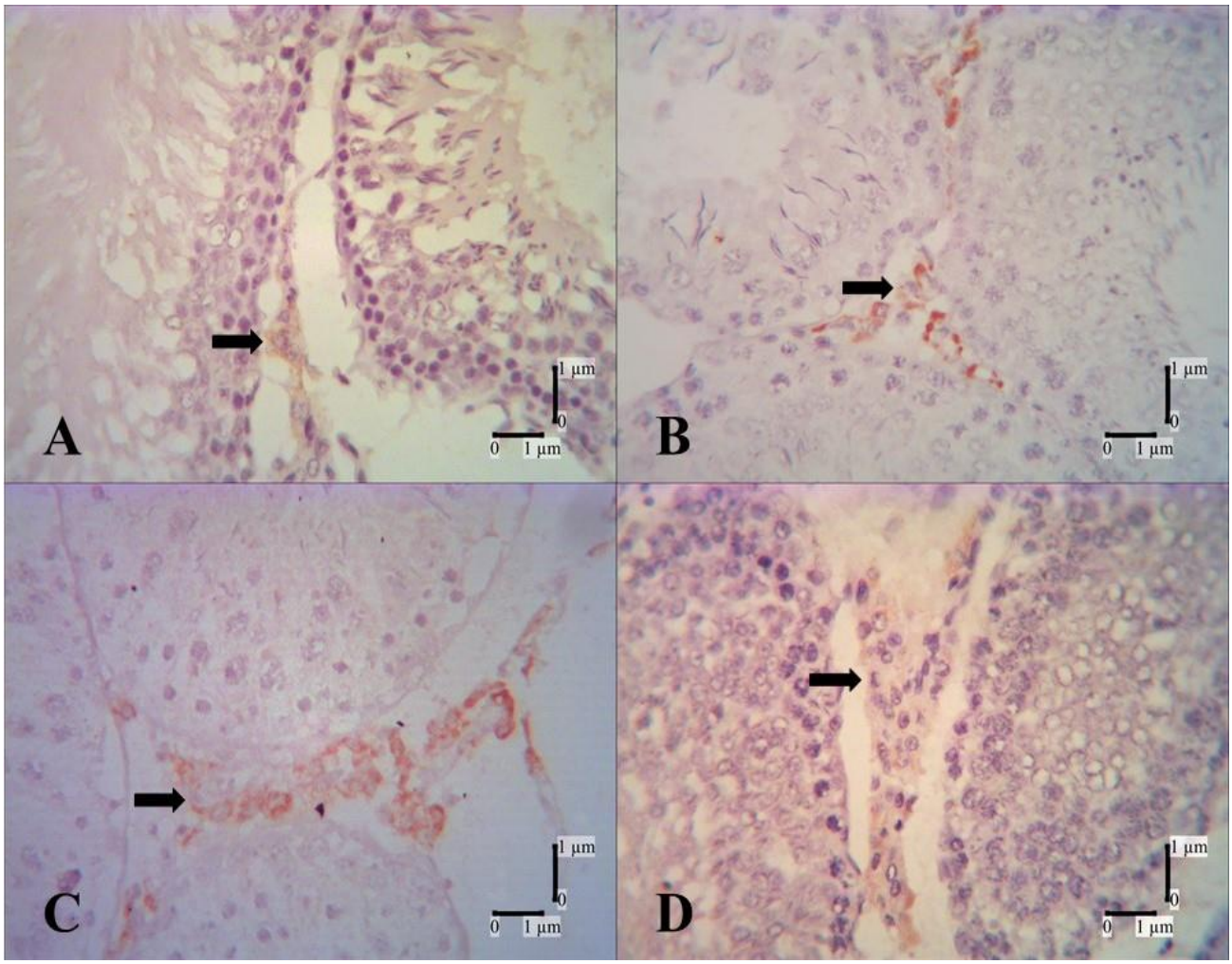


Figure 1. Immunoreactive cells of HSD3B1 with injection dose of 0.2 ml/kg BW mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** the control group; black arrow: Immunoreactive cells of HSD3B1

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.,* 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

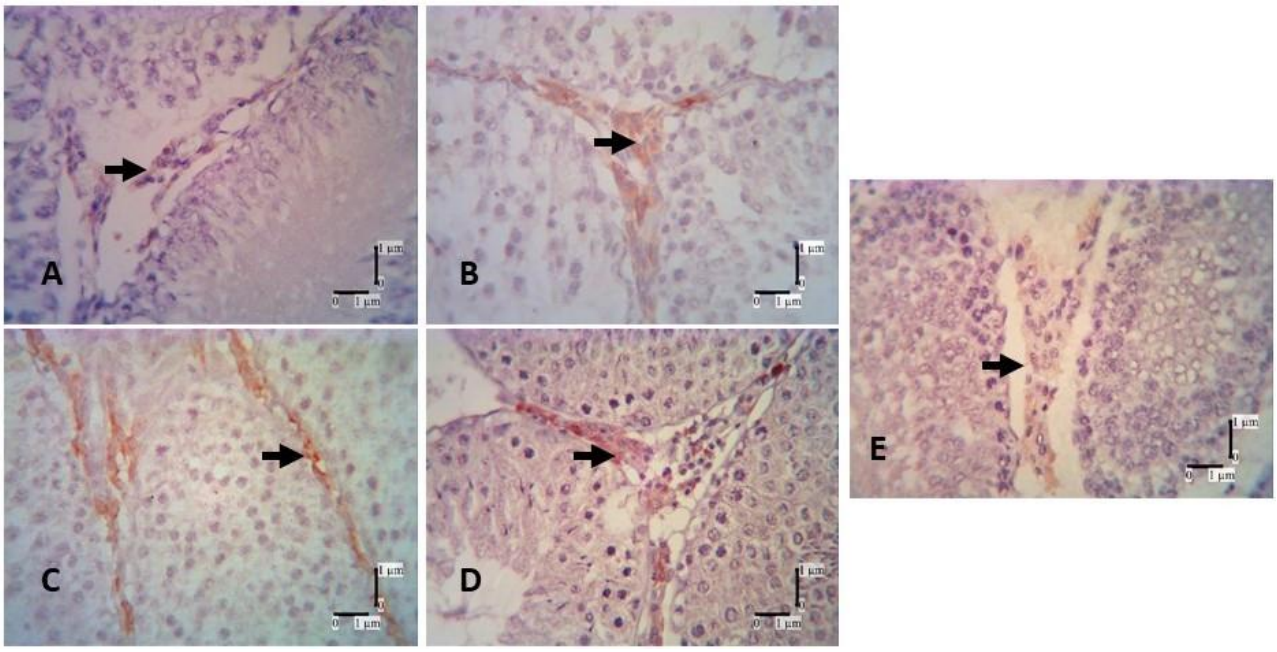


Figure 2. Immunoreactive cells of HSD3B1 with injection dose of 0.5 ml/kg BW mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** One week after the fourth injection, **E:** Control group, black arrow: Immunoreactive cells of HSD3B1

Table 1. Analysis of variance at the number of immunoreactive cells of HSD3B1

Source	Type III Sum of Squares	df	Mean Square	F	p value
Corrected model	7115.575[a]	5	1423.115	9.591	0.000
Intercept	37238.450	1	37238.450	250.973	0.000
Weeks	6189.083	3	2063.028	13.904	0.000
Groups	2551.800	2	1275.900	8.599	0.001
Error	5044.800	34	148.376		
Total	55391.000	40			
Corrected Total	12160.375	39			

[a.] R Squared = 0.585 (Adjusted R Squared = 0.524). R Squared (R^2) showed that the variety of weeks effects on a variety of groups was 58,5%. The significance level less than or equal to 0.05 was considered statistically significant ($p < 0.05$).

Table 2. The mean of immunoreactive cells of HSD3B1 one week after the first, second, third, and fourth injection of mesenchymal stem cells-conditioned medium, and in the control group in rats with testicular dysfunction

Week	Mean
One week after the first injection	21.80[a]
One week after the second injection	30.90[a]
One week after the third injection	41.70[bc]
One week after the fourth injection	52.40[c]

[a,b,c] Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 3. The mean of immunoreactive cells of HSD3B1 in the P1, P2, and control group in rats with testicular dysfunction

Group	Mean
The P1 group	26.93[a]
The P2 group	35.55[ab]
Control group	40.00[b]

The P1 group: mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.2 ml/kg BW; The P2 group: mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.5 ml/kg BW; [a,b] Means within a column with different superscripts differ significantly ($p < 0.05$).

The immunoreactivity of HSD17B1 was detected in Sertoli cells, Leydig cells, and spermatids. The intensity was increased every week. At first, the intensity of immunoreactive cells detected in spermatids and Sertoli were weak at one week after the first injection in the P1 group. The spermatogenic cells in one week after the first and the second injections were still not complete compared to one week after the third injection (Figure 3). The intensity of HSD17B1 increased in the P2 group (Figure 4). One week after the second injection of the P2 group, the spermatogenic cells improved similar to the ones in the control group. One week after the first injection, the intensity was weak. One week after the second and the third injections, the intensity was strong, and one week after the fourth injection, it was very strong. The spermatogenic cells in the group with cisplatin-induced testicular dysfunction were destroyed and lost

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.,* 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

(Figure 5). The immunoreactivity of p450scc was detected in Leydig cells. The immunoreactivity in the P2 group was increased one week after the second injection of mesenchymal stem cells-conditioned medium. One week after the first injection, the intensity was weak. However, one week after the third and the fourth injections was strong. The number of positive cells increased every week (Figure 6). The immunoreactivity of p450scc was detected weak one week after the first injection of 0.2 ml/kg BW mesenchymal stem cells-conditioned medium. Although in one week after the second and the third injections, it was strong (Figure 7). The immunoreactivity of p450scc on the control group was detected in Leydig cells with strong intensity (Figure 8-B). The immunoreactivity of p450scc in the group with cisplatin-induced testicular dysfunction was not expressed. A lot of spermatogenic cells have been damaged (Figure 8-A).

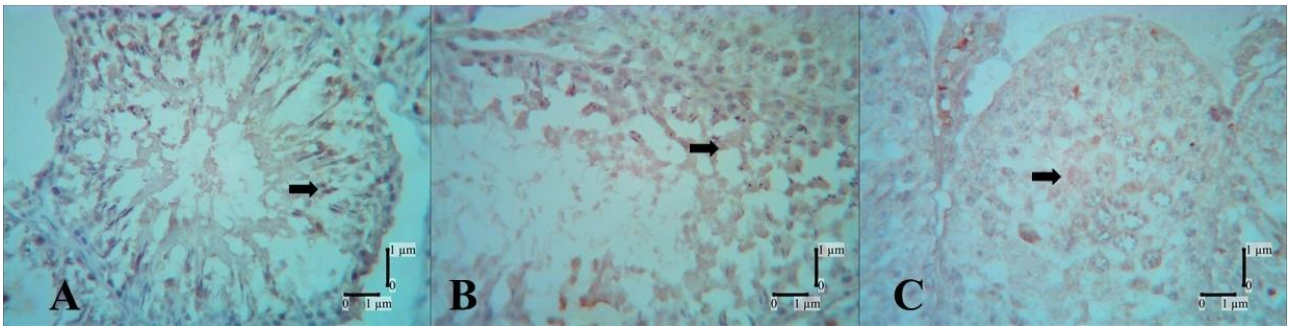


Figure 3. Immunoreactive cells of HSD17B1 of the treated group with an injection dose of 0.2 ml/kg BW of mesenchymal stem cells-conditioned medium. Immunoreactive cells were detected in Sertoli cells and spermatids with a weak intensity one week after the first injection dose of 0.2 ml/kg BW mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, black arrow: IMMUNOREACTIVE cells of HSD17B1

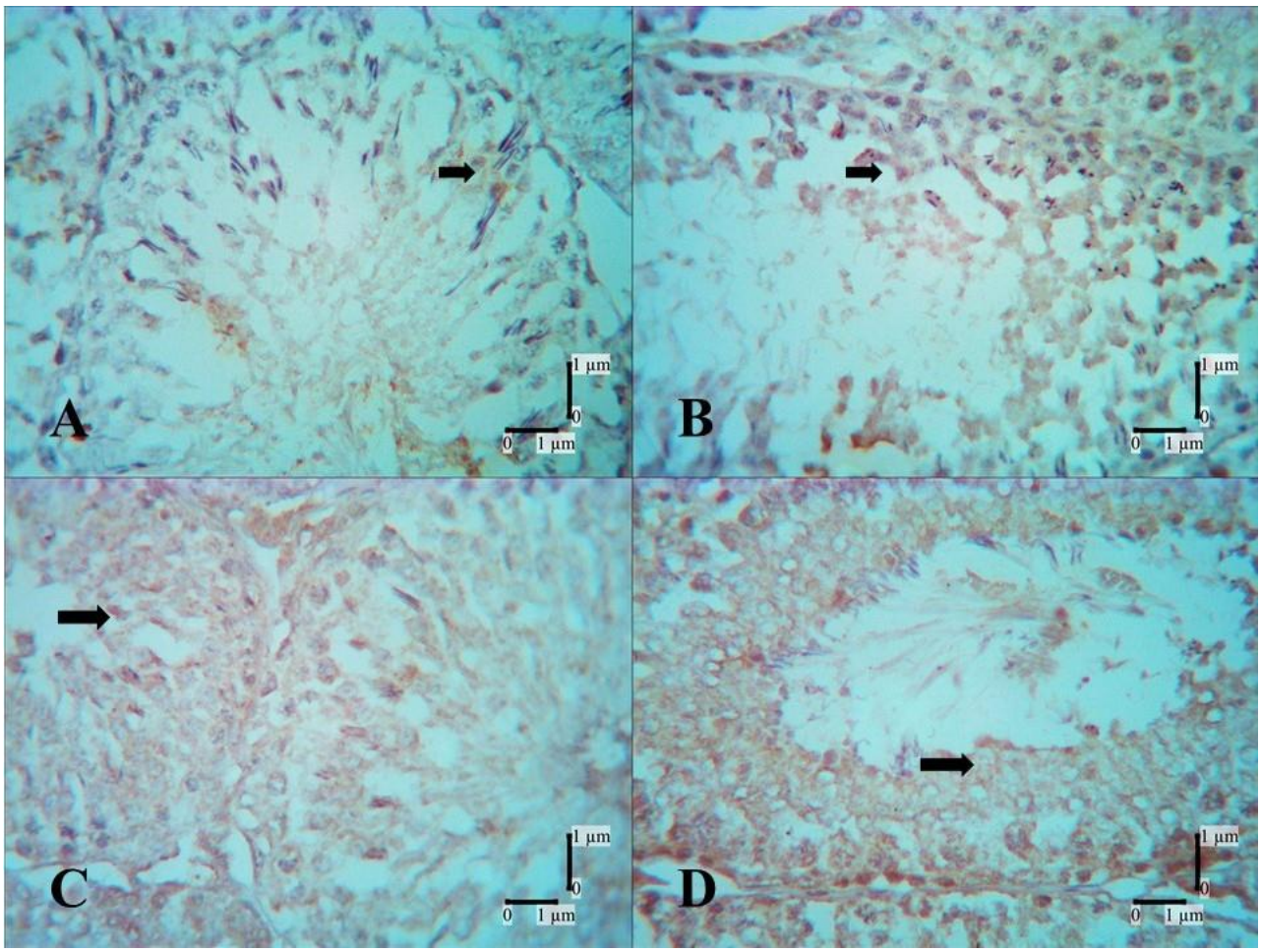


Figure 4. Immunoreactive cells of HSD17B1 in the treated group with an injection dose of 0.5 ml/kg BW of mesenchymal stem cells-conditioned medium treated group. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** One week after the fourth injection, black arrow: Immunoreactive cells of HSD17B1

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.,* 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

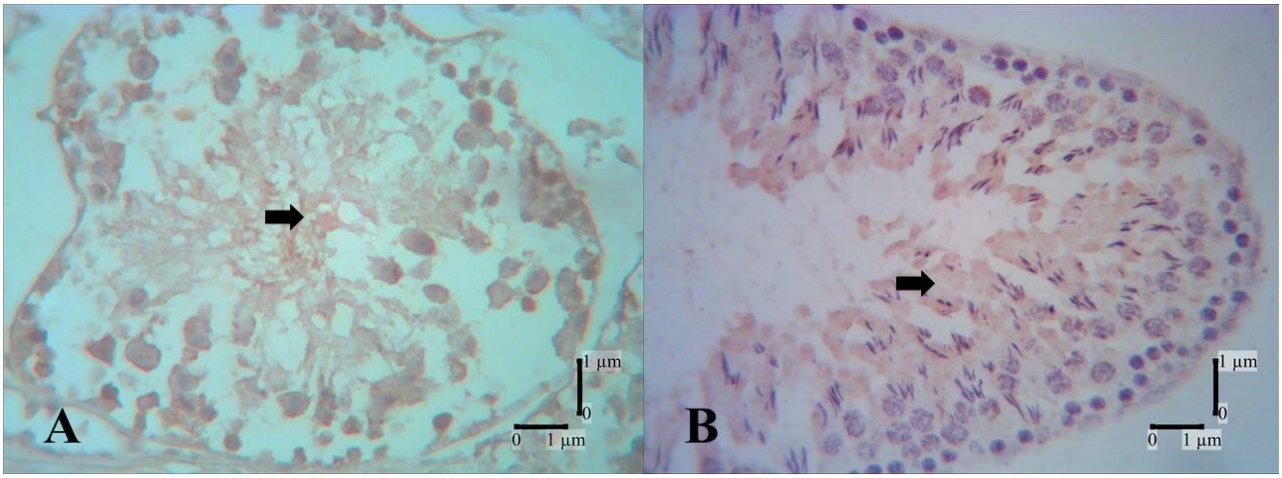


Figure 5. Immunoreactive cells of HSD17B1 in the cisplatin-induced group with the testicular dysfunction and the control group. **A:** Cisplatin-induced testicular dysfunction, **B:** Control group**,** black arrow: Immunoreactive cells of HSD17B1

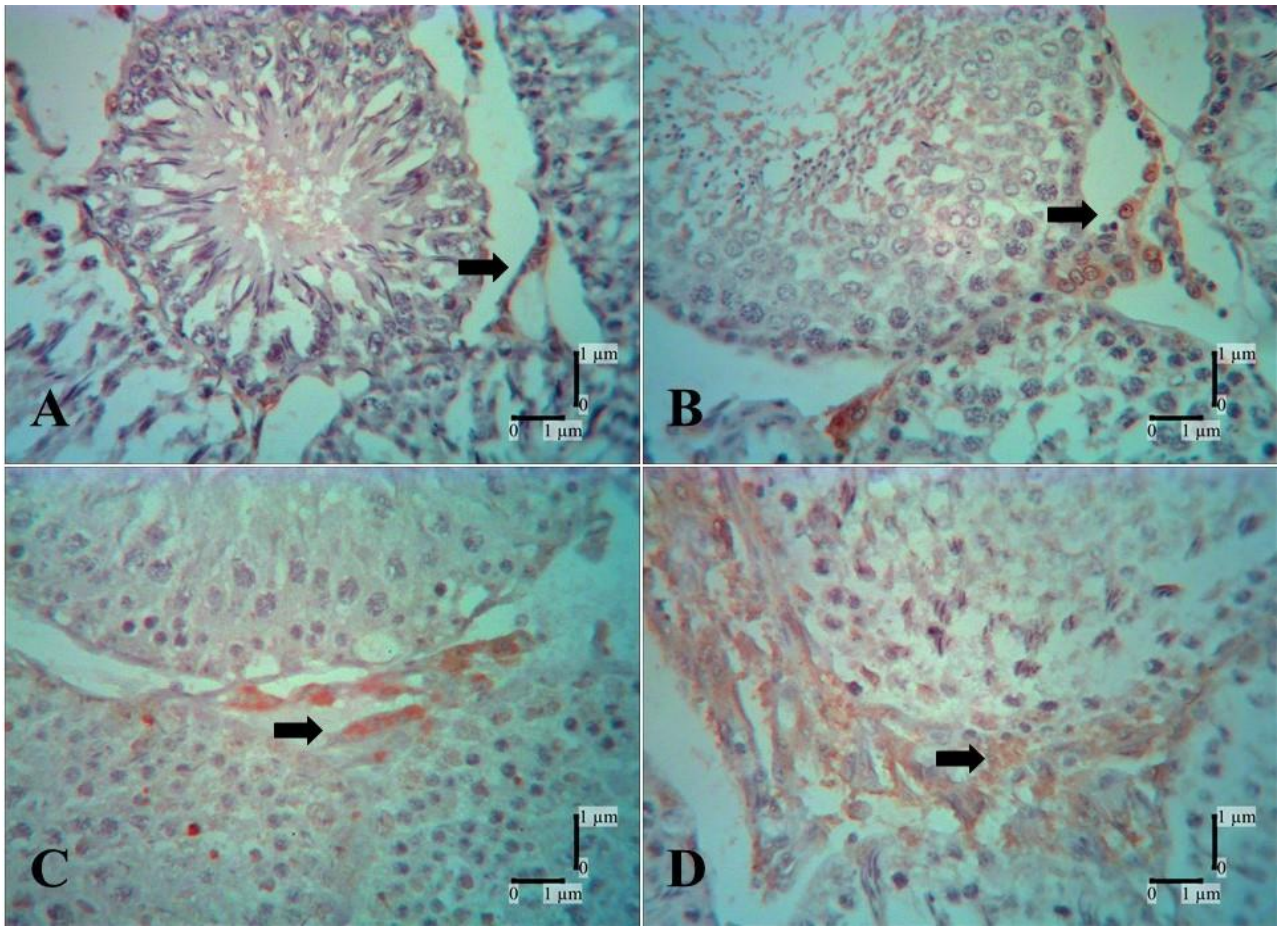


Figure 6. Immunoreactive cells of p450scc in the treated group with an injection dose of 0.5 ml/kg BW of mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** One week after the fourth injection, black arrow: Immunoreactive cells of p450scc.

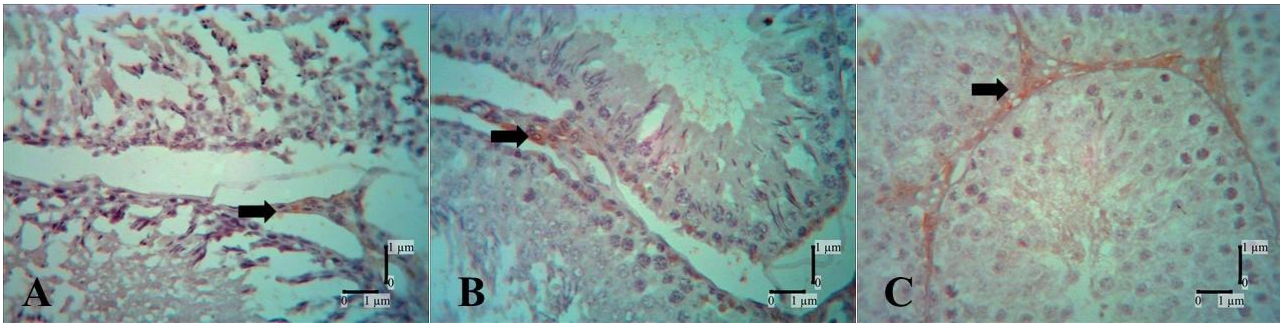


Figure 7. Immunoreactive cells of p450scc in the treated group with an injection dose of 0.2 ml/kg BW of mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, black arrow: Immunoreactive cells of p450scc.

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.,* 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

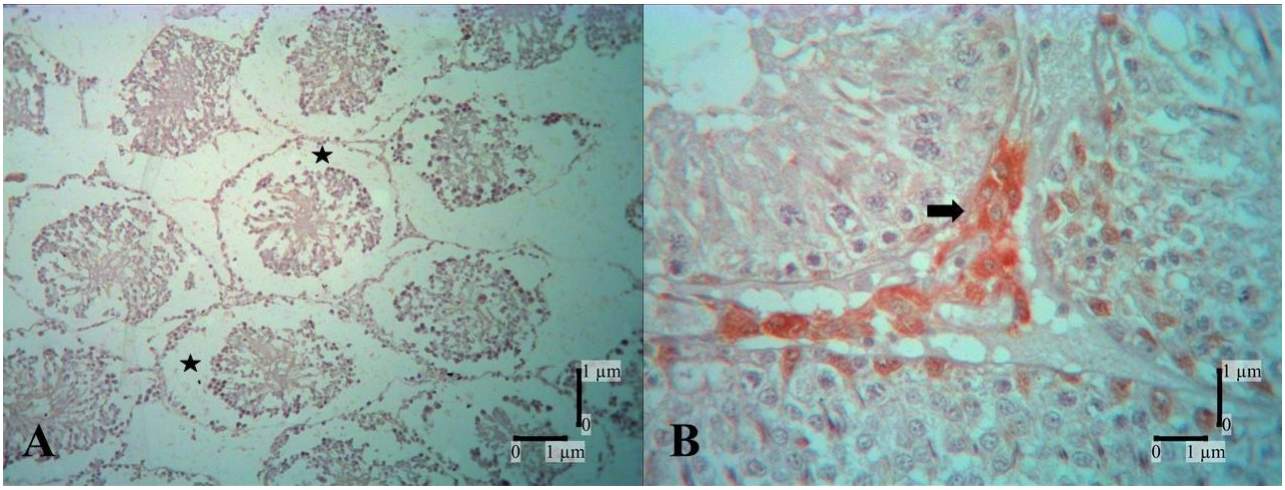


Figure 8. Immunoreactive cells of p450scc in the cisplatin-induced group with the testicular dysfunction and the control group. **A:** Cisplatin-induced testicular dysfunction group. **B:** Control group. Black arrow: Immunoreactive cells of p450scc, black star: Damage of seminiferous tubules.

DISCUSSION

Steroidogenesis involves multiple processes for the biosynthesis of steroid hormones (testosterone) from cholesterol in Leydig cells. Steroid hormones regulate sexual development and are essential for physiological functions. In males, the Leydig cells are considered important for producing the testosterone hormone that is critical for spermatogenesis and maintenance of secondary sexual functions (Wang et al., 2017). Steroidogenesis depends on the steroidogenic enzyme, including P450scc, HSD3B1, HSD17B1, 17a-hydroxylase/C17, 21 lyases (P450c17), and aromatase cytochrome P450 (P450arom) as markers of steroid production (Kobayashi et al., 1998).

The HSD3B1 is a key enzyme necessary for the synthesis of testosterone in Leydig cells in rats. This enzyme has a major catalytic activity in changing pregnenolone, 17-OH-pregnenolone, dehydroepiandrosterone (DHEA), and androstenediol. The deficiency of HSD3B1 affects the gonads (testes in males) and adrenal glands, which reduces the production of the testosterone hormone. The HSD17B1 enzyme is involved in the local regulation of sex steroids (Gunnarsson et al., 2005). The HSD17B1 is involved in the final step of the biosynthesis of testosterone, which catalyzes androstenedione to testosterone hormone (Baker et al., 1997). In immunohistochemistry methods, the immunoreactivity expresses in Leydig cells of interstitial cells of testes (Hanukoglu, 1992). The P450scc catalyzes three chemical reactions, 20a-hydroxylase, 22- hydroxylase, and cuts the side chain of cholesterol into pregnenolone. In the testis, P450scc is detected in Leydig cells (Hanukoglu, 1992; Ulloa-Aguirre and Conn, 2014).

The present study showed that the injection of a mesenchymal stem cells-conditioned medium in rats with cisplatin-induced testicular dysfunction promoted the recovery of testicular degeneration. The immunohistochemistry method detected the expression of HSD3B1, HSD17B1, and p450scc as markers of steroidogenesis. The immunoreactivity of HSD3B1, HSD17B1, and p450scc was shown in Leydig cells, and the intensity increased every week after the injections of mesenchymal stem cells-condition medium. In the immunohistochemical staining, localization of HSD3B1 and HSD17B1 was on steroidogenic cells, selectively expressed in Leydig cells in the interstitial cells of the testis, and it was not found in seminiferous tubules. The upregulation of cellular factors and the testicular microenvironment can occur along with the regeneration and differentiation of Leydig cells via secretion of cellular factors such as epidermal growth factors, insulin-like growth factors-1, and platelet-derived growth factors, that contain in HUMSCs (Lin et al., 1998; Odeh et al., 2014; Zhanget al., 2017). The current study reported the p450scc positive in Leydig cells in both treated groups. The steroidogenic process maybe was developed to make steroid hormones. Stem cell therapy may help to reduce the effect of cancer therapy on testicular dysfunction in the patients coping with cancer (Tamadon et al., 2015). Transplantation of mesenchymal stem cells induced spermatogenesis in azoospermic models (Moreno et al., 2015); differentiated into germ cells, Sertoli cells, and Leydig cells (Lue et al., 2007; Monsefi et al., 2013). Mesenchymal Stem Cells (MSCs) might be able to recover the regeneration of testicular dysfunction by three mechanisms, firstly, MSCs may transform into target cells via appropriate induction conditions. Secondly, growth factors are secreted by MSCs to repair the cell function, and finally, MSCs are incorporated with the endogenous cells repairing the injured tissue function (Mansour et al., 2012; Leatherman, 2013; Tamadon et al., 2015).

Mesenchymal stem cells-conditioned mediums or secretome are factors secreted by stem cells containing growth factors and cytokines, such as vascular endothelial and epidermal growth factors, insulin growth factor, interleukin, tumor necrosis factor, and interferons, that potentially modulates cellular processes, including cell proliferation, differentiation, immunomodulation, migration, angiogenesis, and survival. Mesenchymal stem cells have been used in

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.*, 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

tissue repair, and they can differentiate into Leydig-like cells in vitro (Yazawa et al., 2006). These cellular factors can then promote the regeneration of Leydig cells, and enhance their functions (Ricci et al, 2012; Odeh et al., 2014).

CONCLUSION

The results of the present study showed that the injection of a mesenchymal stem cells-conditioned medium can improve the regeneration of spermatogenic cells, and recover spermatogenesis proved by positive cells of HSD3B1, HSD17B1, and p450scc as markers of steroid production.

DECLARATIONS

Authors' contribution

Linda M. Khasanah contributed to data analysis and the write-up of the manuscript, Teguh Budipitojo created the concepts and designed the experiments, interpreted the data, read and approved the final manuscript. Yuda H. Fibrianto developed the concepts and designed the experiment, and produced secretome. All authors approved the final manuscript and checked the ethical issues.

Competing interests

The authors declared no conflictS of interest.

Acknowledgments

The authors are grateful to the Directorate General of Higher Education (DIKTI), Ministry of Research, Technology and Higher Education of Indonesia (Fund number: 3178/UN1. DITLIT/DIT-LIT/PT/2020).

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Atessahin A, Sahna E, Turk G, Ceribasi AO, Yilmaz S, Yuce A, and Bulmus O (2006). Chemoprotective effect of melatonin against cisplatin-induced testicular toxicity in rats. Journal of Pineal Research, 41: 21-27. DOI: https://www.doi.org/10.1111/j.1600-079X.2006.00327.x

Baker PJ, Sha JH, and O'Shaughnessy PJ (1997). Localisation and regulation of 17beta-hydroxysteroid dehydrogenase type 3 mrna during development in the mouse testis. Molecular and Cellular Endocrinology, 133(2): 127-133. DOI: https://www.doi.org/10.1016/s0303-7207(97)00159-7

Beytur A, Ciftci O, Oguz F, Oguzturk H, and Yılmaz F (2012). Montelukast attenuates side effects of cisplatin including testicular, spermatological, and hormonal damage in male rats. Cancer Chemotherapy and Pharmacology, 69(1): 207-213. DOI: https://www.doi.org/10.1007/s00280-011-1692-y

Gunnarsson C, Hellqvist E, and Stal O (2005). 17β-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. British Journal of Cancer, 92(3): 547-552. DOI: https://www.doi.org/10.1038/sj.bjc.6602375

Hanukoglu I (1992). Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. The Journal of Steroid Biochemistry and Molecular Biology, 43(8): 779-804. DOI: https://www.doi.org/10.1016/0960-0760(92)90307-5

Kobayashi T, Nakamura M, Kajiura-Kobayashi H, Young G, and Nagahama Y (1998). Immunolocalization of steroidogenic enzymes (p450scc, p450c17, p450arom, and 3b-hsd) in immature and mature testes of rainbow trout (*Oncorhynchus mykiss*). Cell and Tissue Research, 292: 573-577. DOI: https://www.doi.org/10.1007/s004410051086

Kusindarta DL, Wihadmadyatami H, Fibrianto YH, Nugroho WS, Susetya H, Musana DK, Wijayanto H, Prihatna SA, and Wahyuni AETH (2016). Human umbilical mesenchymal stem cells conditioned medium promote primary wound healing regeneration. Veterinary World, 9(6): 605-610. DOI: https://www.doi.org/10.14202/vetworld.2016.605-610

Leatherman J (2013). Stem cells supporting other stem cells. Frontiers in Genetics, 4: 1-6. DOI: https://www.doi.org/10.3389/fgene.2013.00257

Lin T, Wang D, Hu J, and Stocco DM (1998). Upregulation of human chorionic gonadotrophin-induced steroidogenic acute regulatory protein by insulin-like growth factor-i in rat leydig cells. Endocrine, 8(1): 73-78. DOI: https://www.doi.org/10.1385/endo:8:1:73

Lue Y, Erkkila K, Liu PY, Ma K, Wang C, Hikim AS, and Swerdloff RS (2007). Fate of bone marrow stem cells transplanted into the testis: Potential implication for men with testicular failure. The American Journal of Pathology, 170(3): 899-908. DOI: https://www.doi.org/10.2353/ajpath.2007.060543

Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, and Blin-Wakkach C (2012). Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. The Journal of Experimental Medicine, 209(3): 537-549. DOI: https://www.doi.org/10.1084/jem.20110994

Monsefi M, Fereydouni B, Rohani L, and Talaei T (2013). Mesenchymal stem cells repair germinal cells of seminiferous tubules of sterile rats. Iranian Journal of Reproductive Medicine, 11(7): 537-544. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3941348/

Moreno I, Míguez-Forjan JM, and Simón C (2015). Artificial gametes from stem cells. Clinical and Experimental Reproductive Medicine, 42(2): 33-44. DOI: https://www.doi.org/10.5653/cerm.2015.42.2.33

Nugroho WS, Kusindarta DL, Susetya H, Fitriana I, Mukyani GT, Fibrianto YH, Haryanto A, and Budipitojo T (2016). The structural and functional recovery of pancreatic b-cell in type 1 diabetes mellitus induced mesenchymal stem cells-conditioned medium. Veterinary World, 9(5): 535-539. DOI: https://www.doi.org/10.14202/vetworld.2016.535-539

Odeh HM, Kleinguetl C, Ge R, Zirkin BR, and Chen H (2014). Regulation of the proliferation and differentiation of leydig stem cells in the adult

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.*, 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

testis. Biology of Reproduction, 90(6): 1-7. DOI: https://www.doi.org/10.1095/biolreprod.114.117473

Padeta I, Nugroho WS, Kusindarta DL, Fibrianto YH, and Budipitojo T (2017). Mesenchymal stem cells-conditioned medium promote the recovery of skin burn wound. Asian Journal of Animal and Veterinary Advances, 12(3): 132-141. DOI: https://www.doi.org/10.3923/ajava.2017.132.141

Prihatno SA, Adi YK, and Budipitojo T (2020). Immunolocalization of IL-6 and IL-10 in the testicular tissue of testicular dysfunction rat treated with secretome. Journal of Advanced Veterinary and Animal Research, 7(3): 514-520. DOI: http://doi.org/10.5455/javar.2020.g448

Prihatno SA, Padeta I, Larasati AD, Sundari B, Hidayati A, Fibrianto YH, Budipitojo T (2018). Effects of secretome on cisplatin-induced testicular dysfunction in rats. Veterinary World, 11(9): 1349-1356. DOI: https://www.doi.org/10.14202/vetworld.2018.1349-1356

Reddy KP, Madhu P, and Reddy PS (2016). Protective effects of resveratrol against cisplatin-induced testicular and epididymal toxicity in rats. Food and Chemical Toxicology, 91: 65-72. DOI: https://www.doi.org/10.1016/j.fct.2016.02.017

Ricci G, Guglielmo MC, Caruso M, Ferranti F, Canipari R, Galdieri M, and Catizone A (2012). Hepatocyte growth factor is a mouse fetal leydig cell terminal differentiation factor. Biology of Reproduction, 87(6): 146. DOI: https://www.doi.org/10.1095/biolreprod.112.104638

Simsek N, Koc A, Karadeniz A, Yildirim ME, Celik HT, Sari E, and Kara A (2016). Ameliorative effect of selenium in cisplatin-induced testicular damage in rats. Acta Histochemica, 118(3): 263-270. DOI: https://www.doi.org/10.1016/j.acthis.2016.02.002

Tamadon A, Mehrabani D, Rahmanifar F, Jahromi AR, Panahi M, Zare S, Khodabandeh Z, Jahromi IR, Tanideh N, Dianatpour M et al. (2015). Induction of spermatogenesis by bone marrow-derived mesenchymal stem cells in busulfan-induced azoospermia in hamster. International Journal of Stem Cells, 8(2): 134-145. DOI: https://www.doi.org/10.15283/ijsc.2015.8.2.134

Ulloa-Aguirre A, and Conn PM (2014). Cellular endocrinology in health and disease. Elsevier Inc, USA, pp. 207-214. Available at: https://www.elsevier.com/books/cellular-endocrinology-in-health-and-disease/ulloa-aguirre/978-0-12-819801-8

Wang Y, Chena F, Yea L, Zirkinb B, and Haolin C (2017). Steroidogenesis in leydig cells: Effects of aging and environmental factors. Reproduction, 154(4): 111-122. DOI: https://www.doi.org/10.1530/REP-17-0064

Yang C, Du YK, Wang J, Luan P, Yang QL, Huang WH, and Yuan L (2015). Transplanted adipose‐derived stem cells ameliorate testicular dysfunction in a d‐galactose‐induced aging rat model. Journal of Cellular Physiology, 230(10): 2403-2414. DOI: https://www.doi.org/10.1002/jcp.24970

Yazawa T, Mizutani T, Yamada K, Kawata H, Sekiguchi T, Yoshino M, Kajitani T, Shou Z, Umezawa A, and Miyamoto K (2006). Differentiation of adult stem cells derived from bone marrow stroma into leydig or adrenocortical cells. Endocrinology, 147(9): 4104-4111. DOI: https://www.doi.org/10.1210/en.2006-0162

Zhang ZH, Xing XY, Ju GQ, Zhong L, and Sun J (2017). Mesenchymal stem cells from human umbilical cord ameliorate testicular dysfunction in a male rat hypogonadism model. Asian Journal of Andrology, 19(5): 543-547. DOI: https://www.doi.org/10.4103/1008-682X.186186

To cite this paper: Khasanah LM, Budipitojo T, and Fibrianto YH (2021). Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium. *World Vet. J.*, 11 (3): 368-376. DOI: https://dx.doi.org/10.54203/scil.2021.wvj48

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 377-383, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats

Mehran Hadi[1], Elham Moghtadaei-Khorasgani[2]*, and Mohammad Hossein Etesamnia[1]

[1]*Graduate in Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*
[2]*Department of Pathobiology, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*

*Corresponding author's Email: moghtadaiee@gmail.com; ORCID: 0000-0002-8666-5104

ORIGINAL ARTICLE
pii: S232245682100049-11
Received: 26 June 2021
Accepted: 19 August 2021

ABSTRACT

Diabetic wounds and *cutaneous* wounds are important issues in medical science. Basil is an herbaceous plant and has compounds such as terpenes, flavonoids, and antioxidant properties. A total of 50 male Wistar rats were allocated into 10 groups including the healthy group without treatment, the healthy group treated with 1% phenytoin, eucerin, 5% basil seed extract, 10% basil seed, diabetic group without treatment, 1% phenytoin, eucerin. After anesthesia of rats, we made a 4 cm^2 wound on the back of the animal, and different histopathological characteristics were examined, and also on days 3, 7, and 21, the area of wounds was analyzed. In the healthy group treated with 10% basil seed extract, it was revealed that the wound size was significantly healed in the research days. In the diabetic rat groups, the decrease in the wound area was not significant and there was no significant difference between groups. Histopathological findings on day 21 in the healthy group treated with 10% basil seed extract revealed proper epidermis formation and relatively dense dermis containing collagen filaments. While in the diabetic groups, healing was slower. The results indicated that basil extract with anti-inflammatory and antioxidant characteristics can quicken the repair of *cutaneous* wounds.

Keywords: Basil, Diabetes, Histopathology, Wound

INTRODUCTION

Diabetes mellitus is the most prevalent endocrine disease that is related to carbohydrate, fat, and protein metabolism disorders. The disease results from the lack of cellular absorption of blood sugar because of diminished insulin or resistance of body cells to insulin (Siljander et al., 2019). Diabetes is a common reason for delayed or compromised wound healing. 15% of individuals with diabetes mellitus develop lower-extremity ulcers (Walko et al., 2015). *Cutaneous* wounds and a decrease in their healing time are considered important concerns in medical and surgical sciences. Wound healing is an active process in which a series of associations take place between diverse cells, cytokine mediators, and extracellular matrix (Foo et al., 2017). which this process includes several stages. However, in general, it is a constant process, so that each stage overlaps with the next stage. These stages include coagulation, inflammation, proliferation, and resuscitation (Reinke and Sorg, 2012). Diabetes is a metabolic illness and leads to complications, such as neurological (neuropathy), ocular (retinopathy), and renal problems (nephropathy, Shahrokh et al., 2021). One of the most significant chronic complications of diabetes is diabetic foot ulcers, which can finally cause amputation. It is now well-known that diabetes leads to a rise in free radicals at the cell cytoplasm (Khaksar et al., 2011) . Free radicals are very reactive because of their single electrons, and their excessive production causes damage in macromolecules such as DNA and proteins (Ibrahim et al., 2018). Research has revealed that antioxidants lessen DNA fragmentation caused by oxidative stress due to diabetes (Guo et al., 2020). It has been revealed that oral administration of vitamins E, C, and A improves plasma levels of antioxidant enzymes in diabetic patients. Various reports indicate that plants have many antioxidant compounds, such as different types of vitamins, carotenoids, and phenols (phenolic acids, flavonoids, and tannins) (Miri et al., 2020). Although diverse approaches are applied today to treat diabetic wounds, so far no efficient and without side effect treatment has been provided (Everett and Mathioudakis, 2018; Pourmahdi and Faedmaleki, 2021). In this regard, medicinal plants are of superior significance, because medicinal plants have rich sources of natural antioxidants that are utilized in traditional medicine to control and treat many skin diseases (Aziz et al., 2017). Currently, there is a serious trend to use medicinal plants because of the lack of side effects and the effective compounds in plants (Amalraj and Gopi, 2017).

Basil is a herbaceous, annual, fragrant plant from the family Lamiaceae, which is currently grown in most parts of Iran. Basil leaves can be utilized in many cases such as treating insomnia, healing of scratches, and as a painkiller (Veith et al., 2019). Basil contains numerous compounds such as terpenes, sesquiterpenoids, and flavonoids. Flavonoids have strong antioxidant characteristics (Denaro et al., 2020). The significance of basil is that besides flavor in food, its main active ingredient has antimicrobial effects and is efficient against different types of Gram-positive, Gram-negative

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

pathogenic bacteria, molds, and yeasts. The antimicrobial impacts of basil extract are associated with its phenolic compounds. The most key phenolic compounds in basil are linalool and methyl chavicol (Adeli-Sardou et al., 2019). Consequently, basil extract because of its plentiful antioxidants and anti-inflammatory agents can be efficient in the healing process by stimulating angiogenesis (Gutiérrez-Grijalva et al., 2018). Therefore, this research aimed to examine the repairing effects of basil seed extract concerning its advantageous effects (anti-inflammatory, anti-apoptotic, and anti-free radical) on *cutaneous* wound healing in diabetic rats.

MATERIALS AND METHODS

Ethical approval

This is experimental laboratory research that was conducted in the Animal Research Laboratory of Shahrekord Azad University in 2019.

Preparing extract

If the substances in plant cells are extracted from plants with solvents such as water or organic solvents, this product is called an extract. If the extract is extracted with water or ethanol, it can be applied in medicines without eliminating the solvent. To prepare the extract, 500 g of plant seeds were added to 1000 ml of 70% ethanol alcohol and incubated in a water bath at 45 ° C for 3 days in a container covered with aluminum foil. The extract was then separated by filter paper and a funnel and concentrated by vacuum distillation at 70 °C as much as possible (Mirjalili et al., 2017).

Laboratory Animals

For this research, 50 male rats with a specific weight range (200-250g) were obtained from the Laboratory Animal Breeding Center of Islamic Azad University, Shahrekord Branch, Iran. The rats were randomly allocated into ten groups of five rats. The rats were maintained in standard temperature conditions at 25-30°C and light conditions of 12 hours of light and 12 hours of darkness. Likewise, in terms of access to water and food and space away from pollution, the animals were in good condition, and to a high degree, all the principles in "Ethical Guide to Animal Research" were applied.

Experimental design and grouping

In this study, 50 male Wistar rats were allocated into 10 groups (healthy group without treatment, healthy group treated with 1% phenytoin, healthy group treated with eucerin, healthy group treated with 5% basil seed extract, healthy group treated with 10% basil seed extract, diabetic group without treatment, diabetic group treated with 1% phenytoin, diabetic group treated with eucerin). To induce type 1diabetes in 5 groups of mice, intraperitoneal injection of streptozotocin made by Sigma Company of Germany was used at a dose of 50 mg/kg (Sadoughi, 2013). After anesthetizing the mice with an intraperitoneal injection of ketamine (50mg/kg) and xylazine (10mg/kg) (Pourmahdi and Faedmaleki, 2021), a wound of 4 cm^2-width was created on the back of all animals in the experimental groups. Different histopathological characteristics were surveyed on days 3, 7, and 21, moreover, wound area was statistically analyzed on the same days according to Firoozeh et al. (2019).

Data analysis method

All values are reported as mean ± S.E.M. and the statistical significance of differences among groups was assessed using one-way ANOVA. Tukey test was used to measure specific differences between pairs of means. A value of $p < 0.05$ was considered significant.

Ethical consideration

Ethics committee approval was received for this study from the ethics committee of Shahrekord University, Iran, is IR.IAU.SHK.REC.10501972024).

RESULTS AND DISCUSSION

Morphological findings in the healthy group treated with 10% basil seed extract indicated a significant tendency to wound healing in the research days. This healing trend was followed in the phenytoin group with almost a similar rate. This wound healing in the 10% extract group on days 7 and 21 was significant ($p < 0.05$), and in the phenytoin-treated

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

group indicated a significant reduction on day 21 ($p < 0.05$). Then, in the group treated with 5% extract and eucerin, the wound area was decreased at a slower rate and did not display a significant decrease ($p > 0.05$). In the diabetic rat groups, the decrease in wound area was small and there was no significant difference between the groups. But in terms of a decrease in the wound area, the 10% basil extract and phenytoin groups had a better improvement (Graphs 1, 2, 3). Histopathological findings in this research indicated that on the third day, almost all groups had significant inflammation, edema, and bleeding. Tissue examination on day seven also revealed that in the healthy group treated with basil, the amount of inflammation was very small, little new blood vessels were formed, and collagen filaments have good density. While in the diabetic group, the number of collagen filaments was low and tissue had edema. On the 21, in the healthy group treated with 10% basil extract, epithelium formation was proper and all-around, and relatively dense collagen filaments were visible in the tissue. While in the diabetic rat group treated with 10% basil extract, the epithelium was not formed properly and the collagen filaments were not in the required order and edema was considerable in the tissue. In general, based on the obtained results, particularly histopathologically, an excellent tendency to collagen formation and the lack of secondary infection in the site was witnessed, which is perhaps because of antimicrobial and anti-inflammatory characteristics and strong phenolic antioxidants in basil (Figure 1, Table 1). Wounds are clefts and modifications in the anatomical structure and activity of the skin. cutaneous wounds are caused by different reasons such as burns, mechanical injuries, etc. When a wound forms on the skin, a cellular reaction arises instantly. After a *cutaneous* wound, leukocytes are the first cells to appear at the wound site. After some hours, granulocytes and macrophages accumulate at the margin of the wound, and after a few days, the proliferation of derm begins and fibroblasts enter the area. Macrophages release compounds that lead to the attraction and proliferation of fibroblasts. Fibroblasts steadily replace most of the leukocytes, and collagen proliferation also enhances. The production of new vessels in wound tissue is essential for wound healing, and this process is affected by the mediators released by macrophages. The contractile phenomenon happens when fibronectin provides an appropriate substrate for cell migration and growth during granulated tissue development and attaches to myofibroblasts. Connects. After healing with collagen filaments, the wound site looks like the skin tissue before the wound is produced, and the blood and oxygen flow in the vessels of the wound site is increased through dilation of the arteries. Many efforts have been made to heal diabetic wounds, including:

In 2017, studied the influence of aqueous extract of Morus nigra fruit on wound healing in type 1 diabetic mice and the findings revealed that its fruit extract can quicken wound healing in diabetic mice. It also increases tissue repair indicators such as epithelial layer development and collagen layer density (Mirjalili et al., 2017). Bagheri et al. (2016) studied the effect of sesame alcoholic extract on the healing of diabetic wounds in male Wistar rats, and the results revealed that sesame alcoholic extract quickens the healing of *cutaneous* wounds in healthy and diabetic individuals.

Although diverse approaches are applied today to treat diabetic wounds, so far no efficient and without side effect treatment has been provided. In this regard, medicinal plants are of superior significance, because medicinal plants have rich sources of natural antioxidants that are utilized in traditional medicine in order to control and treat many skin diseases (Shahrokh et al., 2021). Currently, there is a serious trend to use medicinal plants because of the lack of different side effects and the effective compounds in plants (Denaro et al., 2020). Basil is a herbaceous, annual, fragrant plant from the family Lamiaceae, which is currently grown in most parts of Iran. Basil leaves can be utilized in many cases such as treating insomnia, healing of scratches, and as a painkiller. Basil contains numerous compounds such as terpenes, sesquiterpenoids, and flavonoids. Flavonoids have strong antioxidant characteristics (Salem et al., 2019). The significance of basil is that besides flavor in food, its main active ingredient has antimicrobial effects and is efficient against different types of Gram-positive, Gram-negative pathogenic bacteria, molds, and yeasts (Mostafavi et al., 2019). The antimicrobial impacts of basil extract are associated with its phenolic compounds. The most key phenolic compounds in basil are linalool and methyl chavicol (Filip, 2017). Consequently, basil extract because of its plentiful antioxidants and anti-inflammatory agents can be efficient in the healing process by stimulating angiogenesis (Brereton et al., 2015). Therefore, in this research, the repairing effects of basil seed extract on *cutaneous* wound healing in diabetic and healthy rats were evaluated concerning its advantageous effects (anti-inflammatory, anti-apoptotic, and anti-free radical).

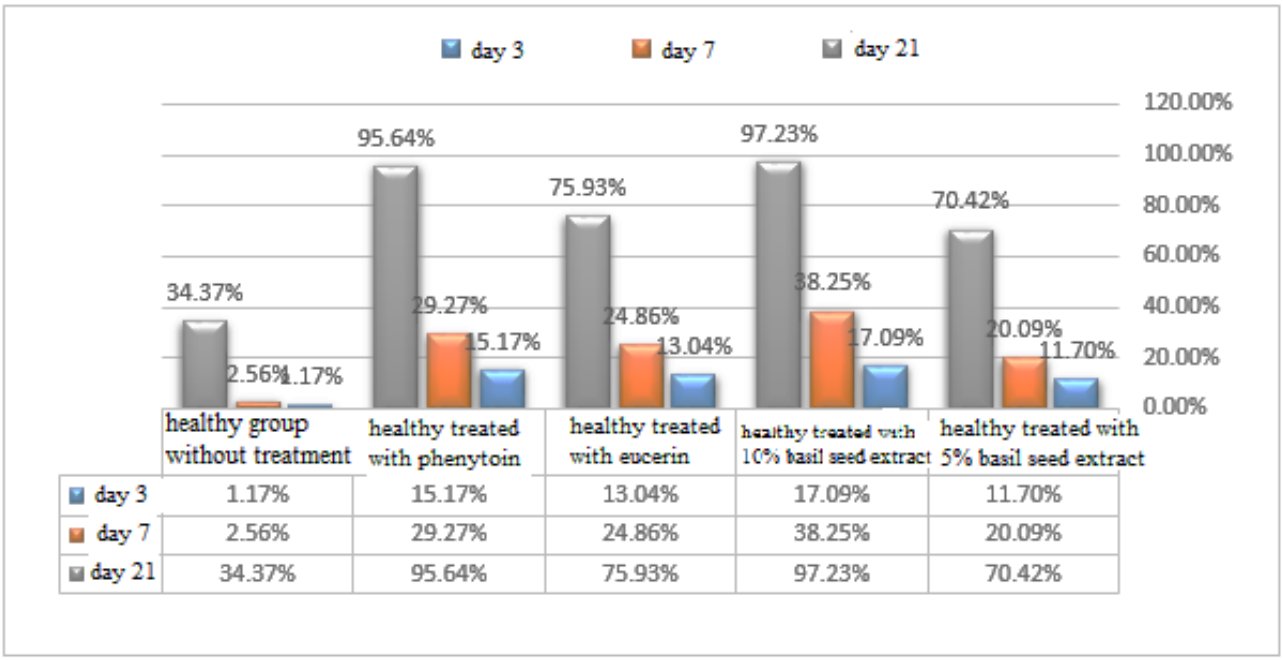
Histopathological findings in this research indicated that on the 3rd day, almost all groups had significant inflammation, edema, and bleeding. Tissue examination on day seven also revealed that in the healthy group treated with basil, the amount of inflammation was very small, little new blood vessels were formed, and collagen filaments have good density whreas the number of collagen filaments was low and tissue had edema in the diabetic group. On day 21, epithelium formation was proper and all-around, and relatively dense collagen filaments were visible in the tissue in the healthy group treated with 10% basil extract. However, in the diabetic rat group treated with 10% basil extract, the

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

epithelium was not formed properly and the collagen filaments were not in the required order and edema was considerable in the tissue.

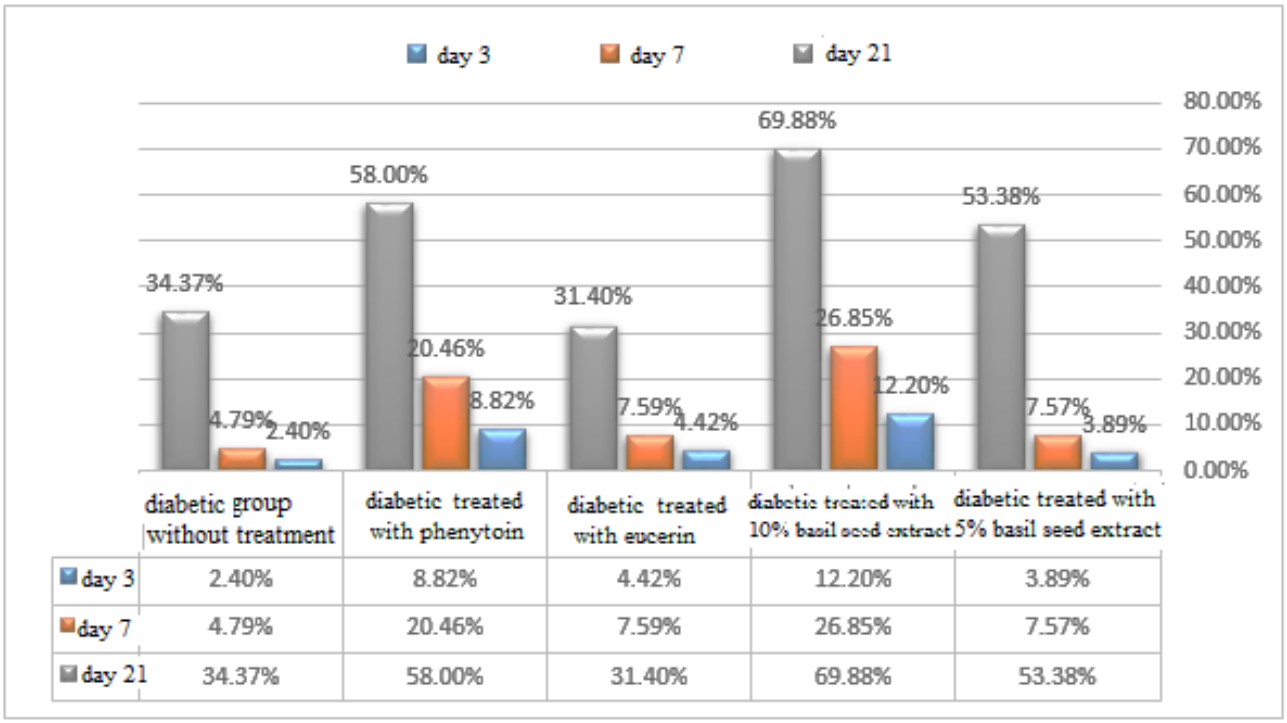
Morphological findings in the healthy group treated with 10% basil seed extract indicated a significant tendency to wound healing in the research days. This healing trend was followed in the phenytoin group with almost a similar rate. This wound healing in the 10% extract group on days 7 and 21 was significant ($p < 0.05$), and in the phenytoin-treated group indicated a significant reduction on day 21 ($p < 0.05$). Then, in the group treated with 5% extract and eucerin, the wound area was decreased at a slower rate and did not display a significant decrease ($p > 0.05$).

In the diabetic rat groups, the decrease in wound area was small and there was no significant difference between the groups. But in terms of a decrease in the wound area, the 10% basil extract and phenytoin groups had a better improvement.



	healthy group without treatment	healthy treated with phenytoin	healthy treated with eucerin	healthy treated with 10% basil seed extract	healthy treated with 5% basil seed extract
day 3	1.17%	15.17%	13.04%	17.09%	11.70%
day 7	2.56%	29.27%	24.86%	38.25%	20.09%
day 21	34.37%	95.64%	75.93%	97.23%	70.42%

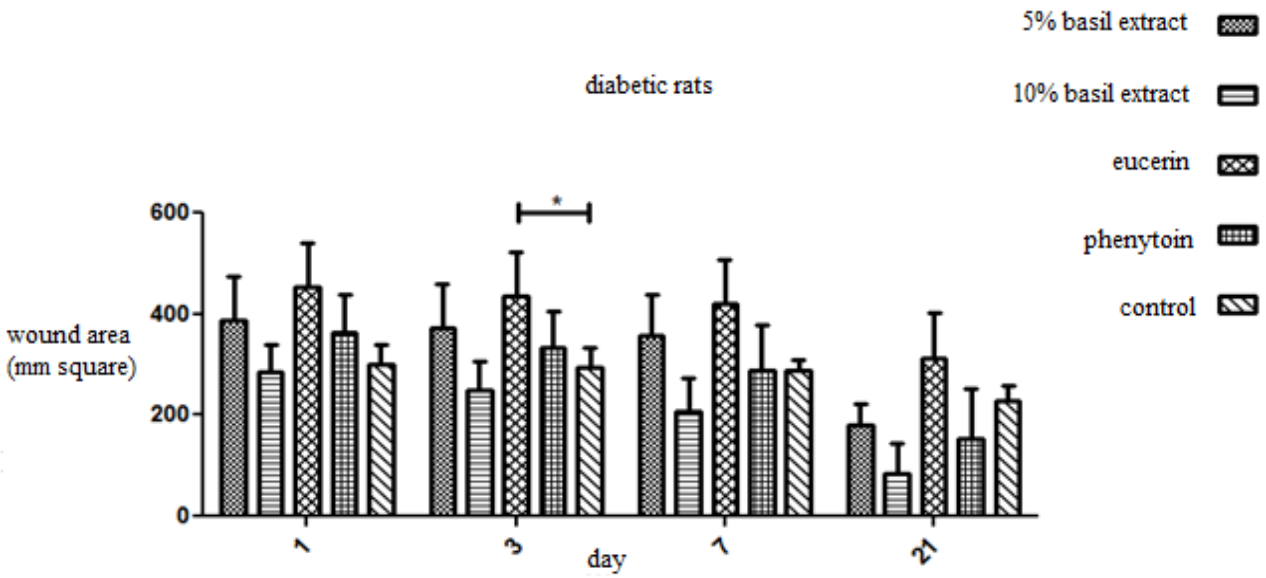
Graph 1. Comparison of the mean decrease in wound area compared to the first day in non-diabetic groups during the study period



	diabetic group without treatment	diabetic treated with phenytoin	diabetic treated with eucerin	diabetic treated with 10% basil seed extract	diabetic treated with 5% basil seed extract
day 3	2.40%	8.82%	4.42%	12.20%	3.89%
day 7	4.79%	20.46%	7.59%	26.85%	7.57%
day 21	34.37%	58.00%	31.40%	69.88%	53.38%

Graph 2. Comparison of the mean decrease in wound area compared to the first day in diabetic groups during the study period

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49



Graph 3. Comparison of the wound area among diabetic rats during the study period

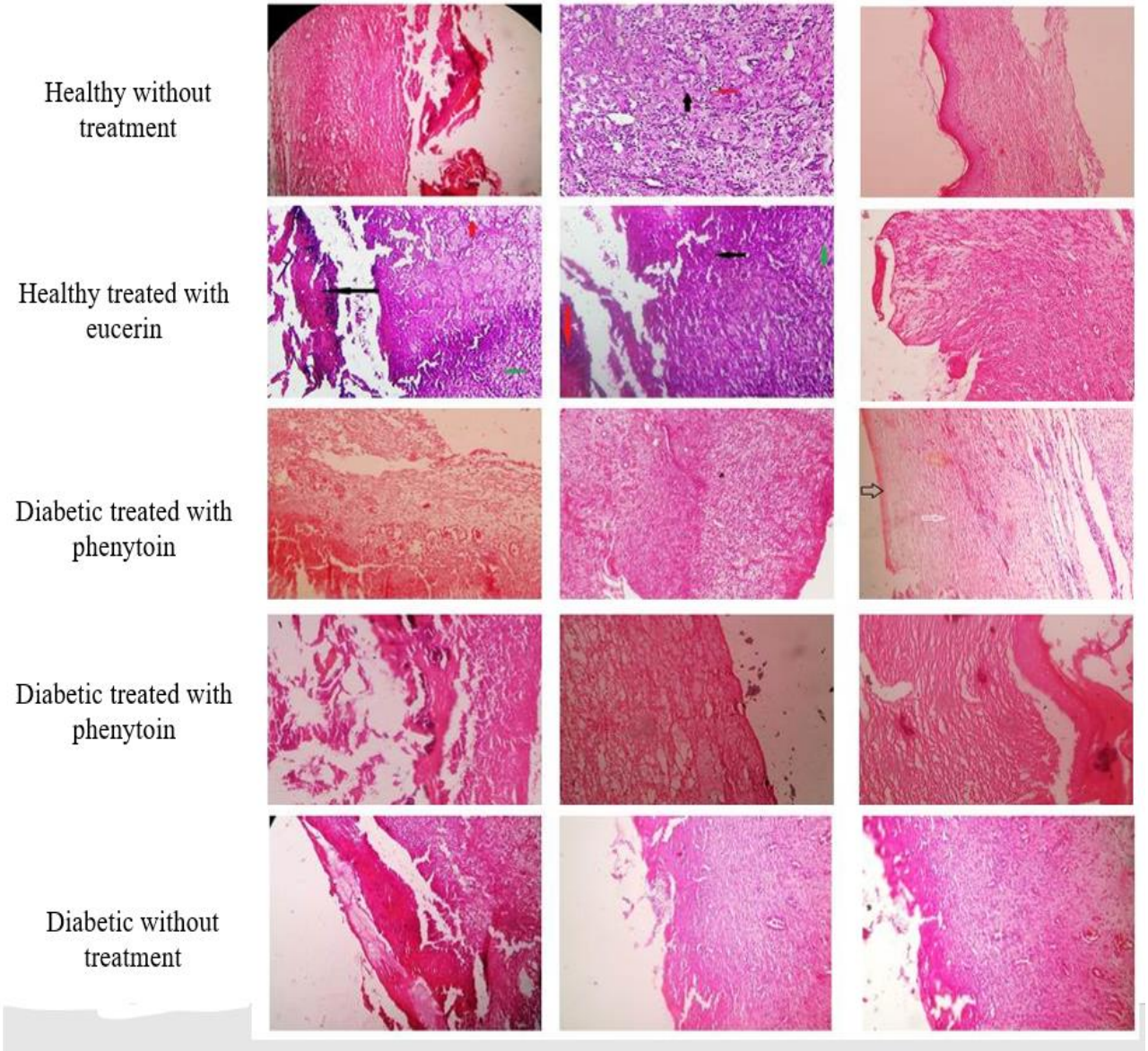


Figure 1. Histological section of wound healed on days 3,7, and 21 post-surgery

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

Table 1. Results of histopathological examinations of wound 3, 7, and 21 days after wound healing

Groups	Day	Inflammation	Bleeding	Angiogenesis	Collagen fiber	Epithelium Fiber	Clot
Healthy without treatment	3	+++	+++	++	+	–	–
	7	++	+++	+	+	–	–
	21	+	+	+	++	+	–
Diabetic without treatment	3	++	++	++	+	–	+
	7	+++	+	+	+	–	++
	21	+	+	+	++	–	–
Healthy phenytoin	3	+	+	++	+	–	++
	7	+	–	++	++	–	–
	21	–	–	–	+++	++	–
Diabetic phenytoin	3	++	++	–	+	–	++
	7	+	+	++	++	–	–
	21	+	+	+	++	–	–
Healthy eucerin	3	++	+	+	+	–	++
	7	++	++	++	++	–	–
	21	+	–	–	++	+	–
Healthy with 5 %basil	3	+	+	++	+	–	++
	7	++	+	+	+	–	–
	21	+	–	–	++	++	–
Diabetic 5%basil	3	++	++	+++	+	–	–
	7	++	+	++	+	–	–
	21	+	–	–	++	+	–
Healthy 10%basil	3	+	+	+	++	–	–
	7	–	–	++	++	–	–
	21	–	–	–	++	++	–
Diabetic 10%basil	3	+	+	+	+	–	+++
	7	+	+	+	+	–	–
	21	+	+	–	+	+	–

+++: Severe ++: Moderate +: Light –: Absence

CONCLUSION

In general, based on the obtained results, particularly histopathologically, an excellent tendency to collagen formation and the lack of secondary infection in the site was witnessed, which is perhaps because of antimicrobial and anti-inflammatory characteristics and strong phenolic antioxidants in basil. Therefore, these seeds can be used in the production of wound healing ointments.

DECLARATION

Authors' contribution

All authors have read and agreed to the published version of the manuscript. Mehran Hadi, Elham Moghtadaei-Khorasgani, and Mohammad Hossein Etesamnia contributed to the design and implementation of the research, the analysis of the results, and the writing of the manuscript.

Competing interests

The authors have no competing interests. In addition, we have no authorship or article publication conflict. Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

Acknowledgments

The authors are grateful to the Faculty of Veterinary Medicine of Shahrekord Azad University.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Adeli-Sardou M, Yaghoobi MM, Torkzadeh-Mahani M, and Dodel M (2019). Controlled release of lawsone from polycaprolactone/gelatin electrospun nanofibers for skin tissue regeneration. International Journal of Biological Macromolecules, 124: 478-491. DOI: https://www.doi.org/10.1016/j.ijbiomac.2018.11.237

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

Amalraj A, and Gopi S (2017). Biological activities and medicinal properties of Asafoetida: A review. Journal of Traditional and Complementary Medicine, 7(3): 347-359. DOI: https://www.doi.org/10.1016/j.jtcme.2016.11.004

Aziz MA, Khan AH, Adnan M, and Izatullah I (2017). Traditional uses of medicinal plants reported by the indigenous communities and local herbal practitioners of Bajaur Agency, Federally Administrated Tribal Areas, Pakistan. Journal of Ethnopharmacology, 198: 268-281. DOI: https://www.doi.org/10.1016/j.jep.2017.01.024

Bagheri M, Molzemi S, Hadizadeh M, and Aminian M (2016). Study of alcohol extract of sesame on wound healing of diabetic male wistar rats. Journal of Neyshabur University of Medical Science, 3(4): 1-9. Available at: http://journal.nums.ac.ir/article-1-202-en.html

Brereton MF, Vergari E, Zhang Q, and Clark A (2015). Alpha-, delta-and PP-cells: Are they the architectural cornerstones of islet structure and co-ordination?. Journal of Histochemistry and Cytochemistry, 63(8): 575-591. DOI: https://www.doi.org/10.1369/0022155415583535

Denaro M, Smeriglio A, Barreca D, De Francesco C, Occhiuto C, Milano G, and Trombetta D (2020). Antiviral activity of plants and their isolated bioactive compounds: An update. Phytotherapy Research, 34(4): 742-768. DOI: https://www.doi.org/10.1002/ptr.6575

Everett E, and Mathioudakis N (2018). Update on management of diabetic foot ulcers. Annals of the New York Academy of Sciences, 1411(1): 153-165. DOI: http://www.doi.org/10.1111/nyas.13569

Filip S (2017). Basil (*Ocimum basilicum L.*) a source of valuable phytonutrients. International Journal of Clinical Nutrition and Dietetics, 3: 118-123. DOI: https://www.doi.org/10.15344/2456-8171/2017/118

Firoozeh E, Khodarahmi P, Naseh V, Shahedi V, Golgoo F, and Mohammad yari S (2019). Synergistic effect of treadmill exercise and green tea extract on cutaneous wound healing in diabetic mice. New Cellular and Molecular Biotechnology Journal, 9(34): 47-56. Available at: http://ncmbjpiau.ir/article-1-1187-en.html

Foo YZ, Nakagawa S, Rhodes G, and Simmons LW (2017). The effects of sex hormones on immune function: A meta-analysis. Biological Reviews, 92(1): 551-571. DOI: https://www.doi.org/10.1111/brv.12243

Guo W, Tan HY, Chen F, Wang N, and Feng Y (2020). Targeting cancer metabolism to resensitize chemotherapy: Potential development of cancer chemosensitizers from traditional Chinese medicines. Cancers, 12(2): 1-21. DOI: https://www.doi.org/10.3390/cancers12020404

Gutiérrez-Grijalva EP, Picos-Salas MA, Leyva-López N, Criollo-Mendoza MS, Vazquez-Olivo G, and Heredia JB (2018). Flavonoids and phenolic acids from oregano: Occurrence, biological activity and health benefits. Plants, 7(1): 2-23. DOI: http://www.doi.org/10.3390/plants7010002

Ibrahim NI, Wong SK, Mohamed IN, Mohamed N, Chin KY, Ima-Nirwana S, and Shuid AN (2018). Wound healing properties of selected natural products. International Journal of Environmental Research and Public Health, 15(11): 2360. DOI: http://www.doi.org/10.3390/ijerph15112360

Khaksar S,Kesmati M, Rezaie A, Rasekh A(2011). Topical Estrogen Accelerates Wound Healing in Diabetic Rats. Iranian Journal of Endocrinology and Metabolism, 12(5):541-551. DOI: https://www.doi.org/10.1007/s00580-010-1151-x

Miri A, Khatami M, Ebrahimy O, and Sarani M (2020). Cytotoxic and antifungal studies of biosynthesized zinc oxide nanoparticles using extract of Prosopis farcta fruit. Green Chemistry Letters and Reviews, 13(1): 27-33. DOI: https://www.doi.org/10.1080/17518253.2020.1717005

Mirjalili MM, Faramarzi S, Esmaeilidehaj M, Zare Mehrjardi F, and Ebrahim Rezvani M (2017). Effect of aqueous extract of *Morus nigra* on skin wound healing in type 1 diabetic rats. Journal of Shahid Sadoughi University of Medical Sciences, 25(4): 264-270. Available at: http://jssu.ssu.ac.ir/article-1-3875-en.html

Mostafavi S, Asadi-Gharneh HA, and Miransari M (2019). The phytochemical variability of fatty acids in basil seeds (*Ocimum basilicum L.*) affected by genotype and geographical differences. Food Chemistry, 276: 700-706. DOI: http://www.doi.org/10.1016/j.foodchem.2018.10.027

Pourmahdi O, and Faedmaleki F (2021). Comparative effect of ivy extract with geranium on shear wound healing in rats. Journal of Knowledge and Health in Basic Medical Sciences, 15(4): 43-50. DOI: http://www.dx.doi.org/10.22100/jkh.v4i15.2446

Reinke JM, and Sorg H (2012). Wound repair and regeneration. European Surgical Research, 49(1): 35-43. DOI: https://www.doi.org/10.1159/000339613

Sadoughi SD (2013). The effect of aqueous extract of Angoze gum on wound healing in streptozotocin-induced diabetic rats. Knowledge Horizon, 19(3): 129-135. Available at: https://iranjournals.nlai.ir/handle/123456789/633651

Salem AM, Ragheb AS, Hegazy M. G, Matboli M, and Eissa S (2019). Caffeic acid modulates miR-636 expression in diabetic nephropathy rats. Indian Journal of Clinical Biochemistry, 34(3): 296-303. DOI: https://www.doi.org/10.1155/2020/3960857

Shahrokh S, Aliye T, Yazdi M, Siavash M, and Aminorroaya A (2021). Bacterial profile and antimicrobial resistance patterns of infected diabetic foot ulcers in Iran: A systematic review and meta-analysis of cross-sectional studies. The International Journal of Lower Extremity Wounds, 1: 12-13. DOI: https://www.doi.org/10.1177/15347346211002715

Siljander H, Honkanen J, and Knip M (2019). Microbiome and type 1 diabetes. EBioMedicine, 46: 512-521. DOI: https://www.doi.org/10.1016/j.ebiom.2019.06.031

Veith AP, Henderson K, Spencer A, Sligar AD, and Baker AB (2019). Therapeutic strategies for enhancing angiogenesis in wound healing. Advanced Drug Delivery Reviews, 146: 97-125. DOI: https://www.doi.org/10.1016/j.addr.2018.09.010

Walko G, Castañón MJ, and Wiche G (2015). Molecular architecture and function of the hemidesmosome. Cell and Tissue Research, 360(2): 363-378. DOI: https://www.doi.org/10.1007/s00441-014-2061-z

To cite this paper: Hadi M, Moghtadaei-Khorasgani E, and Etesamnia MH (2021). The Influence of Basil Seed Hydroethanolic Extract on the Skin Wound Healing in Diabetic Male Rats. *World Vet. J.*, 11 (3): 377-383. DOI: https://dx.doi.org/10.54203/scil.2021.wvj49

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 384-394, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020

Nahed Yehia*, Fatma Amer, Abdelhafez Samir, Mohamed Samy, Ahmed Sedeek, Neveen Rebie, Wafaa Mohammed, and Naglaa Hagag

Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agriculture Research Center, Giza 12618, Egypt

*Corresponding author's Email: nahedyehia@yahoo.com; ORCID: 0000-0002-2823-6467

ORIGINAL ARTICLE
pii: S232245682100050-11
Received: 15 July 2021
Accepted: 30 August 2021

ABSTRACT

Poultry production has been affected by multiple respiratory diseases triggering serious economic losses in Egypt. The current study aimed to investigate the situation and genetic evolution of respiratory diseases in Egypt during 2020. A total of 53 samples were collected from infected flocks suffering from respiratory signs and variable mortality rates from nine governorates in Egypt during 2020. The collected samples were examined for the detection of respiratory disease viruses (Avian influenza virus (AIV (H5N8, H9N2), Infectious bronchitis virus (IBV), and Newcastle disease virus (NDV)) by rRT-PCR. The single infection was confirmed in 90.6% (37.7% I.B, 30.2% AIV (H5N8), 9.4% I.B and 5.7% NDV) and co-infection of HPAIV (H5N8) + I.BV and LPAIV (H9N2) +IBV were detected in 3.8% of nine governorates. The HA gene of HPAIV (H5N8) was cluster to clad 2.3.4.4.1b in a new branch with characteristic specific mutations especially in T140A in antigenic site A and R72S in the receptor-binding site, compared to A/duck/Egypt/F446/2017 with low A.A identity percent with vaccinal strains of H5N1 and H5N2 reaching to 91.9-94% and 84.6%, respectively. The HA gene of AIV (H9N2) belonged to A/quail/Hong Kong/G1/97-like virus clustered with group B with a specific mutation (212I) that may affect the human transmission of the virus. The HVRs of S1 gene of IBV cluster to GI23 (Egy Var I) clad with multiple mutations in HVR1 and HVR2, compared to IBV/CU/4/2014 and low identity percent (68.3-78.8%) with vaccine strains (H120, M41, 4/91). In conclusion, respiratory disease continues to circulate and rapidly evolve in Egypt during 2020.

Keywords: HPAIV (H5N8), IBV, Genetic characterization, LPAIV(H9N2), Respiratory disease

INTRODUCTION

The poultry industry is one of the most significant industries impacting the national economy in Egypt by promoting investment and jobs availability. Moreover, the primary sources of inexpensive animal protein for poor people in Egypt are poultry meat and eggs (Abdelwhab and Hafez, 2011).

Regrettably, the poultry industry in Egypt has been affected mainly by viral respiratory diseases for several years (Haghighat-Jahromi et al., 2008; Sediek, 2013; Awad et al, 2016). The inability to control this disease results in significant economic losses in the poultry sector. Avian influenza (highly pathogenic, low pathogenic), Newcastle Disease Virus (NDV) and Infectious Bronchitis Virus (IBV) alone or mixed infection (with each other or other bacterial infection) are the main cause of respiratory disease affect poultry farm with a high mortality rate (Radwan et al., 2013; Hassan et al., 2016; Samy and Naguib, 2018).

Avian Influenza (AI) was the most severe respiratory disease in Egypt. The Highly Pathogenic Avian Influenza (HPAI) H5N8 was first recorded in 2016 from a wild bird (common coot) (Selim et al., 2017), then observed in domestic birds, including chickens and ducks, as single or co-infected with other respiratory viruses causing severe losses in poultry production (Salaheldin et al., 2018; Yehia et al., 2018; Shehata et al., 2018; Hassan et al., 2019). The low pathogenic avian influenza (LPAI) H9N2 was first recorded in 2010-2011 belonging to G1 lineage (El-Zoghby et al., 2012). It has immunosuppressive effects that lead to increased opportunism of the infection by other viral diseases as mainly observed in co-infection with H9N2 and I.B viruses (Hassan et al., 2017). In addition, the co-infection with other bacterial infections as Escherichia coli and Haemophilus paragallinarum contribute to increasing the virulence of H9N2 because it has an endoproteases enzyme that cleavage precursor HA proteins (Haghighat-Jahromi et al., 2008; Pan et al., 2012; Hassan et al., 2017).

Infectious bronchitis virus is one of the major significant respiratory infections (Hofstad, 1984). It is primarily recorded was in Egypt during the 1950s (Sheble et al., 1986) that related to the Dutch variant D3128 then D274, Mass, 4/91, and D-08880. (Eid, 1998) Novel endemic genotypes associated with Israeli variant 2 and mass serotypes were

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.,* 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

detected in 2006 (Abdel-Moneim et al., 2006a). In 2011, the Egyptian virus was mutated and classified into variant I and variant II (Abdel-Moneim et al., 2012). Both types of IBV virus circulated in Egypt causing outbreaks despite vaccination programs, which all in all lead to high economic losses (Abd El Rahman et al., 2015; Zanaty et al., 2016). The most severe respiratory disease is Newcastle Disease (ND) that causing high mortality in poultry flocks (OIE, 2012). In Egypt, the NDV was first recorded in 1948 (Daubney and Mansy, 1948) then spread rapidly in Egypt despite vaccination programs (Abdel-Moneim et al., 2006b; El-Bagoury et al., 2015).

Virus isolation is the fundamental diagnostic test for avian respiratory viruses, but it appears to be expensive, slow, and labor-intensive (Suarez et al., 2007). Recently, reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR) were rapid diagnostic tests used to detect viral nucleic acid (Spackman et al., 2002).

In the recent year, the respiratory viruses (HPAI (H5N8), H9N2 (LPAI)), IB, NDV has continuous evolution that may be affected on the pathogenicity of the virus and vaccine efficacy as previously recorded (Yehia et al., 2018; Hassan et al., 2017; Zanaty et al., 2016; Abdel-Moneim et al., 2006b). The aim of the current research was to explore the updated status of respiratory disease outbreaks in broiler Egyptian farms during 2020 using rRT-PCR and to study the genetic variation of the selected circulating respiratory viruses.

MATERIALS AND METHODS

Collection and preparation of specimens

In the present study, AIV (H5N8 and H9N2), NDV, and IBV were examined on 53 infected poultry flocks exhibiting severe respiratory manifestation and high mortality during 2020. The oropharyngeal swabs were obtained from disease or freshly dead birds in nine provinces of Egypt, including Alexandria, Giza, Dakhalia, El-sharqia, Qualiobia, El-Monofia, El-Behira, Domiat, and Cairo (Table 1, Figure 1). A total of 45 chicken flocks (including 36 broilers, 5 commercial layers, and 4 breeder farms), 2 flocks of turkeys, and 6 duck farms were examined. The samples from each flock were collected by 10-15 swabs were pooled in 2 ml of phosphate buffer saline (OIE, 2014; Naguib et al., 2017).

RNA extraction, identification, and subtyping of viruses

The QIAamp Viral RNA Mini Kit (Qiagen) was used for the extraction of viral RNA from pooled oropharyngeal swabs following the manufacturer's guidelines, then eluted into 50 µl of nuclease-free water. All samples were tested for the detection of M gene of influenza type A viruses by rRT-PCR (Fereidouni et al., 2012) using the AgPath Real-time Kit (Ambion) and the real-time PCR step one plus System (Applied Biosystems, Foster City, CA, USA). In the next step, subtyping using rRT-PCR targeted HA and NA genes as described by Hoffmann et al. (2016). In addition, all samples were tested for the detection of NDV, IBV using rRT-PCR target matrix gene of avian paramyxovirus-1 (Wise et al., 2004) then the positive samples were tested for the detection of NDV of genotype VII (velogenic strain) (Moharam et al., 2019). Furthermore, rRT-PCR was used for the detection of IBV RNA as described by (Naguib et al., 2017).

Virus isolation

The positive samples were isolated into specific pathogen-free (SPF) embryonated chicken eggs (ECEs) aged 10 days old in allantoic fluid with daily observation. The mortalities were recorded and the allantoic sac was collected and tested by rapid slide haemagglutination (HA) test (OIE manual, 2008).

Sequencing of the viral genome

The HA gene of AI (H5, H9) and HVRs of S1 gene of IBV amplification were examined by PCR using specific primers as described by Hoper et al. (2009), Selim et al. (2013) and Naguib et al. (2015), and high fidelity Phusion® DNA polymerase (Thermo Fisher Scientific, MA, USA). Using QIAquick Gel Extraction Kit, the PCR products were separated and purified from the gel (Qiagen).

Cycle sequencing reactions have been performed on PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Using centri-sep columns (Macherey-Nagel GmbH & Co.) the sequence products were purified then sequenced on an ABI PRISM3130 Genetic Analyzer (Life Technologies). For each sequence, the blast search was performed (http://www.ncbi.nlm.nih.gov/BLAST). Mutation and phylogenetic analysis were conducted using MEGA version 6 program using a bootstrap of 1000 trials of the Clustal W alignment algorithm (Tamura et al., 2013). The amino acid identity was analyzed using DNA star software (DNAStar, Madison, WI). Then, it was published by the national center for Biotechnology Information with the accession number provided in Table 2.

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

Table 1. Epidemiological data results of PCR of collected samples of collected samples

Number	Governorates	Species	Breeds	Age (day)	Results
1	El-Dakhlia	Chicken	Broiler	20	HPAIV(H5N8)
2	El-Dakhlia	Chicken	Layer	275	HPAIV(H5N8)
3	El-Dakhlia	Chicken	Broiler	25	IBV
4	Giza	Chicken	Breeder	90	IBV
5	Alexandria	Chicken	Broiler	30	IBV
6	Alexandria	Chicken	Layer	365	HPAIV(H5N8)
7	Giza	Chicken	Broiler	33	HPAIV(H5N8)
8	Giza	Chicken	Broiler	36	Negative
9	Giza	Turkey		65	HPAIV(H5N8)
10	Giza	Chicken	Broiler	40	NDV
11	El-sharqia	Chicken	Broiler	22	IBV
12	El-Monofia	Chicken	Layer	390	NDV
13	El-sharqia	Chicken	Broiler	15	LPAIV(H9N2)
14	El-Dakhlia	Chicken	Breeder	392	IBV
15	Cairo	Chicken	Broiler	23	IBV
16	Cairo	Chicken	Broiler	34	IBV
17	Domiat	Chicken	Broiler	42	HPAIV(H5N8)
18	El-Dakhlia	Chicken	Broiler	36	IBV
19	El-Dakhlia	Chicken	Layer	120	IBV
20	El-Dakhlia	Chicken	Broiler	22	HPAIV(H5N8)
21	El-Behira	Chicken	Breedrer	196	Negative
22	Giza	Chicken	Broiler	20	IBV
23	Beni-suief	Turkey		60	IBV
24	Beni-suief	Chicken	Broiler	25	IBV
25	El-Dakhlia	Chicken	Broiler	15	IBV
26	El-sharqia	Chicken	Broiler	30	IBV
27	Domiat	Chicken	Broiler	15	IBV
28	Domiat	Chicken	Layer	120	HPAIV(H5N8)
29	Giza	Chicken	Broiler	33	IBV
30	Alexandria	Chicken	Broiler	33	HPAIV(H5N8)+IBV
31	Alexandria	Chicken	Broiler	34	IBV
32	Cairo	Chicken	Breeder	95	IBV
33	Cairo	Chicken	Broiler	22	HPAIV(H5N8)
34	El-Behira	Duck		12	HPAIV(H5N8)
35	Domiat	Chicken	Broiler	18	Negative
36	Giza	Duck		15	LPAIV(H9N2)
37	Giza	Chicken	Broiler	20	IBV
38	Beni-suief	Chicken	Broiler	15	HPAIV(H5N8)
39	El-Monofia	Duck		95	IBV
40	El-Dakhlia	Chicken	Broiler	20	HPAI (H5N8)
41	Cairo	Chicken	Broiler	28	LPAIV(H9N2)
42	El-sharqia	Duck		27	IBV+LPAIV(H9N2)
43	El-Monofia	Chicken	Broiler	18	NDV
44	Domiat	Chicken	Broiler	33	HPAIV(H5N8)
45	Domiat	Chicken	Broiler	23	IBV+LPAIV(H9N2)
46	El-sharqia	Duck		33	HPAI (H5N8)+IBV
47	El-Dakhlia	Chicken	Broiler	15	HPAIV(H5N8)
48	Beni-suief	Chicken	Broiler	35	Negative
49	Cairo	Chicken	Broiler	34	HPAIV(H5N8)
50	El-Behira	Chicken	Broiler	15	HPAIV(H5N8)
51	El-Dakhlia	Duck		17	LPAIV(H9N2)
52	El-Behira	Chicken	Broiler	28	LPAIV(H9N2)
53	El-sharqia	Chicken	Broiler	18	Negative

Table 2. Accession number of HA gene of HPAI (H5N8 and H9N2) and S1 gene of infectious bronchitis virus samples

Number	Code	GenBank Accession number		
		HA(H5)	HA(H9)	S1
45	IBV/EGY/CH/AY1/2020, A/chicken/FW1/2020	-	MW227513	MW240842
30	IBV/EGY/CH/AY2/2020 A/chicken/Egypt/FN1/2020	MW227501	-	MW240843
3	IBV/EGY/CH/AY3/2020	-	-	MW240844
11	IBV/EGY/CH/AY4/2020	-	-	MW240845
15	IBV/EGY/CH/AY5/2020	-	-	MW240846
39	IBV/EGY/CH/AY6/2020	-	-	MW240847
13	A/chicken/FW2/2020	-	MW227514	-
36	A/chicken/FW3/2020	-	MW227515	-
28	A/chicken/Egypt/FN2/2020	MW227502	-	-
40	A/chicken/Egypt/FN3/2020	MW227503	-	-

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

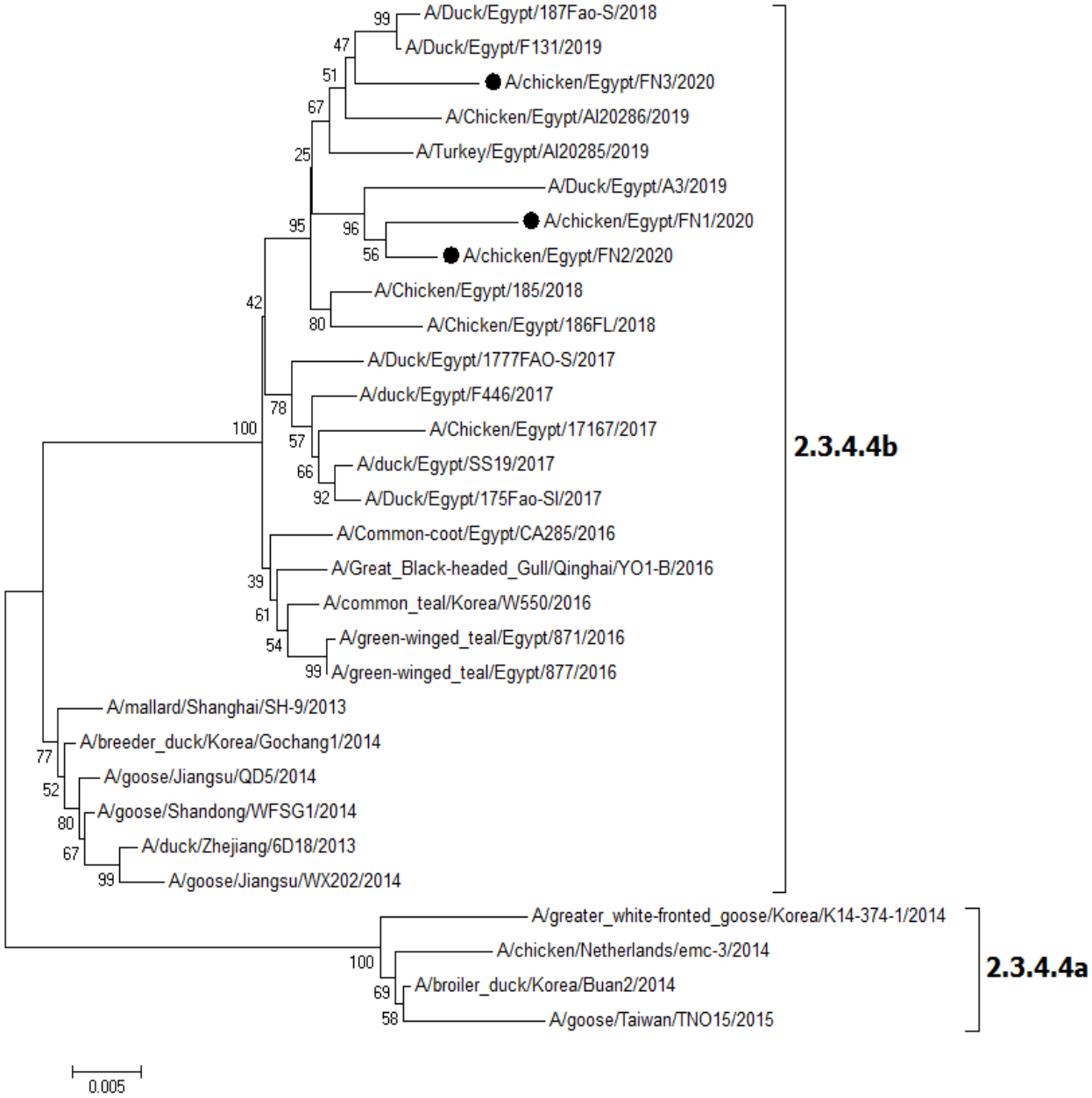


Figure 1. Phylogenetic tree of HA gene of highly pathogenic avian influenza (H5N8)

RESULTS

Result of real time-PCR

The findings indicated that 48 samples out of 53 tested samples were positive for RT-PCR for different respiratory diseases (20 samples were positive for IBV, 16 samples for HPAI (H5N8), and 5 samples for LPAI (H9N2), 3 samples for NDV (velogenic strain), 2 samples for HPAI (H5N8) +IBV, and 2 samples for LPAI (H9N2)+I.BV) as mentioned in Table.1.

Genetic characterization of the HPAI (H5N8) Virus HA gene

Phylogenetic analysis of full HA sequence of four selected AI (H5N8) isolates was named A/ CHICKEN/EGYPT/FN1/2020, A/ CHICKEN/EGYPT/FN2/2020 from co-infected farms and A/ CHICKEN/EGYPT/FN3/202, CHICKEN/EGYPT/FN4/2020 from other infected flocks belonged to clade 2.3.4.4b. They cluster with Egyptian viruses isolated in 2019 making a new subgroup with bootstrapping 95 as shown in Figure 1. They have five nucleotide mutations, compared to A/duck/Egypt/F446/2017, which is different from Egyptian viruses isolated in 2017 and 2018.

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

By mutation analysis in antigenic site A, one mutation (T140A) was detected in the A/chicken/Egypt/FN1/2020 and A/chicken/Egypt/FN2/2020 similar to A/Chicken/Egypt/186FL/2018. In addition, the receptor-binding site in A/chicken/Egypt/FN3/2020 had R72S was similar to A/Chicken/Egypt/185/2018 and A/Chicken/Egypt/186FL/2018. The S94R was also found only in the A/chicken/Egypt/FN3/2020. The A.A. identity of the current strains and H5N1 vectormune vaccine (A/mute_swan/Hungary/4999/2006), B.E.S.T Vaccine (A/duck/China/E319-2/03) was within the range of 91.9-94% and H5N2 CEVac Flukem (A/chicken/Mexico/232/1994) was 84.6% (Figure 2).

Genetic characterization of the H9N2 Virus HA gene

The phylogenetic analysis of the full HA sequence of three selected AI H9 isolate was named A/ CHICKEN/EGYPT/FW1/2020 from co-infected farms and A/CHICKEN/EGYPT/FW2/2020 and A/ CHICKEN/EGYPT/FW3/2020 from single infected flocks belonged to the A/quail/Hong Kong/G1/97-like virus lineage clustered with group B (Figure 3). The viruses in the current study were closely related to other Egyptian strains with identities of 94.9- 98.2% (Figure 4). Considering mutation analysis, mutations in S16N, M58K, T121I/V, I134M, T145S, N179T as specific to all Egyptian viruses were recorded and N41G, I75V, V212I, T413N resemble Egyptian viruses in 2018-2019, compared to the A/quail/Hong Kong/G1/97. In addition, R180K/Q was specific for viruses in the present study. The amino acid sequences at the cleavage site of HA contained a low pathogenic RSSR/GLF motif.

Genetic characterization of S gene of infectious bronchitis virus

The phylogenetic analyses of HVRs of S1 genes of six selected viruses were named IBV/EGY/CH/AY1/2020, IBV/EGY/CH/AY2/2020, and IBV/EGY/CH/AY3/2020 from co-infected flocks and IBV/EGY/CH/AY4/2020, IBV/EGY/CH/AY5/2020, and IBV/EGY/CH/AY6/2020 from single infected flocks. The viruses in this study were clustered to clad GI23 (Egy Var I) (Figure 5).

By mutation analysis of HVRs, the recorded mutation was compared with the reference strain of IBV/CU/4/2014. The HVR1 of three Egyptian viruses (IBV-EGY-CH-AY1-2020, IBV-EGY-CH-AY2-2020, IBV-EGY-CH-AY3-2020) had three A.A. and the other two viruses (IBV-EGY-CH-AY4-2020, IBV-EGY-CH-AY6-2020) had four A.A. However, the IBV-EGY-CH-AY5-2020 had two A.A. The HVRII had 7A.A. mutation in all viruses except IBV-EGY-CH-AY2-2020, which had six amino acid mutations, and IBV-EGY-CH-AY1-2020, IBV-EGY-CH-AY5-2020 which had four A.A. (Figure 6). The S1 gene identity of amino acids revealed that viruses in the current study related to vaccine seeds used commonly in Egypt (M41, H120, 4/91) within the range of 68.3-78.8% (Figure 7).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		97.2	98.5	98.5	98.7	99.4	99.6	99.4	98.9	99.6	99.4	99.4	99.2	91.9	94.0	84.6	1
2	2.9		97.6	97.6	96.8	97.4	97.6	97.6	97.4	97.6	97.7	97.7	97.4	91.7	93.8	84.9	2
3	1.5	2.5		100.0	98.1	98.7	98.9	98.7	98.1	98.9	98.7	98.7	98.5	91.7	94.0	84.6	3
4	1.5	2.5	0.0		98.1	98.7	98.9	98.7	98.1	98.9	98.7	98.7	98.5	91.7	94.0	84.6	4
5	1.3	3.3	1.9	1.9		98.7	99.1	98.9	98.1	98.9	98.7	98.7	98.5	91.5	93.4	84.4	5
6	0.6	2.7	1.3	1.3	1.3		99.4	99.2	99.1	99.8	99.6	99.6	99.4	91.7	93.8	84.4	6
7	0.4	2.5	1.1	1.1	0.9	0.6		99.8	98.9	99.6	99.4	99.4	99.2	91.7	94.0	84.6	7
8	0.6	2.5	1.3	1.3	1.1	0.8	0.2		98.7	99.4	99.2	99.2	99.1	91.7	94.0	84.6	8
9	1.1	2.7	1.9	1.9	1.9	0.9	1.1	1.3		99.2	99.2	99.2	99.2	91.9	94.0	84.2	9
10	0.4	2.5	1.1	1.1	1.1	0.2	0.4	0.6	0.8		99.8	99.8	99.6	91.9	94.0	84.6	10
11	0.6	2.3	1.3	1.3	1.3	0.4	0.6	0.8	0.8	0.2		100.0	99.4	91.9	94.0	84.6	11
12	0.6	2.3	1.3	1.3	1.3	0.4	0.6	0.8	0.8	0.2	0.0		99.4	91.9	94.0	84.6	12
13	0.8	2.7	1.5	1.5	1.5	0.6	0.8	0.9	0.8	0.4	0.6	0.6		91.9	94.0	84.6	13
14	8.2	8.4	8.4	8.4	8.6	8.4	8.4	8.4	8.2	8.2	8.2	8.2	8.2		97.0	87.4	14
15	6.1	6.3	6.1	6.1	6.7	6.3	6.1	6.1	6.1	6.1	6.1	6.1	6.1	2.9		87.6	15
16	16.4	15.9	16.4	16.4	16.6	16.6	16.4	16.4	16.8	16.4	16.4	16.4	16.4	12.9	12.9		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 A-duck-Egypt-F446-2017
2 A-broiler-duck-Korea-Buan2-2014
3 A-goose-Jiangsu-QD5-2014
4 A-goose-Shandong-WFSG1-2014
5 A-Chicken-Egypt-17167-2017
6 A-Common-coot-Egypt-CA285-2016
7 A-duck-Egypt-SS19-2017
8 A-Duck-Egypt-175Fao-SI-2017
9 A-Chicken-Egypt-185-2018
10 A-Turkey-Egypt-AI20285-2019
11 A-chicken-Egypt-FN1-2020
12 A-chicken-Egypt-FN2-2020
13 A-chicken-Egypt-FN3-2020
14 A-mute-swan-Hungary-4999-H5N1-vectoremu
15 A-duck-China-E319-2-03-H5N1-B.E.S.T-Vac
16 A-chicken-Mexico-232-1994-H5N2

Figure 2. Amino acid identities and divergence of HA gene of H5N8 viruses compared to other selected strains and vaccinal strains

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

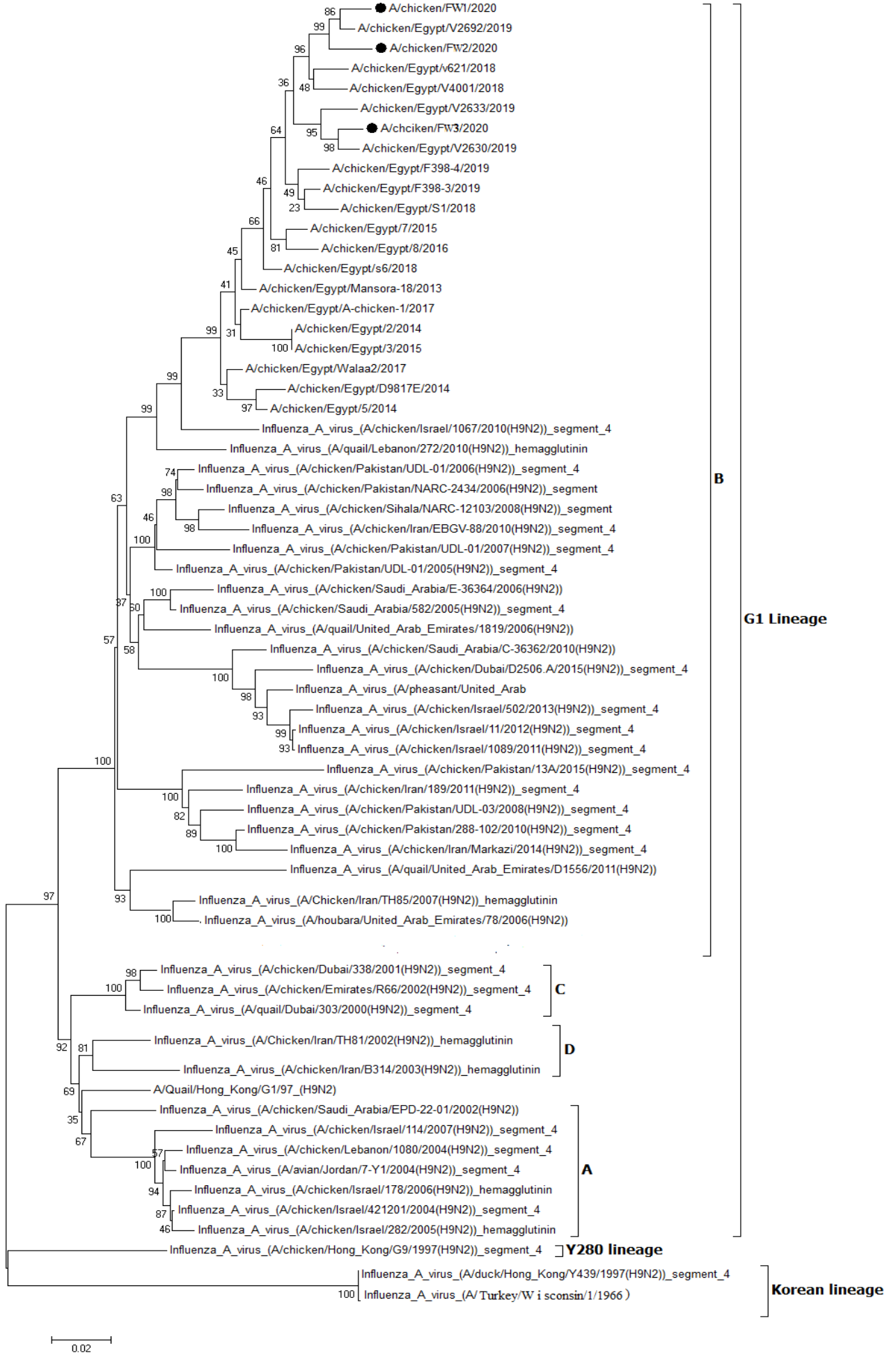


Figure 3. Phylogenetic tree of HA gene of H9N2

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1		85.3	88.7	92.5	92.1	92.7	93.3	91.3	90.5	87.9	88.1	87.1	89.3	88.9	88.3	87.9	87.5	87.5	88.5	1
2	14.8		87.5	84.2	83.6	84.6	84.2	83.6	84.0	83.6	82.4	82.2	83.4	83.2	82.6	82.8	82.4	82.4	82.8	2
3	10.8	12.3		89.5	89.3	89.1	89.5	87.7	89.3	87.1	87.1	86.3	87.5	87.5	87.3	87.1	86.5	86.5	87.9	3
4	6.4	16.5	10.1		98.2	93.3	93.3	91.3	90.7	87.9	88.1	87.9	89.3	88.9	88.3	87.9	87.7	87.7	88.9	4
5	6.9	17.2	10.3	0.8		92.9	93.1	91.1	90.5	87.7	88.1	87.7	89.1	88.7	88.1	87.7	87.5	87.5	88.7	5
6	6.0	15.5	10.4	5.5	6.0		93.1	90.9	91.3	88.3	87.9	87.1	89.3	88.7	88.1	87.9	87.3	87.3	88.7	6
7	5.5	16.3	10.1	5.8	6.0	5.8		92.7	92.7	89.3	89.1	89.3	90.9	90.5	89.9	89.5	89.5	89.5	90.3	7
8	7.8	17.2	12.2	8.0	8.2	8.3	6.4		95.8	92.5	92.3	91.7	94.3	93.7	92.5	92.5	91.7	91.7	92.7	8
9	8.7	16.9	10.5	8.9	9.1	7.8	6.4	3.3		93.7	93.3	93.1	95.8	95.2	93.9	93.7	93.1	93.1	93.7	9
10	11.8	17.5	13.2	12.2	12.5	11.3	10.3	6.9	5.9		98.4	95.2	96.2	96.8	97.4	97.8	94.9	94.9	97.6	10
11	11.5	19.0	13.2	12.0	12.0	11.8	10.6	7.1	6.4	1.4		95.4	96.0	96.8	97.4	98.2	94.9	94.9	97.6	11
12	12.8	19.3	14.2	12.2	12.5	12.8	10.3	7.8	6.6	4.6	4.4		95.2	95.8	95.6	95.6	97.6	97.6	95.8	12
13	10.1	17.7	12.5	10.5	10.8	10.1	8.5	4.9	3.6	3.1	3.3	4.2		97.8	96.2	96.0	94.9	94.9	96.2	13
14	10.6	18.0	12.7	11.0	11.3	10.8	8.9	5.5	4.4	2.7	2.7	3.8	1.4		97.2	97.0	95.8	95.8	97.0	14
15	11.3	18.8	12.9	11.7	12.0	11.5	9.6	6.9	5.7	2.3	2.3	4.2	3.1	2.3		97.8	95.6	95.6	97.8	15
16	11.8	18.5	13.2	12.2	12.5	11.8	10.1	6.9	5.9	2.1	1.6	4.2	3.3	2.5	1.9		95.6	95.6	97.2	16
17	12.3	19.0	13.9	12.5	12.7	12.5	10.1	7.8	6.6	4.8	4.8	2.1	4.4	3.8	4.2	4.2		99.6	95.8	17
18	12.3	19.0	13.9	12.5	12.7	12.5	10.1	7.8	6.6	4.8	4.8	2.1	4.4	3.8	4.2	4.2	0.0		95.8	18
19	11.1	18.5	12.2	11.0	11.3	10.8	9.2	6.6	5.9	2.1	2.1	4.0	3.1	2.5	1.9	2.5	4.0	4.0		19
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	

Divergence

1 A-Quail-HongKong-G1-97
2 A-duck-HongKong-Y439-1997
3 A-chicken-HongKong-G9-1997
4 A-chicken-Israel-421201-2004
5 A-chicken-Israel-178-2006
6 A-Chicken-Iran-TH81-2002
7 A-chicken-Dubai-338-2001
8 A-chicken-Saudi
9 A-chicken-Israel-1067-2010
10 A-chicken-FW1-2020
11 A-chicken-FW2-2020
12 A-chciken-FW3-2020
13 A-chicken-Egypt-2-2014
14 A-chicken-Egypt-7-2015
15 A-chicken-Egypt-v621-2018
16 A-chicken-Egypt-V4001-2018
17 A-chicken-Egypt-V2630-2019
18 A-chicken-Egypt-V2633-2019
19 A-chicken-Egypt-V2692-2019

Figure 4. Amino acid identities and divergence of HA gene of H9N2 viruses compared to other selected strains and vaccinal strains

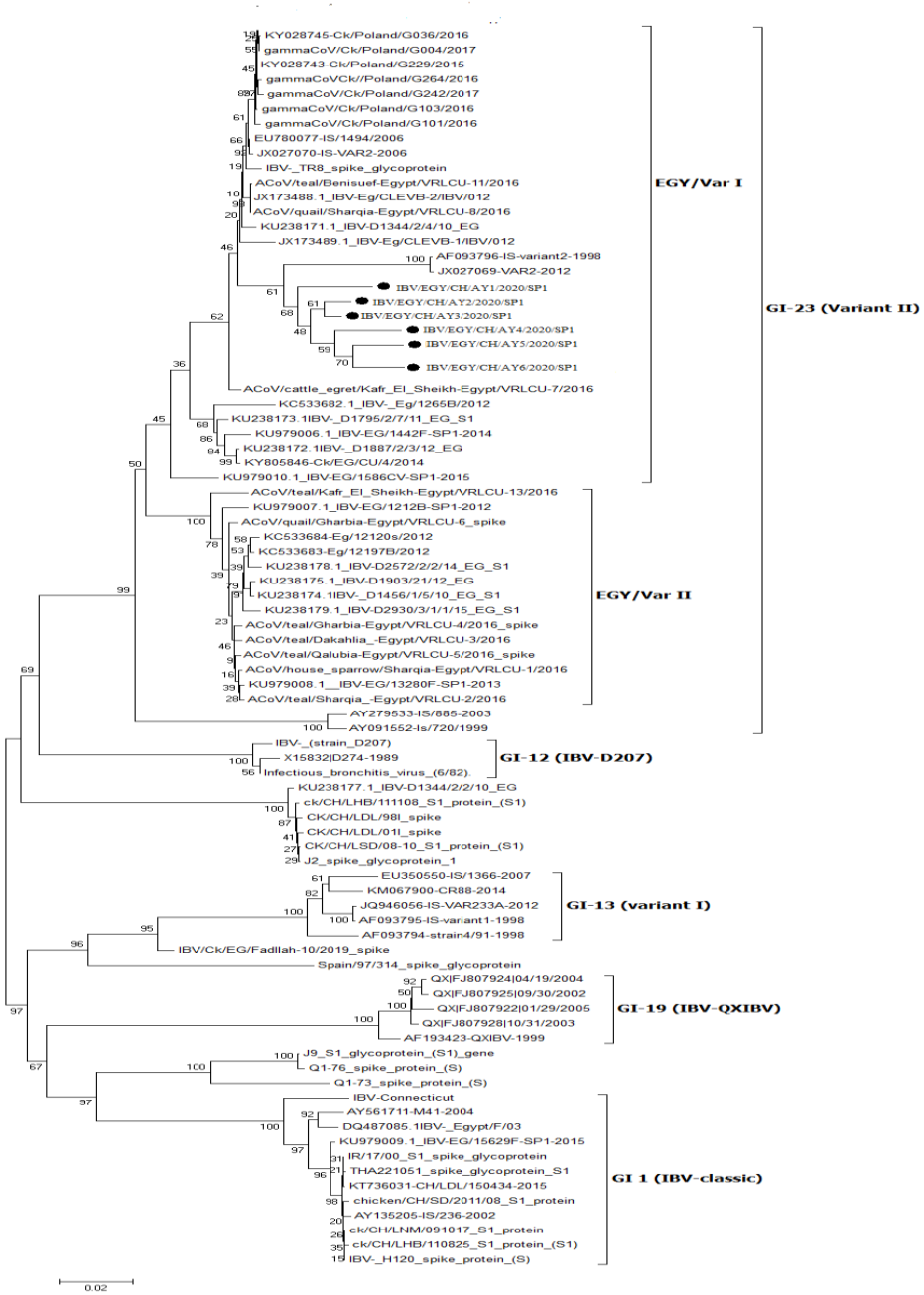


Figure 5. Phylogenetic tree of partial S1 gene of infectious bronchitis virus

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

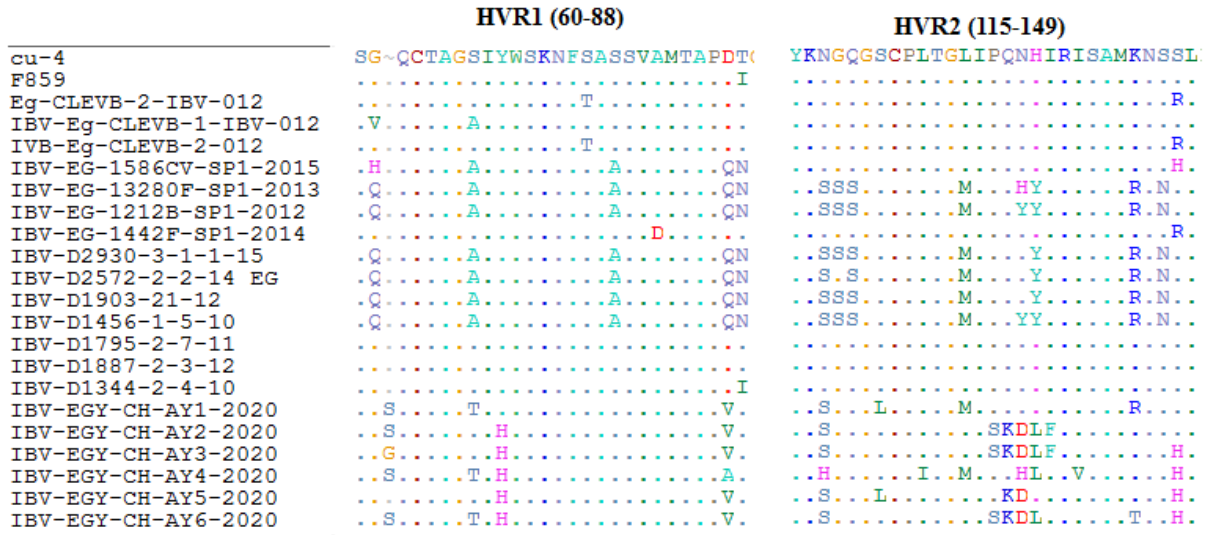


Figure 6. Amino acid mutation in HVRS in S1 gene of infectious bronchitis virus

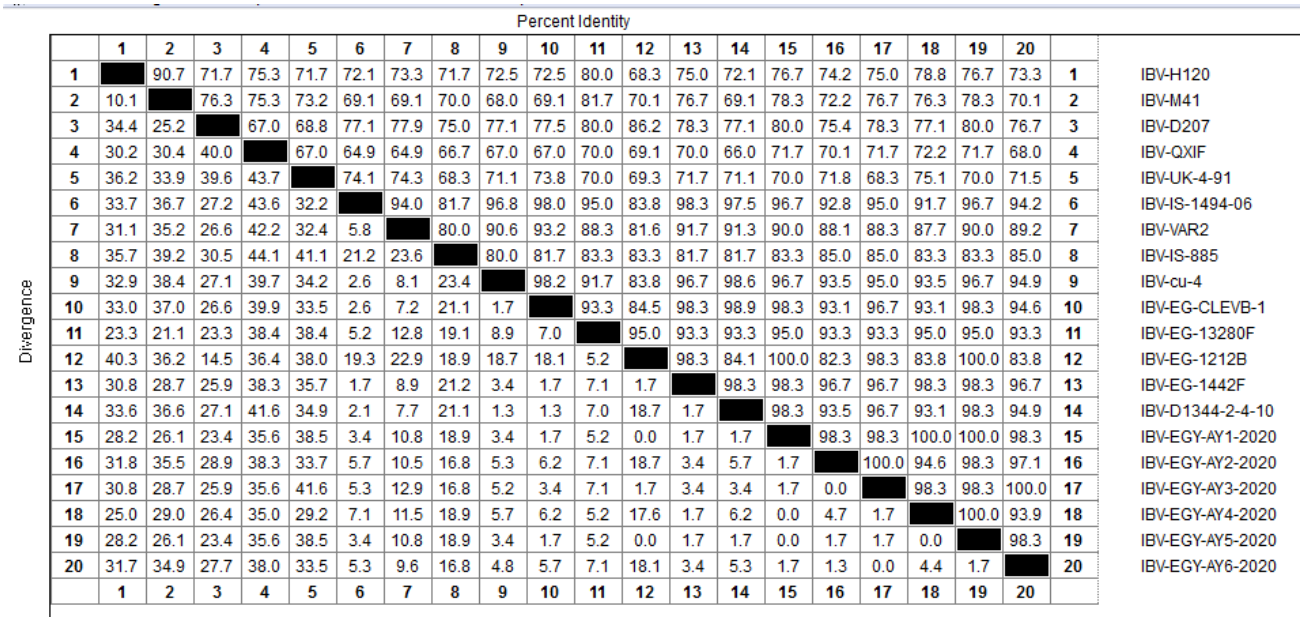


Figure 7. Amino acid identities and divergence of partial S1 gene of infectious bronchitis virus sequenced viruses compared to other selected strains and vaccinal strains

DISCUSSION

In Egypt during the last years, the outbreaks due to respiratory disease with high mortality rates have increased and affected poultry production. Many avian viral pathogens were the main cause of this problem (Malik et al., 2004; Roussan et al., 2008). The avian influenza virus (HP, LP), IBV, and NDV were the main detected viruses alone or as co-infected in broiler chickens (Hassan et al., 2016). The current study aimed to identify the incidence of viral respiratory disease in broiler chickens, especially AIV (HP, LP), IBV, and NDV in Egypt during 2020, and study the molecular characterization and evolution of detected viruses.

In the present study, multiple respiratory viruses as single or mixed infections were detected. The AIV subtypes and IBV were recorded in 48 out of 53 farms. Single infection represented 90.6% (37.7% I.B, 30.2% H5N8, 9.4% I.B and 5.7% NDV). Furthermore, co-presence of HPAI (H5N8) and IBV, unique detection of these co-infected flocks, and LPAI (H9N2) and IBV, were detected in 3.8% as previously recorded by El-Shall et al. (2019). The occurrence of IBV and HPAI (H5N8) in Egyptian poultry with a high mortality rate in different vaccinated poultry flocks has previously been reported by a number of researchers (Abdel-Moneim et al., 2006a, Abd El Rahman et al., 2015; Yehia et al., 2020). It was indicated the high incidence of respiratory disease in broiler chicken during 2020 in single infection more than co-infected with other viruses.

In recent years, the avian influenza situation in Egypt has been more complicated due to the detection and circulation of many serotypes, including HPAI (H5N1), HPAI (H5N8), and LPAI (H9N2) (Shehata et al., 2019). The

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

phylogenetic analysis of four selected samples of the HA gene of HPAI H5N8 revealed that the four samples were clustered to clad 2.3.4.4.1b as previously recorded by Shehata et al. (2018) and El-Shall et al. (2019) in a new subgroup with characteristic specific mutations due to multiple mutations that may affect the pathogenicity and vaccine efficacy.

Considering mutation analysis, a change was detected in the (T140A) antigenic site A in two samples of A/chicken/Egypt/FN1/2020 and A/chicken/Egypt/FN2/2020 as previously reported by Yehia et al. (2020) that may be due to excessive use of avian influenza vaccine H5N1 and H5N2. In addition, the R72S in A/chicken/Egypt/FN3/2020 in the receptor-binding site was detected as previously mentioned by Nabil et al. (2020).

The A.A. identity was very low in H5N1 and H5N2 vaccine reaching 91.9-94% and 84.6%, respectively, similar to previous studies (Kandeil et al., 2018; Yehia et al., 2020). It explained where the outbreaks occurred in the vaccinated flocks and caused high mortality.

The phylogenetic analysis of three LPAI (H9N2) selected strains revealed that all viruses in the current study belonged to A/quail/Hong Kong/G1/97-like virus lineage clustered with group B as previously recorded (Kareem et al., 2015; El-Shall et al., 2019). Regarding mutation analysis, the LPAI (H9N2) Egyptian viruses had six A.A. mutation-specific. In addition, I75V, N41G, and T413N mutation specific to 2018-2019 Egyptian viruses and 212I mutation that recorded previously had an important effect on the increased pathogenicity of the H9N2 in mice as previously recorded (Yang et al., 2014) that may affect the human transmission of the virus. So, there is a need to conduct further studies to detect the effect of these mutations on the pathogenicity of the virus and vaccine efficacy in poultry.

In the current study, a high number of infected vaccinated flocks with IBV viruses by RT-PCR was recorded. Therefore, partial S1 gene sequencing is important to identify the IBV strains. Furthermore, the partial IBV S1 gene was sequenced, and the analysis revealed that six selected viruses were related to clad GI23 (Egy Var I) as previously indicated (Hassan et al., 2016; Zanaty et al., 2016; Abozeid et al., 2017). The Egyptian strains in this study had low identity percent with vaccine strains H120, M41, 4/91 ranging 68.3-78.8% that lead to the appearance of infection in vaccinated farms as previously recorded (Abd El Rahman et al., 2015; Sultan et al., 2019). Accordingly, future studies should be conducted to evaluate vaccine efficacy and update the vaccine used to give high protection against newly mutated strains.

The S1 subunit, which comprises three major hypervariable regions (HVRs) in the first 395 amino acids. The HVRs contain major antigenic sites between 38 and 67 amino acids for HVR-1, 97 and 141 amino acids for HVR2, and 274 and 387 amino acids for HVR-3 (Moore et al., 1997). The present study indicated multiple A.A. mutations in the HVR as previously recorded (Hassan et al., 2016; Zanaty et al., 2016; Abozeid et al., 2017) that may be due to widespread use of heterologous vaccine leading to vaccination pressure, which increases the pathogenicity of the virus and changes in tissue tropism (Cavanagh et al., 1992).

CONCLUSION

Infectious bronchitis virus and highly pathogenic avian influenza (H5N8) are the main causes of respiratory disease in Egyptian flocks alone or co-infected with other viruses with a high mortality rate. Circulation of avian influenza (H5N8) was detected with a mutation in the antigenic site and receptor binding site, low pathogenic avian influenza (H9N2) with a specific mutation, and infectious bronchitis viruses were rapidly evolved in hypervariable regions. The infectious bronchitis virus and highly pathogenic avian influenza H5N8 viruses were distinct from vaccine strains so it is important to surveillance of respiratory disease viruses and study the genetic evolution and its effect on pathogenicity and vaccine efficacy.

DECLARATIONS

Authors' contribution

Nahed Yehia contributed to molecular characterization and data analysis and the write-up of the manuscript and Fatma Amer contributed to sample collection and diagnosis.

Competing interests

The authors have not declared any conflict of interest.

Consent to publish

All the authors approved and agreed to publish the manuscript.

Ethical consideration

All authors approved the final draft of the manuscript for publication. Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

REFERENCES

Abd El Rahman S, Hoffmann M, Lueschow D, Eladl A, and Hafez HM (2015). Isolation and characterization of new variant strains of infectious bronchitis virus in Northern Egypt. Advanced Animal Veterinary Science, 3(7): 362-371. Available at: https://pubmed.ncbi.nlm.nih.gov/1965995/

Abdel-Moneim AS, Afifi MA, and El-Kady MF (2012). Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. Archive of Virology, 157: 2453-2457. Available at: https://pubmed.ncbi.nlm.nih.gov/22903394/

Abdel-Moneim AS, El-Kady MF, Ladman BS, and Gelb J (2006a). S1 gene sequence analysis of a nephropathogenic strain of avian infectious bronchitis virus in Egypt. Virology Journal, 3: 78. DOI: https://www.doi.org/10.1186/1743–422X-3–78

Abdel-Moneim AS, El-Sawah AA, and Kandil MA (2006b). Characterization of variant strain of Newcastle disease virus in Egypt. BS. Journal of Veterinary Medical Research, 16: 12-17. Available at: https://jvmr.journals.ekb.eg/article_77914.html

Abdelwhab EM and Hafez HM (2011). An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: Epidemiology and control challenges. Epidemiology & Infection, 139(5): 647-657. Available at: https://pubmed.ncbi.nlm.nih.gov/21281550/

Abozeid H, Paldurai A, Khattar SK, Afifi MA, El-Kady MF, El-Deeb AH, and Samal SK (2017). Complete genome sequences of two avian infectious bronchitis viruses isolated in Egypt: Evidence for genetic drift and genetic recombination in the circulating viruses. Infection, Genetics and Evolution, 53: 7-14. Available at: https://pubmed.ncbi.nlm.nih.gov/28495648/

Anis A, AboElkhair M, and Ibrahim M (2017). Characterization of highly pathogenic avian influenza H5N8 virus from Egyptian domestic waterfowl in 2017. avian pathology, 47(4):400-409. Available at: https://pubmed.ncbi.nlm.nih.gov/29701481/

Awad MA, Ali BA, Abd El-Hamid SH, El-Naggar LA, Sediek ME, El-Shall AN, and El-Samahy SH (2016). Epidemiological Studies on H5N1 and H9N2 Avian Influenza Viruses during late 2013 and 2015 in Egypt. Alexandria Journal of Veterinary Sciences, 1(2): 164-173. Available at: https://www.bibliomed.org/?mno=221099

Cavanagh D, Davis PJ, and Cook JKA (1992). Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. Avian Pathology, 21: 401-408. Available at: https://pubmed.ncbi.nlm.nih.gov/18670955/

Daubney R and Mansy W (1948). The occurrence of Newcastle disease in Egypt. Journal of Comparative Pathology and Therapeutic, 58: 189-200. Available at: https://www.sciencedirect.com/science/article/abs/pii/S0368174248800196

Eid AM. (1998). Infectious bronchitis virus infection in Egypt. Proceedings of the International Symposium on infectious bronchitis and pneumovirus infections in Poultry. Rauischholzhausen, Germany. 145-156.

El-Bagoury GF, Samar F, ElHabbaa AS, and Suzan SE (2015). Isolation, identification, and pathotyping of Newcastle disease virus from chickens in Egyptian. Benha Veterinary Medicine Journal, 29(1): 196-204. Available at: https://bvmj.journals.ekb.eg/article_31819.html

El-Shall NA, Sedeik ME, El-Nahas AF, Abdel-salam RA, and Awad AM (2019). Epidemiological Surveillance of Some Avian Respiratory Viral Diseases in Broiler Chickens. Advanced Journal of Veterinary Science, 61(1): 185-194. Available at: https://www.researchgate.net/publication/332984305_Epidemiological_Surveillance_of_Some_Avian_Respiratory_Viral_Diseases_in_Broiler_Chickens_Article_History

El-Zoghby EF, Arafa AS, Hassan MK, Aly MM, Selim A, Kilany WH, Selim U, Nasef SM Aggor G, Abdelwhab EM et al. (2012). Isolation of H9N2 avian influenza virus from bobwhite quail (Colinus virginianus) in Egypt. Archives of Virology, 157: 1167-1172. Available at: https://pubmed.ncbi.nlm.nih.gov/22426861/

Fereidouni SR, Harder TC, Gaidet N, Ziller M, Hoffmann B, Hammoumi S, and Starick E (2012). Saving resources: Avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. Journal of Virological Methods, 186(1): 119-125. DOI. https://doi.org/10.1016/j.jviromet.2012.08.002

Haghighat-Jahromi M, Asasi K, Nili H, Dadras H, and Shooshtari AH (2008). Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Archives of Virology, 153: 651-655. DOI: https://doi.org/10.1007/s00705-008-0033-x

Hassan KE, Ali A, Dahshan AHM, El-Sawah AA, Shany SAS, and El-Kady MF (2016). Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poultry Science, 95(6): 1271-1280. Available at: https://pubmed.ncbi.nlm.nih.gov/26976895/

Hassan KE, Ali A, Shany SA, and El-Kady (2017). Experimental co-infection of infectious bronchitis and low pathogenic avian influenza H9N2 viruses in commercial broiler chickens. Research in Veterinary Science, 115: 356-362. Available at: https://pubmed.ncbi.nlm.nih.gov/28692924/

Hassan KE, El-Kady MF, El-Sawah AAA, Luttermann C, Parvin R, Shany S, Beer M and Harder T (2019). Respiratory disease due to mixed viral infections in poultry flocks in Egypt between 2017 and 2018: Upsurge of highly pathogenic avian influenza virus subtype H5N8 since (2018). Transboundry and Emerging Disease, 68, 21-36. Available at: https://onlinelibrary.wiley.com/doi/full/10.1111/tbed.13281

Hoffmann B, Hoffmann D, Henritzi D, Beer M, and Harder TC (2016). Riems influenza a typing array (RITA): An RT-qPCR-based low density array for subtyping avian and mammalian influenza a viruses. Scientific Reports, 6: 27211-2721. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4891686/

Hofstad MS, Barnes HJ, Calnek BW, Reid WM, and Yoder HW (1984). Diseases of Poultry, Iowa State University Press, Ames, IA. pp. 429-443 Available at: https://www.thepoultrysite.com/publications/diseases-of-poultry/194/infectious-bronchitis-ib

Hoper D, Hoffmann B, and Beer M (2009). Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes. Journal of Clinical Microbiology, 47: 674-679. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2650901/

Kandeil A, Sabir JSM, Abdelaal A, Mattar EH, ElTaweel AN, Sabir MJ, Khalil AA, Webby R, Kayali G, and Ali MA (2018). Efficacy of commercial vaccines against newly emerging avian influenza H5N8 virus in Egypt Scientific Reports, 8: 9697. Available at: https://repository.msa.edu.eg/xmlui/handle/123456789/2270

Kareem EH, Salama AS, Shany AA, Al-Hussien MD, Azza AE, and El-kady M (2015). Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poultry Science, pp. 1-10. Available at: https://pubmed.ncbi.nlm.nih.gov/26976895/

Malik YS, Patnayak DP, and Goyal SM (2004). Detection of three avian respiratory viruses by single-tube multiplex reverse transcription polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation, 16(3): 244-248. Available at: https://pubmed.ncbi.nlm.nih.gov/15152843/

Moharam I, Abd el Razik A, Sultan H, Ghezlan M, Meseko C, Franzke K, and Grund C (2019). Investigation of suspected Newcastle disease outbreaks in Egypt uncovers a high virus burden in small-scale holdings and the presence of multiple pathogens. Avian Pathology, Pp. 1-31. Available at: https://pubmed.ncbi.nlm.nih.gov/31090444/

Moore KM, Jackwood MW, and Hilt DA (1997). Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. Archives of Virology., 142: 2249-2256. Available at: https://pubmed.ncbi.nlm.nih.gov/9672590/

Nabil NM, Erfan AM, Tawakol MM, Haggag NM, Naguib MM, and Samy A (2020). Wild Birds in Live Birds Markets: Potential Reservoirs of Enzootic Avian Influenza Viruses and Antimicrobial Resistant Enterobacteriaceae in Northern Egypt. Pathogens, 9 (3). 169-184. DOI: https://www.doi.org/10.3390/pathogens9030196

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

Naguib MM, Arafa AS, El-Kady MF, Selim AA, Gunalan V, Maurer-Stroh S, and Harder TC (2015). Evolutionary trajectories and diagnostic challenges of potentially zoonotic avian influenza viruses H5N1 and H9N2 co-circulating in Egypt. Infection, Genetics, and Evolution, 34: 278-291. Available at: https://pubmed.ncbi.nlm.nih.gov/26049044/

Naguib MM, El-Kady MF, Lüschow D, Hassan KE, Arafa AS, El-Zanaty A and Harder TC (2017). New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle Disease viruses. Journal of Virological Methods, 245: 19-27. Available at: https://pubmed.ncbi.nlm.nih.gov/28336367/

Pan Q, Liu A, Zhang F, Ling Y, Ou C, Hou N, and He C (2012). Co-infection of broilers with Ornithobacterium rhinotracheale and H9N2 avian influenza virus. BMC Veterinary Research, 8: 104-104. Available at: https://pubmed.ncbi.nlm.nih.gov/22748160/

Radwan MM, Darwish SF, El-Sabagh IM, El-Sanousi AA, and Shalaby MA (2013). Isolation and molecular characterization of Newcastle disease virus genotypes II and VIId in Egypt between 2011 and 2012. Virus Genes, 47(2): 311-316. Available at: https://pubmed.ncbi.nlm.nih.gov/23842723/

Roussan DA, Haddad R, and Khawaldeh G (2008). Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. Poultry Science, 87(3): 444-448. Available at: https://pubmed.ncbi.nlm.nih.gov/18281569/

Salaheldin AH, El-Hamid HS, Elbestawy AR, Veits J, Hafez HM, Mettenleiter TC, and Abdelwhab EM (2018). Multiple Introductions of Influenza A(H5N8) Virus into Poultry, Egypt (2017). Emerging Infectious Diseases, 24(5): 943-946. Available at: https://pubmed.ncbi.nlm.nih.gov/29248796/

Samy A and Naguib MM (2018). Avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: Field and experimental findings. Veterinary Sciences, 5(1): 23. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5876583/

Sediek ME (2013). Molecular characterization and serotyping of infectious bronchitis virus isolated from broiler chicken farms in Egypt during (2013). International Journal of Agriculture Innovations and Research, 3(1): 286-292. Available at: https://www.sciencedirect.com/science/article/pii/S2314459913000288

Selim AA, Erfan AM, Hagag N, Zanaty A, Samir A, Samy M, Abdelhalim A, Arafa AA, Soliman MA, Shaheen M et al. (2017). Highly pathogenic Avian Influenza virus (H5N8) (clade 2.3.4.4) infection in migratory birds, Egypt. Emerging Infectious Disease Journal, 23(6): 1048-1051. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5443452/

Selim K, Arafa A, Hussein A, and El-Sanousi AA (2013). Molecular characterization of infectious bronchitis viruses isolated from broiler and layer chicken farms in Egypt during, 2012. International Journal of Veterinary Science and Medicine, 1: 102-108. Available at: https://www.sciencedirect.com/science/article/pii/S2314459913000288

Sheble A, Sabry MZ, Davelaar FG, Burger AG, Khafagy AR, Moustafa MM, and Henna M (1986). Present status of infectious bronchitis in Egypt. Journal of the Egyptian Veterinary Medical Association, 46: 393-411.

Shehata AA, Sedeik ME, Elbestawy AR, Zain El-Abideen MA, Ibrahim HH, Kilany WH, and Ali A (2019). Co-infections, genetic, and antigenic relatedness of avian influenza H5N8 and H5N1 viruses in domestic and wild birds in Egypt. Poult Science, 98 (6): 2371-2379. DOI: https://www.doi.org/10.1007/s00705-020-04621-7

Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, and Suarez DL (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology, 40: 3256-3260. Available at: https://pubmed.ncbi.nlm.nih.gov/12202562/

Suarez DL, Das A and Ellis E (2007). Review of rapid molecular diagnostic tools for avian influenza virus. Avian Disease, 51: 201-208. Available at: https://pubmed.ncbi.nlm.nih.gov/17494554/

Sultan HA, Ali A, El Feil WK, Bazid AHI, Zain El-Abideen MA, and Kilany WH (2019). Protective Efficacy of Different Live Attenuated Infectious Bronchitis Virus Vaccination Regimes Against Challenge with IBV Variant-2 Circulating in the Middle East. Frontiers in Veterinary Science, 6: 341. DOI: https://www.doi.org/10.3389/fvets.2019.00341

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular and Biological Evolution, 30: 2725-2729. Available at: https://pubmed.ncbi.nlm.nih.gov/24132122/

Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ and Spackman E (2004). Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNAin clinical samples. Journal of Clinical Microbiology, 42(1): 329-338. Available at: https://pubmed.ncbi.nlm.nih.gov/14715773/

World organization for animal health (OIE) (2015). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter 2.3.4. Avian influenza. Accessed November 2015. Available at: https://www.oie.int/doc/ged/d12009.pdf

World organization for animal health (OIE) (2008). Update on highly pathogenic avian influenza in animals (type H5 and H7), Pp. 465-476. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf

World organization for animal health (OIE) (2012). Newcastle disease. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter, 2.3.14. Available at: https://www.oie.int/ international-standardsetting/terrestrial-manual/access-online.

Yang J, Zhang T, Guo L, Hu Y, Li J, Su H, Xiao Y, Ren X, Dong J, Sun L, Xiao Y, Li L, Yang F, Wang J, Yuan H, and Jin Q (2014). Mutations of novel influenza A(H10N8) virus in chicken eggs and MDCK cells. Emerging Infectious Disease; 20(9): 1541-1543. Available at: https://pubmed.ncbi.nlm.nih.gov/25148105/

Yehia N, Naguiba MM, Ruiyun Li, Hagag N, El-Husseiny M, Mosaad Z, Nour A, Rabea N, Hasan WM, Hassan MK et al. (2018). Multiple introductions of reassorted highly pathogenic avian influenza viruses (H5N8) clade 2.3.4.4b causing outbreaks in wild birds and poultry in Egypt. Infection, Genetics, and Evolution, 58: 56-65. Available at: https://pubmed.ncbi.nlm.nih.gov/29248796/

Yehia N, Wafaa H, Ahmed S, and Elhusseiny M (2020). Genetic variability of avian influenza virus subtype H5N8 in Egypt in 2017 and 2018. Archives of Virology, 165, 1357–1366. DOI: https://www.doi.org/10.1007/s00705-020-04621-7

Zanaty A, Arafa AS, Hagag N, and El-Kady M (2016). Genotyping and pathotyping of diversified strains of infectious bronchitis viruses circulating in Egypt. World journal of virology, 5(3): 125-134. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4981825/

To cite this paper: Yehia N, Amer F, Samir A, Samy M, Sedeek A, Rebie N, Mohammed W, and Hagag N (2021). Concurrent Respiratory Disease in Broiler Chickens in Egypt during 2020. *World Vet. J.*, 11 (3): 384-394. DOI: https://dx.doi.org/10.54203/scil.2021.wvj50

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 395-401, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh

Md. Abdus Sabur, Mouri Rani Das, Md Bashir Uddin, Md. Mahfujur Rahman, Md. Rafiqul Islam, Md. Shahidur Rahman Chowdhury, and Md. Mukter Hossain*

Department of Medicine, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet-3100, Bangladesh
*Corresponding author's Email: mukter.vetmed@sau.ac.bd; ORCID: 0000-0002-8506-1202

ORIGINAL ARTICLE
pii: S232245682100051-11
Received: 27 June 2021
Accepted: 10 August 2021

ABSTRACT

The present study aimed at the molecular detection of *Salmonella* species from feces of goats and the characterization of the isolated *Salmonella* by biochemical and antimicrobial sensitivity techniques. A total of 220 goat feces samples were collected, of which 27 (12.27%) were positive for *Salmonella* by conventional culture methods and 20 (9.09%) by biochemical and PCR techniques. The prevalence was higher in goats under one year of age (20%), compared to older animals aged one to two years (7.8%) and more than two years of age (4.7%), respectively. Moreover, the prevalence of diarrheic goats was significantly higher (38.46%) than healthy animals (2.76%). DNA was extracted from *Salmonella* strains and amplified by PCR using the specific primers of *Salmonella* invasion gene (*invA gene*). The antibiotic sensitivity test indicated that Ciprofloxacin (100 percent sensitivity), Gentamycin (100 percent sensitivity), and Neomycin (100 percent sensitivity) were the most effective antibiotics for the majority of *Salmonella* isolates. On the other hand, *Salmonella* isolates were found to have substantially high resistance to Erythromycin (100%), Amoxicillin (100%), Trimethoprim-Sulfamethoxazole (81.48%), Streptomycin (62.96%), and Tetracycline (55.56 percent). Since the rate of *Salmonella* carriers was relatively high, eating goat meat could increase the risk of foodborne salmonellosis.

Keywords: Antibiotic sensitivity, Goat isolation, PCR detection, *Salmonella*

INTRODUCTION

Capra aegagrus hircus, popularly the black Bengal goat domesticated in Bangladesh, preliminary reared for chevon by millions of poor women and landless peoples to increase poverty (Rahmanet al., 2017). Salmonellosis is one of the most important foodborne zoonoses all over the world. The pathogen has been isolated from the feces, lungs, and liver of goats worldwide (Ziinoet al., 2009). *Salmonella* can infect a wide range of animals, including poultry and other birds, horses, cattle, pigs, sheep, goats, dogs, cats, and reptiles (Songer and Post, 2004). These Gram-negative bacteria are mainly transmitted through contaminated food and water and are clinically characterized by septicemia and enteritis. However, the possibility of the upper respiratory tract bacterial transmission in the animal has been reported (Garg and Sharma, 1979). Salmonellosis in goats could occur at all ages during the year in both males and females and is responsible for the considerable loss of kids and may even cause abortion in adults (Arrudaet al., 2004). Salmonellosis is the most frequent disease in goats among the most common bacterial zoonotic diseases characterized by diarrhea (Radostits et al., 2007; Kahn et al., 2010). Detection of these organisms could be a serious public health concern (Adesiji et al., 2011).

The conventional laboratory cultural methods take a long time to achieve a positive or negative result. Molecular techniques, such as genetic probes and Polymerase Chain Reaction (PCR) enable rapid, sensitive, and pathogen identification in the atmosphere with precision (Josephson et al., 1991). Without displaying any clinical symptoms of salmonellosis, the infected animals may shed the *Salmonella* organism in their feces. As a result, a rapid, specific, and responsive *Salmonella* detection method is critical for animal and human health and as well as the diagnostic industry. Polymerase chain reaction detection of Salmonella in clinical samples from animals is faster than traditional culture techniques, with a sensitivity and specificity of 100 percent as compared to culture techniques. The method could be applied for rapid routine diagnosis (Stone et al., 1994). The objective of the present study was to estimate the prevalence of *Salmonella* in both diarrheic and healthy goats and to confirm it by PCR detection.

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

MATERIALS AND METHODS

Ethical statement

This study was approved by the Ethical Committee of Sylhet Agricultural University, Sylhet-3100, Bangladesh, as well as by mutual (verbal) understanding of the respective farm owners.

Geo-location of the study area

The present study was conducted in the Sylhet district of Bangladesh, which is located in the northeastern part of Bangladesh. The average maximum and minimum temperatures were 38°C and 7°C, respectively. The present study was conducted over a 12-month period from January to December 2018 and the data regarding climatic conditions were retrieved from Regional Meteorological Centre, Sylhet, Bangladesh.

Animals and samples

A total of 180 black Bengal goats of different ages from the Government Goat Development Farm, Sylhet, and 40 goats from individual farmers in Sadar Upazilla, Sylhet, constituted the study population. Fecal samples were collected directly from the rectum of apparently healthy goats and stored refrigerated at 4°C until processed for nucleic acid purification within 24 hours of collection. A cross-sectional study was conducted to isolate and identify the *Salmonella* from the feces of goats.

Salmonella isolation and identification

The conventional bacteriological methods were used to isolate the *Salmonella* from the samples as described (Khan et al., 2021). The samples were inoculated into the nutrient broth and incubated at 37°C for 24 hours. Fecal samples were subjected to initial nutrient pre-enrichment and incubated, and 0.5 mL was transferred to 10 mL Tetrathionate Broth (Merck) and incubated at 37°C for 24 hours. Pre-incubated brilliant green and Xylose Lysine Deoxycholate (XLD) agar was streaked with a loop of each enrichment broth and incubated. The plates were then analyzed for the existence of *Salmonella* colonies. Suspected colonies were inoculated into TSI (Triple Sugar Iron agar), peptone water, Simmon's Citrate, Urea medium, and MR-VP.

DNA extraction and PCR amplification

The DNA extraction was performed according to the manufacturer's instruction using the Addprep genomic DNA extraction kit (Addbio Inc. Ltd., Korea). These eluted DNA samples were stored at -80°C until further analysis. The PCR analysis was performed to detect the *Salmonella* invasion gene (*invA* gene) according to the manufacturer's instruction (Addbio Inc. Ltd., Korea). PCR assay performed in the thermal cycler TC1000G PCR System® (DLAB Scientific Inc., USA) with a heated lid. The cycling conditions included 50°C for 3 minutes (UDG Reaction), 95°C for 10 minutes (Initial Denaturation), 35 cycles of 95°C for 30 seconds (denaturation), 68°C for 45 seconds (annealing), and 72°C for 5 minutes for final extension (Khan et al., 2021). For the detection of *Salmonella*, the primers of invS-F (5'-TAA TGCCAGACGAAAGAGCGT-3') and invS-R (5'-GATATTGGTGTTTATGGG GTCGTT-3') were used (Khan et al., 2021). All reaction mixtures, including the negative control and *Salmonella* positive DNA, were tested in duplicate in the same run of PCR assay. PCR products were analyzed on 1.8% agarose gels stained with RedSafe™ (iNtRON Biotechnology, Korea) Nucleic Acid Staining Solution (20,000×), photographed, and stored as a digital image.

Antimicrobial sensitivity testing

The qualitative agar diffusion method (Kirby-Bauer method) was used for antibiotic sensitivity testing employing Mueller Hinton agar. The *in vitro* antibiotic sensitivity test was determined by the standard disc diffusion procedure according to the Clinical and Laboratory Standard Institute (CLSI, 2012). The antibiotic discs used in the present study were Erythromycin (ERY), Gentamycin (GEN), Streptomycin (STR), Amoxicillin (AMX), Ciprofloxacin (CIP), Tetracycline (TET), Neomycin (NM), and Trimethoprim-Sulfamethoxazole (SXT). The MIC (MIC50 and MIC90) was determined for each of the antibiotics used and the sensitivity or resistance was determined according to the protocol described (CLSI, 2012).

Statistical analysis

Microsoft Excel was used for the descriptive statistics. Chi-square tests were used to assess the significance of differences in prevalence between age, sex, and health status of the animals. P values less than 0.05 were considered significant using Chi-square tests (SPSS Inc., Chicago, IL, USA).

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

RESULTS

Prevalence of *Salmonella* in goats

From January to December 2018, a total of 220 goat feces samples (180 from Government Goat Development Farm, Sylhet, Bangladesh, and 40 from different private goat farms in Sadar Upazilla in Sylhet) were collected for isolation and identification and molecular detection of *Salmonella* and antibiotic sensitivity testing. Among 220 samples, *Salmonella* isolates were detected in 27 (12.27%) samples using the conventional cultural method. The isolates were followed by biochemical identification and PCR assays in which 20 (9.09%) samples indicated *Salmonella* positive (Table 1). Furthermore, the prevalence of *Salmonella* in the personal private farm was lower (3.89%) than the Goat development farm, Sylhet, which was estimated at 7.20% (Table 2).

Cultural and morphological characterization

All *Salmonella* colonies in Mac Conkey agar plates appeared as non-lactose fermenters, colorless and transparent (Figure 1C), and *Salmonella* turbidity produced on nutrient broth (Figure1A). The isolates of *Salmonella* grown in *Salmonella-Shigella* (SS) agar plates indicated characteristic black-centered colonies (Figure 1B), but isolates were absent in the black dot. In microscopic analysis, the thin smears prepared with the colony from SS agar for Gram's staining showed Gram-negative, pink-colored, tiny rod-shaped appearance arranged in single or paired or short-chain (Figure 1D).

Biochemical characterization

Due to the cultural and morphological properties, all suspected *Salmonella* colonies were subjected to selected biochemical tests, including indole formation (Figure 2E), methyl red (Figure2B), and Voges Proskauer reaction (Figure 2C), citrate utilization, and triple sugar iron agar. Of the 27 suspected *Salmonella* colonies, 20 were confirmed by biochemical test results. On TSI slants, most of the *Salmonella* isolates indicated fermentation of glucose (Figure 2A), gas production from glucose, H_2S formation, but none of the isolates fermented either lactose or sucrose (Figure 2D).

Antimicrobial susceptibility

The antibiotic sensitivity test indicated that the highest number of *Salmonella* isolates were sensitive to Ciprofloxacin (100%), Gentamycin (100%), and Neomycin (100%). Alternatively, there was significantly high resistance in *Salmonella* isolates to Erythromycin (100%), Amoxicillin (100%), Trimethoprim-Sulfamethoxazole (81.48%), Streptomycin (62.96%) followed by Tetracycline (55.56%, Table 3).

Detection of *Salmonella* by the polymerase chain reaction

All *Salmonella* suspected cultures subjected to PCR amplification generated a product of approximate molecular size 100 base per (bp) (*invA* gene) according to the manufacturer's instruction (AddBio Inc., Korea). A 100 bp DNA marker was used as a molecular weight marker (AddBio Inc., Korea). The band size detected in isolated *Salmonella* was consistent as analyzed by agarose gel electrophoresis (Figure3). The estimated prevalence of *Salmonella* by using PCR was 9.09% (20/220) in goat feces.

Table 1. Overall prevalence of *Salmonella* isolates in goats according to biochemical identification at Sylhet district of Bangladesh

Factors	Animal	Number of examined animals	Positive fecal samples	Prevalence	p value
Age	Up to 1 year	45	9	20%	< 0.01
	1 - 2 years	90	7	7.8%	
	> 2 years	85	4	4.7%	
Total		220	20	9.09%	
Sex	Male	150	9	6.0%	0.321
	Female	70	11	15.71%	
Total		220	20	9.09%	
Health status	Apparentlyhealthy	181	5	2.76%	< 0.01
	Diarrheic	39	15	38.46%	
Total		220	20	9.09%	

Level of significance $p < 0.05$

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

Table 2. Farm-level prevalence of *Salmonella* isolates in goats according to conventional cultural methods and biochemical identification in Goat development farm, Sylhet

Farm type	Total number of animal	Number of positive by conventional culture methods	Number of positive by biochemical tests	Prevalence	
				Conventional methods	Biochemical /PCR tests
SGDF*	180	16	13	8.89%	7.20%
IPF	40	11	07	27.5%	3.89%
Total	220	27	20	12.27%	9.09%

***Goat development farm, Sylhet, IPF: Individual private farm

Table 3. Overall susceptibility and resistance patterns of *Salmonella* isolates to selected antibiotics according to clinical and laboratory standard institute

Antimicrobial drug	Antimicrobial class	Abbreviation	Susceptible	Resistant*
Amoxicillin	β-lactams	AMX	-	100%
Ciprofloxacin	Quinolones	CIP	100%	-
Trimethoprim-Sulfamethoxazole	Folate pathway inhibitors	SXT	18.52%	81.48%
Erythromycin	Macrolids	ERY	-	100%
Gentamicin	Aminoglycosides	GEN	100%	-
Neomycin	Aminoglycosides	NM	100%	-
Streptomycin	Aminoglycosides	STR	37.04%	62.96%
Tetracycline	Tetracyclines	TET	44.44%	55.56%

**Salmonella* isolates that indicate moderate resistance to some antibiotics were considered resistant according to the CLSI recommendations.

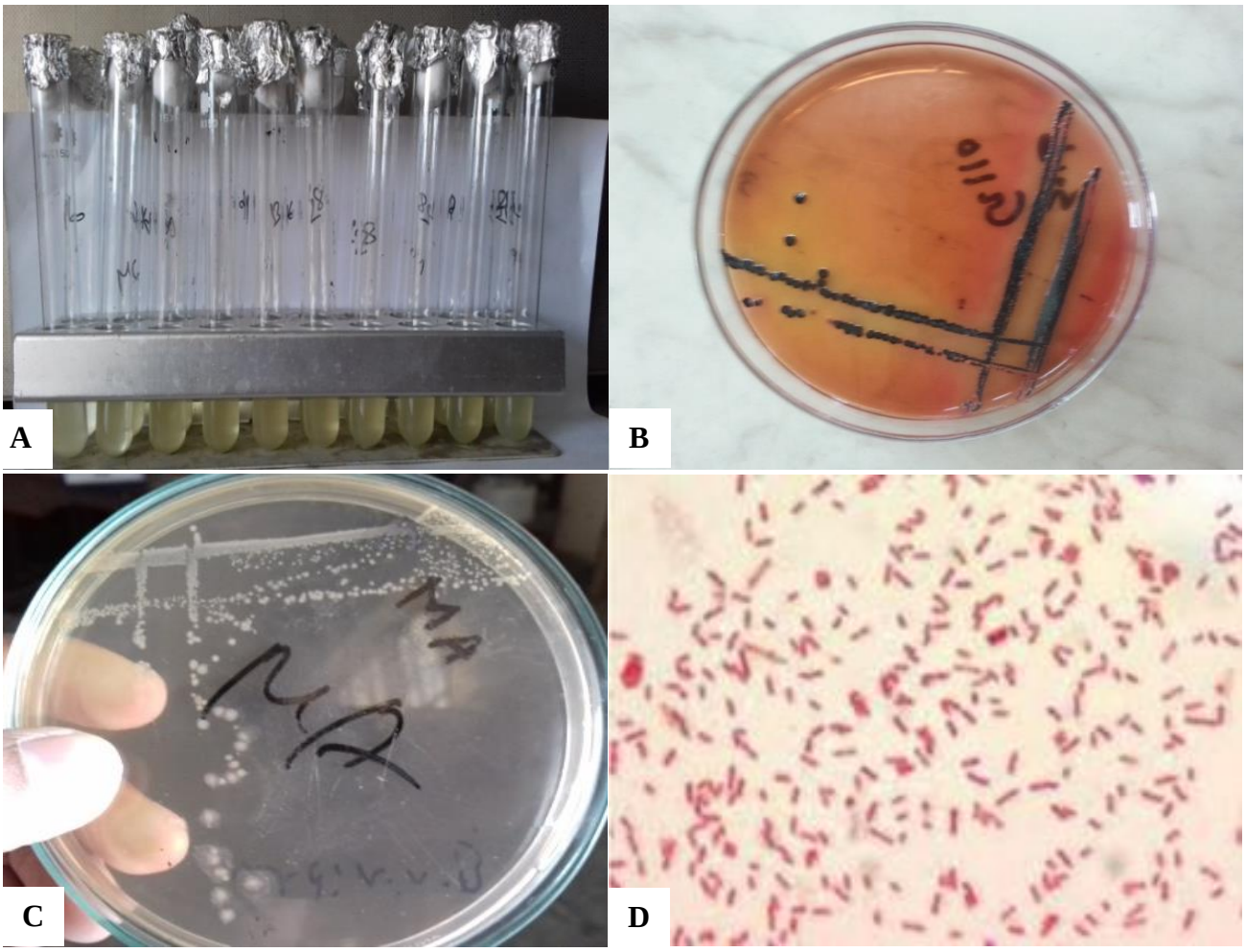


Figure1. A: Nutrient broth *Salmonella* produced turbidity, **B:** Black centered colony on *Salmonella-Shigella* (SS) agar, **C:** Colorless colonies on MacConkey agar, **D:** Gram staining under the microscope revealed Gram-negative, small rods arranged in single, paired, or clustered characteristics

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

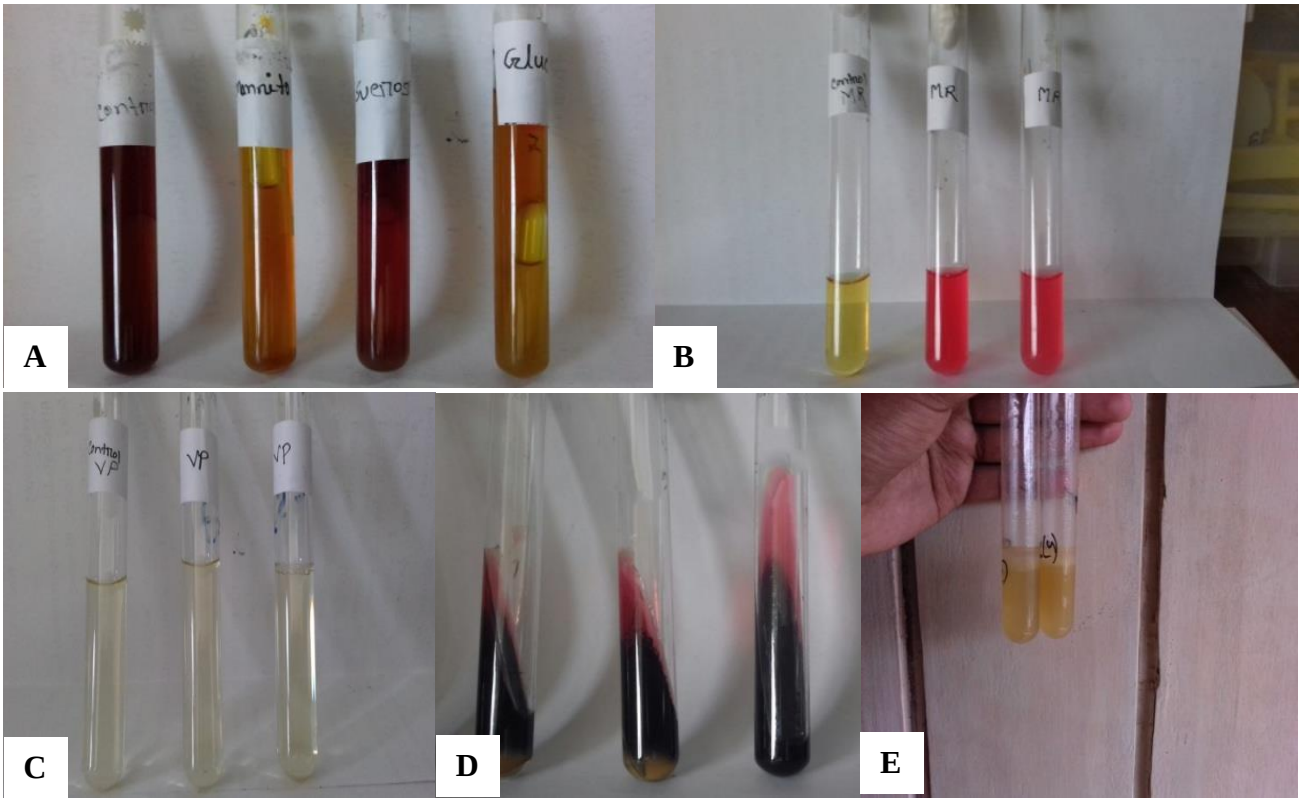


Figure 2. A: Sugar fermentation (Glucose and Mannitol +ve with the production of acid and gas, Sucrose –ve), **B:** MR Test (positive, red color), **C:** VP Test (Negative, no color change), **D:** TSI test (positive, Slant: red, butt: black); **E:** Indole Test (Negative, no color change)

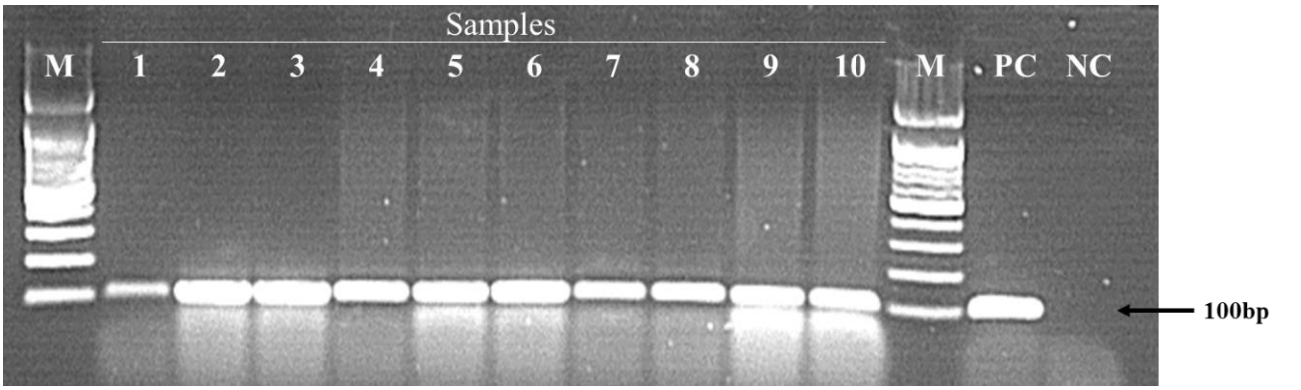


Figure 3. Results of PCR under UV illuminator (bands corresponding to the 100 bases per marker line indicated representative of *Salmonella* positive samples (1~10). M: Marker, NC: Negative control, PC: Positive control

DISCUSSION

The isolation and identification procedure included cultural examination, morphological examination, staining properties, biochemical tests, and molecular detection by PCR technique. In the present study, a total number of 220 feces samples from goats were collected by Sylhet Goat Development Farm (SGDF) and Individual Private Farm (IPF) in Sylhet. The bacteriological examination revealed the isolation of *Salmonella* organisms from goats with an estimated prevalence of 12.27% (27/220). Several studies reported a similar prevalence of *Salmonella* in goat feces (Teklu and Negussie, 2011; Saha et al., 2013; Bosilevac et al., 2015). The prevalence of *Salmonella* was higher in young (up to 1 year) and sick (diarrheic) animals, estimated to be 20% and 38.46%, respectively. The age-related result of the present study is consistent with the findings of other researchers (Saha et al., 2013; Mahmood et al., 2014). The findings also coincide with the results of Hunduma et al. (2010),who stated diarrhea as the major problem in goats with a higher prevalence of 42.2%.

A total of 20 (9.09%) fecal samples were PCR positive for *Salmonella*, which is higher than others who reported only 1.05% PCR positive cases (Esmaeili and Rahmani, 2016). Similar studies were conducted on cattle in Bangladesh and found 8.50% positive by biochemical and PCR (Khan et al., 2021). In contrast, Teklu and Negussie (2011)found a higher prevalence of *Salmonella* in sheep and goats in an export abattoir in Ethiopia. They reported that 7.7% and 11.7%

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

of the sheep and goats were positive for *Salmonella*. Gallegos-Robles et al. (2009) isolated and detected the *Salmonella* from cattle feces using microbiological and PCR methods, estimated the prevalence at 55% and was much higher than in the present study. For the detection of *Salmonella* genus using the PCR technique, it was found that all PCR products of the isolated positive control resulted in 100 bases per amplified fragment. The *invA* gene has been reported to be present in all strains and clinical isolates of *Salmonella* (Dahshan et al., 2010). The detection of *Salmonella* in clinical samples by PCR results in a faster than conventional culture methods (Stone et al., 1994). The conventional method of isolating *Salmonella* is more laborious and requires more manpower (Van der Zee and Veld, 2000). There are a very small number of viable organisms in the feces that may fail to grow in artificial laboratory media. Molecular tests were most successful than conventional micro-biological techniques (Jungkind, 2001).

The antibiotic disk diffusion indicated that some isolates were resistant to Streptomycin (62.96%), Amoxicillin (100%), Erythromycin (100%), trimethoprim/sulfamethoxazole (81.48%), and Tetracycline (55.56%). On the other hand, the highest number of *Salmonella* isolates was sensitive to Ciprofloxacin (100%), Gentamycin (100%), and Neomycin (100%). Isolation of *Salmonella* from food and water to antibiotic resistance is of great importance in the case of public health. While these infections are caused by animal feces, it is also essential to identify the antibacterial agents that are used to treat or prevent infections, as well as the promoters that are created (Graham et al., 2007). It has been reported that a high percentage of *Salmonella* isolates from healthy and diseased animals were resistant to two or more antimicrobial agents (Esaki et al., 2004), which explains the high spread of these organisms through feces of apparently healthy animals and their wide dissemination in the environment. There have been reports of the release of *S. Typhimurium* in a pig plant (Tanaka et al., 2014). Although the animals were found to be healthy, the release was very high several days after inoculation; therefore even normal feces could be a source within the herd infection. It has been reported that multi-resistant S. *Enteritidis* (resistant to two or more antimicrobial agents) with different patterns could reach up to 51.6% (de Oliveira et al., 2005).

The most common patterns of resistance were sulfamethoxazole, streptomycin, and tetracycline while ciprofloxacin resistance was the least common. In Japan, *S. Typhimurium* isolated from various animal species indicated that 20% of the isolates were resistant to ampicillin and 24% to tetracyclines (Esaki et al., 2004). In 2008, 31 Salmonella strains were isolatedfrom 12 different serovars forms in cattle, and the transmission of microbial resistance from *S. Heidelberg* to *S. Typhimurium* and bacteriophages that are resistant to several beta-lactam antibiotics and tetracycline blaCMY-2, tet (A), and tet (B), was demonstrated (Zhang and LeJeune, 2008). Later, 58% resistance to trimethoprim/sulfamethoxazole and 56% to tetracycline, followed by ampicillin and amoxicillin were reported (Yang et al., 2010). In Chile, in a preliminary study, 20.5% of the *Salmonella* strains isolated mainly from pigs, indicated multidrug resistance (MDR),with oxytetracycline being the drug with the highest resistance (69.1%, Junodet al., 2013).

CONCLUSION

From the findings of the present study, it could be concluded that *Salmonella* is an important cause of diarrhea in goats with salmonellosis in Bangladesh. Therefore, a rapid and proper diagnosis could prevent harm inflicted on the livestock industry. In this regard, the most accurate and quick diagnostic methods are required. The molecular basis of *Salmonella* identification techniques, such as the use of the *invA* gene-specific PCR method, could be useful in diagnostic and research laboratories. Ciprofloxacin, Gentamycin, and Neomycin might be the best choice among the antibiotics available on the market. The variation in antibacterial susceptibility or resistance pattern was also observed in the present study. This study suggests the strategic use of antibiotics for the control of *Salmonella* infections in animals.

DECLARATIONS

Acknowledgment

The authors acknowledge the Sylhet Agricultural University Research System (SAURES) for funding this research, and the respective farm owners for data support.

Competing interests

The authors have declared no conflict of interest regarding the publication of this research.

Authors' contribution

This manuscript is from the master's degree thesis of Md. Abdus Sabur. Md. Mukter Hossain designed the study. Md. Abdus Sabur and Mouri Rani Das conducted the experiments. All authors were involved in data interpretation, write up and final approval of the manuscript.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

REFERENCES

Adesiji YO, Alli OT, Adekanle MA, andJolayemi JB (2011). Prevalence of *Arcobacter*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* species in retail raw chicken, pork, beef and goat meat in Osogbo, Nigeria. Sierra Leone Journal of Biomedical Research, 3: 8-12. DOI: https://www.doi.org/10.4314/sljbr.v3i1.66644

Arruda SGB, Biscontini TMB, and Stamford TLM (2004). Microbiological characterization of goat meat subjected to different forms of management. Hygiene Alimentar, 18: 58-62. DOI: https://www.doi.org/10.12750/JET.2013.28.4.337

Bosilevac JM, Gassem MA, Al-Sheddy IA, Almaiman SA, Al-Mohizea IS, Alowaimer A, andKoohmaraie M (2015). Prevalence of *Escherichia coli* O157:H7 and *Salmonella* in camels, cattle, goats, and sheep harvested for meat in Riyadh. Journal of Food Protection, 78: 89-96. DOI: https://www.doi.org/10.4315/0362-028X.JFP-14-176

Clinical and Laboratory Standards Institute (CLSI) (2012). Performance standards for antimicrobial disk susceptibility tests; approved standard, 11th Edition CLSI document M02- A11. Wayne, PA: Clinical and Laboratory Standards Institute. https://www.clsi.org

DahshanH, Shahada F, Chuma T, Moriki H, and Okamoto K (2010). Genetic analysis of multidrug-resistant *Salmonella enteric serovar* Stanley and Typhimurium from cattle. Veterinary Microbiology,145:76-83. DOI: https://www.doi.org/10.1016/j.vetmic.2010.02.035

de Oliveira DS, Siqueira F, Ruschel L, andBrandelli A (2005). Antimicrobial resistance in *Salmonella Enteritidis* strains isolated from broiler carcasses, food, human and poultry-related samples. International Journal of Food Microbiology, 97: 297-305. DOI: https://www.doi.org/10.1016/j.ijfoodmicro.2004.04.022

Esaki H, Morioka A, Ishihara K, Kojima A, Shiroki S, Tamura Y, and Takahashi T (2004). Antimicrobial susceptibility of *Salmonella* isolated from cattle, swine and poultry (2001-2002): Report from the Japanese Veterinary antimicrobial resistance monitoring program. Journal of Antimicrobial Chemotherapy, 53: 266-270. DOI: https://www.doi.org/10.1093/jac/dkh081

Esmaeili H, and Rahmani HK (2016). Detection of *Salmonella* carriers in sheep and goat flocks of Bushehr and Lorestan Provinces, Iran. Journal of Medical Bacteriology, 5: 50-53. Available at: https://jmb.tums.ac.ir/index.php/jmb/article/view/256

Gallegos-Robles MA, Morales-Loredo A, Alvvarez-Ojeda G, OsunaGarcia JA, Martinez IO, Morales-ramos LH, and Fratamico P (2009). PCR detection and microbiological isolation of *Salmonella spp*. from fresh beef and cantaloupes. Journal of Food Science, 74: 37-40. DOI: https://www.doi.org/10.1111/j.1750-3841.2008.01006.x

Garg DN, and Sharma VK (1979). The detection of nasal carriers of *Salmonella* and other enterobacteria amongst young farm animals. Zentralblatt Bakteriologie Orig A, 243: 542-546. Available at: https://pubmed.ncbi.nlm.nih.gov/384721/

Graham JP, Boland JJ, and Silbergeld E (2007). Growth promoting antibiotics in food animal production: An economic analysis. Public Health Reports, 122: 79-87. DOI: https://www.doi.org/10.1177/003335490712200111

Hunduma D, Tigre W, Wagari M, and Regassa F (2010). Preliminary study on major health problems of the newly introduced boer goat breed in Ethiopia. World Applied Sciences Journal, 11: 803-807. Available at: https://www.cabi.org/ISC/abstract/2011319568

Josephson KL, Phillai SD, Way J, Gebra CP, and Pepper IL (1991). Fecal coliforms in soil detected by polymerase chain reaction and DNA-DNA hybridizations. Soil Science Society of America Journal, 55: 1326-1332. DOI: https://www.doi.org/10.2136/sssaj1991.03615995005500050022x

Jungkind D (2001). Automation of laboratory testing for infectious diseases using the polymerase chain reaction: Our past, our present, our future. Journal Clinical Virology, 20: 1-6. DOI: https://www.doi.org/10.1016/S1386-6532(00)00148-7

Junod T, Lopez-Martin J, and Gadicke P (2013). Antimicrobial susceptibility of animal and food isolates of *Salmonella enterica*. Revista Medica De Chile, 141: 298-304. DOI: https://www.doi.org/10.4067/S0034-98872013000300003

Kahn CM, Dana GA, Pete DC, Peter RD, Katherine EQ, Philip TR, Jagdev MS, Roger KW, and Tracee T (2010). The merck veterinary manual. 10th Edition. Merck and CO., Inc. USA. pp183, 287, 1898. 201. Available at: https://www.elsevier.com/books/the-merck-veterinary-manual/major/978-0-911910-93-3

Khan MNK, Das MR, Sabur MA, Rahman MM, Uddin MB, Cho HS, and Hossain MM (2021). Isolation, identification, molecular detection and sensitivity to antibiotics of *Salmonella* from cattle faeces. Bulgarian Journal of Veterinary Medicine, 24: 57-66. DOI: https://www.doi.org/10.15547/bjvm.2019-0061

Mahmood AK, Khan MS, Khan MA, Khan MA, and Bilal M (2014). Prevalence of *Salmonella* in diarrheic adult goats in field conditions. The Journal of Animal and Plant Sciences, 24(1): 98-102. Available at:http://thejaps.org.pk/docs/v-24-1/15.pdf

Radostits OM, Gay CC, Hinchcliff KW, and Constable PD (2007). In: Veterinary Medicine, a textbook of the diseases of cattle, horses, sheep, pigs, and goats. Saunders, Elsevier Ltd, 10th Edi, pp. 314, 316, 321, 898, 1541. Available at: https://www.elsevier.com/books/veterinary-medicine/radostits/978-0-7020-2777-2

Rahman MM, Hossain MM, and Miah MAM (2017). Poverty reduction and livelihood improvement of women through goat rearing. Asian Journal for Poverty Studies, 3: 59-66. Available at: https://ejournal.unib.ac.id/index.php/ajps/article/view/2672

Saha GK, Paul AK, Samad A, Islam MA, and Khan MSR (2013). Epidemiological investigation and antibiotic sensitivity of salmonellosis in goats at the selected areasof Bangladesh. Journal of Animal Reproduction and Biotechnology, 28: 337-342. Available at: http://www.e-jarb.org/journal/view.html?volume=28&number=4&spage=337

Songer JG, and Post KW (2004). Veterinary microbiology: Bacterial and fungal agents of animal disease. 1st ed. St. Louis, MO: Elsevier Saunders, pp. 131-136. Available at: https://www.elsevier.com/books/veterinary-microbiology/songer/978-0-7216-8717-9

Stone GG, Oberst RD, Hays MP, McVey S, and Chengappa MM (1994). Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. Journal of Clinical Microbiology, 32: 1742-1749. DOI: https://www.doi.org/10.1128/JCM.32.7.1742-1749.1994

Tanaka T, Imai Y, Kumagae N, Sasaki T, and Ochiai N (2014). Quantitative microbiological evaluation of *Salmonella* Typhimuriumshed in diarrhea, loose, and normal stools of infected pigs. Open Journal of Veterinary Medicine, 4: 58-66. DOI: https://www.dx.doi.org/10.4236/ojvm.2014.44007

Teklu A, and Negussie H (2011). Assessment of risk factors and prevalence of *Salmonella* slaughtered small ruminants and environment in an export abattoir, Modjo, Ethiopia. American-Eurasian Journal of Sustainable Agriculture, 10(6): 992-999.

Van der Zee H, and Veld JHJH (2000). Method for the rapid detection of *Salmonella*, In: *Salmonella* in domestic animals, Wray C. and Wray A. (eds) CABI Publishing, Wallingford, UK, pp. 373-391. Available at: https://www.cabi.org/vetmedresource/ebook/20002210430

Yang B, Qu D, Zhang X, Shen J, and Cui S (2010). Prevalence and characterization of *Salmonellaserovars*in retail meats of marketplace in Shaanxi, China. International Journal of Food Microbiology, 141: 63-72. DOI: https://www.doi.org/10.1016/j.ijfoodmicro.2010.04.015

Zhang Y, and LeJeune J (2008). Transduction of blaCMY-2, tet(A), and tet(B) from *Salmonella* enterica subspecies enteric serovar Heidelberg to S Typhimurium. Veterinary Microbiology, 129: 418-425. DOI: https://www.doi.org/10.1016/j.vetmic.2007.11.032

Ziino G, Giuffrida A, Bilei S, and Panebianco A (2009). Bacteria isolated from 25 hydatid cysts in sheep, cattle, and goats. Veterinary Record, 165: 234-236. DOI: https://www.doi.org/10.1136/vr.165.8.234

To cite this paper: Sabur MA, Das MR, Uddin MB, Rahman MM, Islam MR, Chowdhury MSR and Hossain MM (2021). Molecular Detection and Antibiotic Sensitivity of *Salmonella* Species Isolated from Goat Feces in Sylhet District of Bangladesh. *World Vet. J.*, 11 (3): 395-401. DOI: https://dx.doi.org/10.54203/scil.2021.wvj51

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 402-415, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019

Loubna Ghallache[1], Abdellah Mohamed-Cherif[2], Bernard China[3], Faiza Mebkhout[4,5], Nesrine Boilattabi[6], Alaoua Bouchemal[7], Ahmed Rebia[5], Ammar Ayachi[1], Djemel Khelef[2], Kamel Miroud[8], and Khatima Ait-Oudhia[2,9*]

[1] *Environment, Health, and Animal Production Laboratory, Institute of Veterinary and Agronomic Sciences, Batna, Algeria*
[2] *High National Veterinary School, Algiers, rue Issad Abbes, El Alia, Oued Smar, Algiers. Algeria*
[3] *Sciensano, The Belgian Institute of Health, 1050 Brussels, Belgium*
[4] *Institute of Veterinary Sciences. University Saad Dahleb Blida 1, Algeria*
[5] *ITELV, Les zouines. BP 03/A, 16000. Birtouta, Algérie*
[6] *Laboratory of Cellular and Molecular Biology. FSB. USTHB, Algiers, Algeria*
[7] *CNIAAG. BP N°10 Birtouta Algiers, Algeria*
[8] *Laboratoire Epidémio-surveillance, santé, productions et reproduction, expérimentation et thérapie cellulaire des animaux domestiques et sauvages (ESSPRETCADS) ». Institut des Sciences Vétérinaires. Université Chadli Bendjedid El-Tarf, Algeria*
[9] *Laboratory of Biotechnology in Animal Reproduction, Blida, Algeria*

*Corresponding author's Email: khatima.aitoudhia@gmail.com; ORCID: 0000-0002-2514-5615

ORIGINAL ARTICLE
pii: S232245682100052-11
Received: 13 July 2021
Accepted: 28 August 2021

ABSTRACT

Mastitis in cows is a major problem in dairy farms leading to a decrease in the quantity and quality of milk. The aim of the present study was to examine the association between the presence of *Escherichia coli (E. coli)* in milk and the subclinical mastitis, and to characterize the antibiotic resistance profiles of the isolated *E. coli*. In the current study, a total of 360 cow raw milk samples from three dairy farms of the region of Algiers were analyzed. The analysis period lasted from Spring 2017 to Winter 2019. The California Mastitis Test (CMT) was applied to detect subclinical mastitis. The *E. coli* strains were isolated from milk using conventional bacteriological methods. The antibiotic resistance profile of the isolated *E. coli* strains to 12 different antibiotics was tested using the disk diffusion method. On *β*-lactamase-producing strains, a double diffusion test was applied to identify the Extended-spectrum *β*-lactamase (ESBL) phenotype. Finally, the *ctXx-M* genes were amplified by PCR. Two-thirds (66.4%) of the milk samples were positive for the CMT test. A total of 97 *E. coli* strains were isolated from the milk samples, their resistance to antibiotics was tested, and 3.1% of the strains were resistant to trimethoprim-sulfamethoxazole, 6.2% to chloramphenicol, 12.3% to gentamicin, 13.4% to colistin, 23.3% to amoxicillin/clavulanate, 31.9% to kanamycin, 39.2% to enrofloxacin, 51.5% to cefotaxime, 52% to tetracycline, 57.7% to ampicillin, 74.3% to nalidixic acid, and 75.3% to amoxicillin. Furthermore, most of the *E. coli* strains (92.8%) were resistant to more than one antibiotic with a Multiple Antibiotic Resistance index ranging from 0 to 0.8. The 50 strains resistant to cefotaxime were analyzed for an ESBL phenotype. 39 of them (78%) were positive to the double-disk synergy test. Among the 39 ESBL positive strains, 27 (69.2%) were confirmed for the presence of a CTX-M gene by PCR. The present study showed that multiple drug-resistant *E. coli*, including ESBL-carriers, were frequently isolated from the milk of dairy cows in Algeria. The results underlined that the use of antibiotics on farms must be reasoned to avoid the spread of resistant strains in animals and human populations.

Keywords: Antibiotic Resistance, Cows, CTX-M gene, *Escherichia coli*, Milk, Subclinical Mastitis

INTRODUCTION

Algerian people are great milk consumers (120 l/inhabitant/year) but the local production is insufficient to fulfill this demand. The imported milk is expensive (around 1 trillion USD per year) for Algeria (MADR, 2003), therefore, Algerian authorities encourage local milk production. It is, thus, necessary to increase the local milk production yield. According to the Ministry of Agriculture and Rural Development (MADR), Algeria has a herd of 900000 dairy cows, including 23000 high potential dairy cattle (MADR, 2003). To achieve the goal of increasing the quantity and quality of milk production in Algeria, it is important to study the physiology of milk production and identify the causes that may affect it.

However, mastitis is the most expensive and the most important disease in the dairy industry. It is defined as an inflammation of one or more quarters of the mammary gland, characterized by physical, chemical, and microbiological changes in milk secretion as well as pathological changes in breast tissue (Sharif and Muhammad, 2009). The economic losses are due to the low yield of infected udders, veterinary treatments, milk seizures as well as the premature culling of cows (Peton and Le Loir, 2014).

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

Clinical mastitis infections are those with symptoms like udder swelling or redness that are clearly visible. On the other hand, subclinical mastitis infections do not cause any visible changes in milk or udder appearance, making it difficult to detect. Nevertheless, subclinical mastitis infections affect the dairy producer's bottom line by reducing milk production, decreasing milk quality, and suppressing reproductive performance.

Regarding the incidence of subclinical mastitis, an average annual cost of around 5000 euros has been estimated in a herd of 100 dairy cows in the Netherlands (Halasa et al., 2009). However, the presence of pathogens and/or toxins in milk as well as residues of antibiotics resulting from the treatment of mastitis can seriously compromise public health. These infections are mainly caused by coliform bacteria *Escherichia coli* (*E. coli*), staphylococci, and streptococci (Baazise, 2006). The *E. coli* is the signature of fecal contamination although *Staphylococci* and *Streptococci* are members of the skin microbiota. To fight mastitis, massive doses of antibiotics are used selecting resistant and multi-resistant bacteria in milk.

The present study was dedicated to the characterization of antibiotic-resistant *E. coli* in the milk of dairy cows in dairy farms of the Algiers region from Spring 2017 to Winter 2019.

MATERIALS AND METHODS

Ethical approval

Since the experiments were performed on naturally collected milk, no ethical question was raised by this study. The samples were taken under the supervision of a veterinarian.

Sampling

A total of 360 milk samples (50 mL) coming from 149 different cows in three different farms (Figure 1) located in the region of Algiers were analyzed. The dairy farms in Algeria were rather small (less than 100 head of cattle) and the production system is mostly semi-intensive (MADR, 2003). Except for farm 2, the cows had access to meadows. The characteristics of the sampled farms are listed in Table 1. In order to examine a possible evolution in time, certain cows were sampled several times at different time periods. Therefore, the number of milk samples was higher than the number of cows.

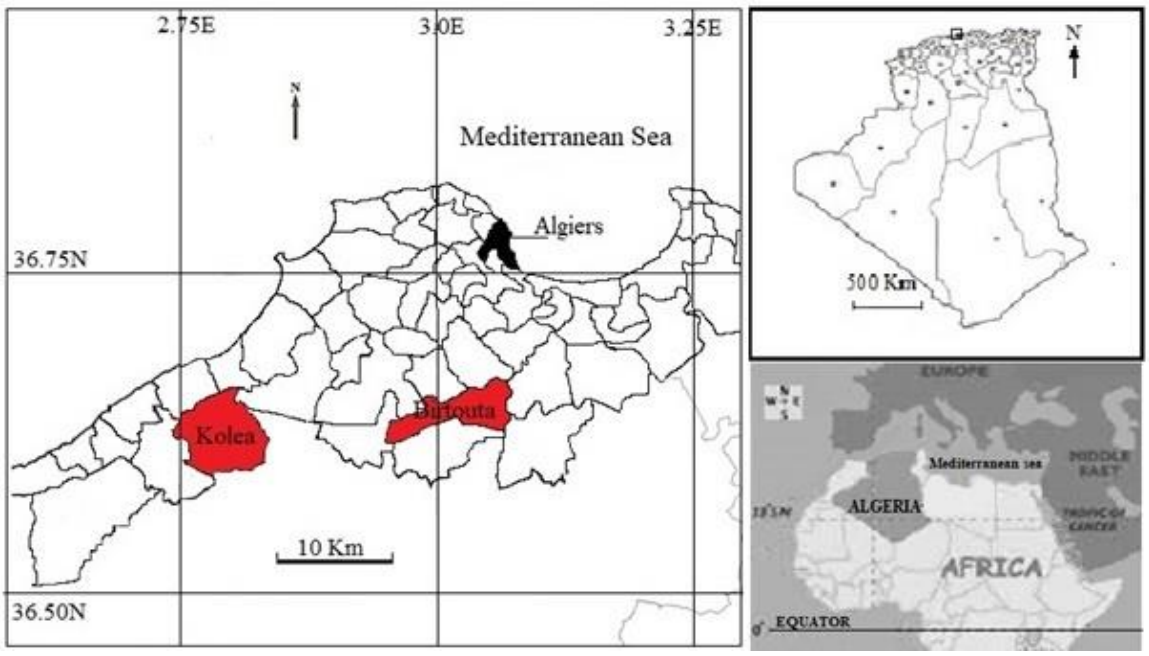


Figure 1. Localization of the sampled farms of the Algiers region

Table 1. Characteristics of the farms of the Algiers region

Farm	Name	Location (town and GPS coordinates)	Number of sampled cows	Number of milk samples	Period of sampling
Farm 1	DOUMA	Kolea, 36°36'05.3"N 2°47'00.5"E)	32	120*	March to May 2017
Farm 2	CNIAAG	Birtouta 36°39'21.8"N 3°01'27.3"E	76	180	April to July 2019
Farm 3	ITELV	Birtouta 36°39'13.9"N 3°03'05.4"E	41	60	April to December 2019
Total			149	360	March 2017 to December 2019

*: In this farm a sample of the bulk milk tank was also analyzed.

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

California mastitis test

The California Mastitis Test (CMT) was employed on farms to identify subclinical mastitis by an indirect estimation of the Somatic Cell Count (SCC) in milk (Leach et al., 2008). A four-well plastic paddle was used, one well per udder quarter of the cow to be tested. Foremilk was discarded, and then about 50-100 ml of the milk was collected into each well. An equal volume of test reagent was added (COVETO, Montaigu, France), and gently manually agitated. The reaction was scored on a scale of 0 (mixture remained unchanged) to 3 (almost-solid gel forms), with a score of 2 or 3 being considered as a positive result. A cow was considered positive if at least one quarter was positive.

Microbiological analysis

In this phase of the study, 1 ml of each milk sample was used to inoculate a sterile tube containing 9 ml of Brain Heart Infusion Broth (BHIB, Oxoid, Basingstoke, UK). The tubes were incubated for 24 hours at 37°C. For the isolation of *E. coli*, a droplet of the enrichment medium was used to inoculate a Tryptone Bile X-glucuronide (TBX) Agar (Oxoid CM0945, Basingstoke, UK) plate using a sterile loop. The plates were incubated 24 hours at 37°C. The characteristic colonies were picked and streaked on a selective medium Basing (Hektoen Agar, Oxoid PO0142, Basingstoke, UK) to obtain a pure culture. For bacterial identification, catalase, oxidase, and coagulase tests were performed (Pilet and Bourdon, 1979).

The antibiotic resistance of the isolated *E. coli* strains was analyzed using the disk diffusion method (Brown and Kothari, 1975). The diameters of inhibition were measured and compared according to the instructions by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (SFM, 2019) to determine the phenotype (sensitive, intermediate, or resistant). The used antibiotics are listed in Table 2. The antibiotics were chosen following the recommendations of the Algerian Ministry of Health (MSPRH, 2014).

Multiple Antibiotic Resistance (MAR) index was defined as diving the number of resistant antibiotics by the number of tested antibiotics (Krumperman, 1983). For ESBL producers, the double-disk synergy test was used as previously described (Drieux et al., 2008). Commercially available disks (Oxoid, Basingstoke, UK) of Cefotaxime (CTX, 30 µg) together with 30 µg Amoxicillin-clavulanate (AMC) were placed 25 mm apart from each other on the surface of inoculated Muller-Hinton Agar (MHA) plate and incubated at 37°C for 24 hours. The test was considered positive when the decreased susceptibility to CTX and/or ceftazidime was associated with a clear augmentation of inhibition zone in front of the AMC antibiotic disk forming a characteristic "champagne cork" shape (Okoko et al., 2020). As a confirmation test, CTX (30 µg) disk and AMC (30 µg) were placed 30 mm apart on the surface of inoculated MHA, and incubated for one hour. Then, the AMC disk was removed and replaced by a CTX disk (30µg), and the plate was incubated at 37°C for 24 hours following the recommendations of the Algerian Ministry of Health (MSPRH, 2014).

Molecular detection of resistance genes

The *E. coli* strains that were positive for the double-disk synergy test (ESBL+) were analyzed for the presence of *bla*CTX-M genes using PCR as previously reported by Tabar et al. (2016). The forward and reverse primers used for PCR were *bla*-CTX-M-F 5´-ACCGCCGATAATTCGCAGAT-3´ and *bla*-CTX-M-R 5´-GATATCGTTGGTGGTGCCATA -3, respectively (Tabar et al., 2016). Briefly, 1 ml of an overnight *E. coli* culture in BHI (Bio-Rad, France) was used to prepare DNA by the boiling method (Djahnit et al., 2018). To amplify the CTX-M gene by PCR, 5 µl of the DNA was used. The master mix consisted of 4 µl of 5X PCR Mix (miniPCR, USA), 0.2 µl of each primer (40 µM, Eurogentec, Belgium), and 10.6 µl of DNAse-RNAse free sterile water (miniPCR, USA). The PCR temperature cycles were applied once (94ºC for 5 minutes), followed by 40 times (94ºC for 30 seconds, 58.0ºC for 30 seconds, and 72ºC for 30 seconds), and finally once (72°C for 5 minutes). The PCR products were analyzed by 2% gel agarose electrophoresis (Green and Sambrook, 2019). A 584 bp product was expected. Two controls were used, namely a CTX-M15+ *E. coli* strain (DSM22664, DSMZ, Germany) and a CTX-M-2+ *E. coli* strain (provided by the Belgian National reference center of antibiotic-resistant Gram-negative bacilli, Yvoir, Belgium).

Statistical analysis

The statistical analyses were performed using the following free online statistics software. MedCalc (version 20.009, Schoonjans, 2017) was used for the odds ratios. For the independence Chi-Square test, BiostatGV (version 2020) was used (Huet et al., 2021). The confidence interval for the proportions was calculated using Sample size.net (version 2021, Kohn and Senya, 2021). For the hierarchical clustering, Wessa (version 1.2.1.) was used (Wessa, 2021) with the option complete linkage. All the statistics used in this work were described in the textbook by Thrusfield and Christley (2018).

The association strength between the presence of *E. coli* in milk samples and the CMT test results was performed using the Odds Ratio (OR) calculation (Thrusfield and Christley, 2018). A case sample was defined as a sample contaminated by *E. coli* and a control sample as an *E. coli* free sample. The association among antibiotic resistance profiles was calculated using the independence Chi-square test (Thrusfield and Christley, 2018). In order to accept or

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

reject the null hypothesis, the following rules were adopted; p value < 0.01 was considered as a highly significant difference, p value < 0.05 was considered as a significant difference, and a p value ≥ 0.05 was considered as non-significant.

Hierarchical clustering analysis of the *E. coli* strains was made using the complete linkage method (Guess and Wilson, 2002). Briefly, a table was made with one row for each strain and one column for each antibiotic. The result of the antibiotic resistance was scored as zero for sensitive, one for resistant, and two for intermediate. The table was then used to introduce the data (as a Comma-separated Value (CSV) file) in a statistic software in R language (Wessa, 2021). The dendrogram was generated, and the length of the bars (height) indicated the distance among the strains.

Table 2. The list of used antibiotics with the disk loads and the used cut-offs

Antibiotic	Diskload (µg)	Cut-off (mm)
Ampicillin (AMP)	10	13-17
Amoxicillin (AMX)	20	13-17
Amoxicillin/Clavulanate (AMC)	30 (20-10)	13-18
Nalidixic acid (NAL)	30	13-19
Cefotaxim (CTX)	30	22-26
Gentamicin (GEN)	10	12-15
Trimethoprim- Sulfamethoxazole (SXT)	25 (1.25-23.75)	10-16
Chloramphenicol (CHL)	30	12-18
Colistin (CST)	25	10-11
Tetracycline (TET)	30	14-19
Kanamycin (KAN)	10	13-18
Enrofloxacin (ENR)	5	16-21

RESULTS

California mastitis test

In farm 1 (Douma), 120 milk sampling were collected from 32 cows. Of 120 milk samples, 78 (65%, CI 95%: 55.8-73.5) were positive to the CMT test, moreover, 31 cows (96.9%) showed at least one positive sample. In order to evaluate an evolution in time, one cow was sampled six times, 25 cows were sampled four times, two cows were samples three times, three cows were sampled two times, one cow was sampled once, and the bulk tank milk was also analyzed. Considering the obtained results, 14 out of 32 cows (43.75%, CI 95%: 26.36-62.34) presented 100% of the positive samples, 3 cows presented 75% of the positive samples, 1 cow presented 66.67% of the positive samples, 4 cows presented 50% of positive samples, 1 cow presented 33.3% of positive samples, 7 cows presented 25% of positive samples, and only 2 cows presented no positive sample. Concerning the evolution of the positivity of the samples in time, no clear pattern appeared. In farm 2 (CNIAAG), 180 milk samples coming from 78 cows were analyzed, 90 samples (50%, CI 95%: 42.5-57.5) were positive to the CMT test. Moreover, 57 out of 78 cows (73.1%, CI 95%: 61.8-82.5) showed at least one positive sample. Three cows were sampled four times, 37 cows were sampled three times, 19 cows were sampled two times and 19 cows were sampled once. Twenty-five out of 78 cows (32.1%) presented 100% of the positive samples, one (1.3%) cow presented 75% of the positive samples, one (1.3%) cow presented 66.67% of the positive samples, 7 (9%) cows presented 50% of positive samples, 17 (21.8%) presented 33.3% of positive samples, and 31 (39.7%) presented no positive samples. In farm 3 (ITELV), 60 milk sampling were performed on 40 cows; 51 samples (85%, CI 95%: 73.4-92.9) were positive. Thirty-three of the cows (82.5%, CI 95%: 67.2-92.7) showed at least one positive sample. Two cows were sampled three times, 16 cows were sampled twice, and 22 cows were sampled once. Furthermore, 31 out of 41 cows (77.5%) showed 100% of positive samples, 2 presented 50% of positive samples and 7 cows generated no positive results.

Globally, 239 out of 360 (66.39%, CI 95% 61.25-71.25) samples were positive to the CMT test, and121 cows out of 150 (80.67%. CI 95%: 73.43-86.65) showed at least one positive sample. These results indicated a severe problem of subclinical mastitis in these farms.

Escherichia coli isolation

The results of the research on *E. coli* in the 360 milk samples (Table 3) indicated an overall prevalence of 26.9% (97/360). Therefore, the next step was to investigate if there was a correlation between CMT results and *E. coli* isolation

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

results (Table 4). The Odds ratio was significantly upper than one indicating an association between the presence of *E. coli* in the milk sample and the presence of a positive CMT test ($p < 0.05$).

Antibiotic sensitivity testing

The main purpose of this study was to examine the antibiotic resistance of isolated *E. coli*. The obtained results of the first serial of 12 antibiotics are summarized in Figure 2. Of the 97 tested *E. coli* strains, 1 was resistant to 10 different antibiotics, containing Ampicillin (AMP), Amoxicillin (AMX), Amoxicillin/Clavulanate (AMC), Cefotaxim (CTX), Trimethoprim-sulphamethoxazole (SXT), Chloramphenicol (CHL), Colistin sulfate (CST), Tetracycline (TET), Kanamycin (KAN), Nalidixic acid (NAL), five strains (5.2%) were resistant to eight different antibiotics (AM, AMX, AMC, CTX, CHL, K, NAL, Enrofloxacin (ENR)), nine strains (9.3%) were resistant to seven antibiotics, 22 strains (22.7%) were resistant to six antibiotics, 19 (19.6%) were resistant to five antibiotics, 11 (11.3%) were resistant to four antibiotics, eight strains (8.2%) were resistant to three antibiotics, 12 (12.4%) were resistant to two antibiotics, four (4.1%) were resistant to one antibiotic and only three strains (3.2%) were sensitive to all the tested antibiotics. Among them, two strains were isolated from two different cows of farm 2 (CNIAAG) and one stain from farm 1 (DOUMA). In other words, the MAR index was in the range of 0-0.8.

Interestingly, out of 97 strains, 85 (97.6%) different resistant types (resist types) were observed. Some clusters per farm were found as expected, but since it does not always indicate that similar resist types can be observed in different farms (Figure 3). The antibiotic resistances of the *E. coli* isolated were compared per antibiotic and among the farms in Table 5. There was a significant difference among AMP, AMC, CTX, CHL, TET, KAN, and ENR ($p < 0.01$).

The association among the resistances was also investigated (Figure 4). The results indicated that the AMP resistance profile was statistically associated with the AMC resistance, the CTX resistance, the TET resistance, the KAN resistance, and the ENR resistance ($p < 0.05$). The AMX resistance was associated with the KAN resistance, the NAL resistance, and the ENR resistance ($p < 0.01$). The AMC resistance was associated with CTX and CST resistance ($p < 0.05$). The CTX resistance was associated with the CST and the KAN resistance ($p < 0.05$). The GEN resistance was associated with the SXT resistance ($p < 0.01$). The SXT resistance was associated with the CHL ($p < 0.01$). The KAN resistance was associated with the NAL and with the ENR resistance ($p < 0.05$). The NAL resistance was associated with the ENR resistance ($p < 0.01$).

Extended-spectrum beta-lactamases

Among the *E. coli* tested samples, some seemed to potentially have an Extended Spectrum □-lactamase phenotype (ESBL). The double-disk synergy test has been performed on the 50 *E. coli* strains resistant to CTX, among which 39 strains presented a characteristic phenotype (Figure 5). The gene CTX-M encoding CTX-M □-lactamase was amplified by PCR on the 39 potentially ESBL + *E. coli*. 27 out of the 39 (69.2%) tested *E. coli* strains were CTX-M positive (Figure 6).

Table 3. *Escherichia coli* isolated strains from milk samples found in the different farms of the Algiers region

Farm*	**Number of milk samples**	**Number of *E.coli* contaminated milk (%)**
Farm 1(DOUMA)	120	45 (37.5%)
Farm 2 (CNIAAG)	180	30 (16.7%)
Farm 3 (ITELV)	60	22 (36.7%)
Total	360	97 (26.9%)

*: The farms are described in material and methods

Table 4. Association between the presence of *Escherichia coli* in milk samples and the results of California Mastitis test for cows of the studied farms of the Algiers region

	Case (*E. coli* +)	**Control (*E. coli* -)**
Exposed (CMT +)	74	147
Not exposed (CMT-)	23	116
Exposure rate (%)	76.3	55.9
Odds	3.2	1.3
Odds ratio (CI 95%)	2.5 (1.5-4.3)	
P-value	<0.05	

CMT: California Mastitis Test, CI: Confidence Interval, *E. Coli: Escherichia coli*

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

Table 5. Comparison of the antibiotic resistance of *Escherichia coli* per antibiotic in the different farms of the Algiers region

Antibiotic*	Farm	S	I	R	p-value (Khi2 test)
AMP	CNIAAG	3	1	27	<0.01 SS
	DOUMA	31	4	10	
	ITELV	0	1	20	
AMX	CNIAAG	4	2	25	0.1 NS
	DOUMA	12	3	30	
	ITELV	1	0	20	
AMC	CNIAAG	9	10	12	<0.01 SS
	DOUMA	32	2	11	
	ITELV	7	14	0	
CTX	CNIAAG	11	0	20	<0.01 SS
	DOUMA	24	0	21	
	ITELV	1	11	9	
GEN	CNIAAG	28	1	2	0.2 NS
	DOUMA	37	0	8	
	ITELV	19	0	2	
SXT	CNIAAG	23	8	0	0.07 NS
	DOUMA	27	15	3	
	ITELV	19	2	0	
CHL	CNIAAG	21	9	1	<0.01 SS
	DOUMA	41	0	4	
	ITELV	20	0	1	
CST	CNIAAG	23	0	8	0.04 SI
	DOUMA	38	3	4	
	ITELV	20	0	1	
TET	CNIAAG	17	3	11	<0.01 SS
	DOUMA	12	11	22	
	ITELV	2	0	19	
KAN	CNIAAG	17	1	13	<0.01 SS
	DOUMA	40	1	4	
	ITELV	4	2	15	
NAL	CNIAAG	4	2	25	0.2 NS
	DOUMA	12	3	30	
	ITELV	2	0	19	
ENR	CNIAAG	4	14	13	<0.01 SS
	DOUMA	24	9	12	
	ITELV	1	6	14	

S: sensitive; **R:** resistant; **I:** intermediate; **SI:** significant; **SS:** very significant; **NS:** not significant. * AMP: Ampicillin, AMX: Amoxicillin, AMC: Amoxicillin/Clavulanate, NAL: Nalidixic acid, CTX: Cefotaxim, GEN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, CHL: Chloramphenicol, CST: Colistin, TET: Tetracycline, KAN: Kanamycin, ENR: Enrofloxacin

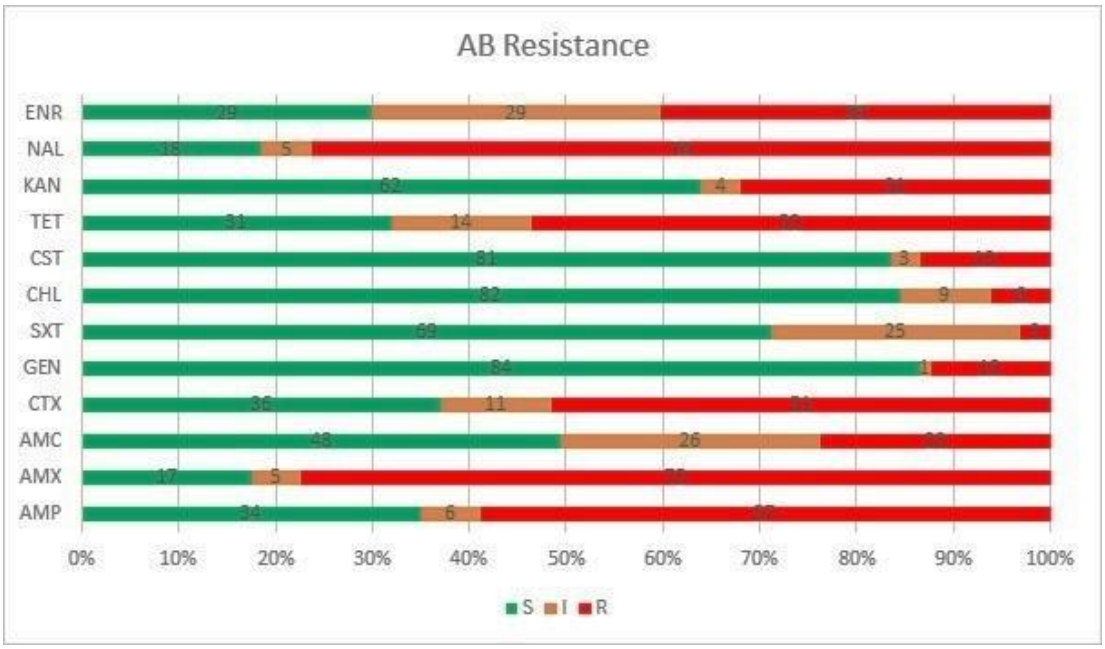


Figure 2. Resistance profile for 12 different antibiotics of the 97 *Escherichia coli* isolates from the farms of the Algiers region. AMP: Ampicillin, AMX: Amoxicillin, AMC: Amoxicillin/Clavulanate, NAL: Nalidixic acid, CTX: Cefotaxim, GEN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, CHL: Chloramphenicol, CST: Colistin, TET: Tetracycline, KAN: Kanamycin, ENR: Enrofloxacin, S: Sensitive, R: Resistant, I: Intermediate.

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

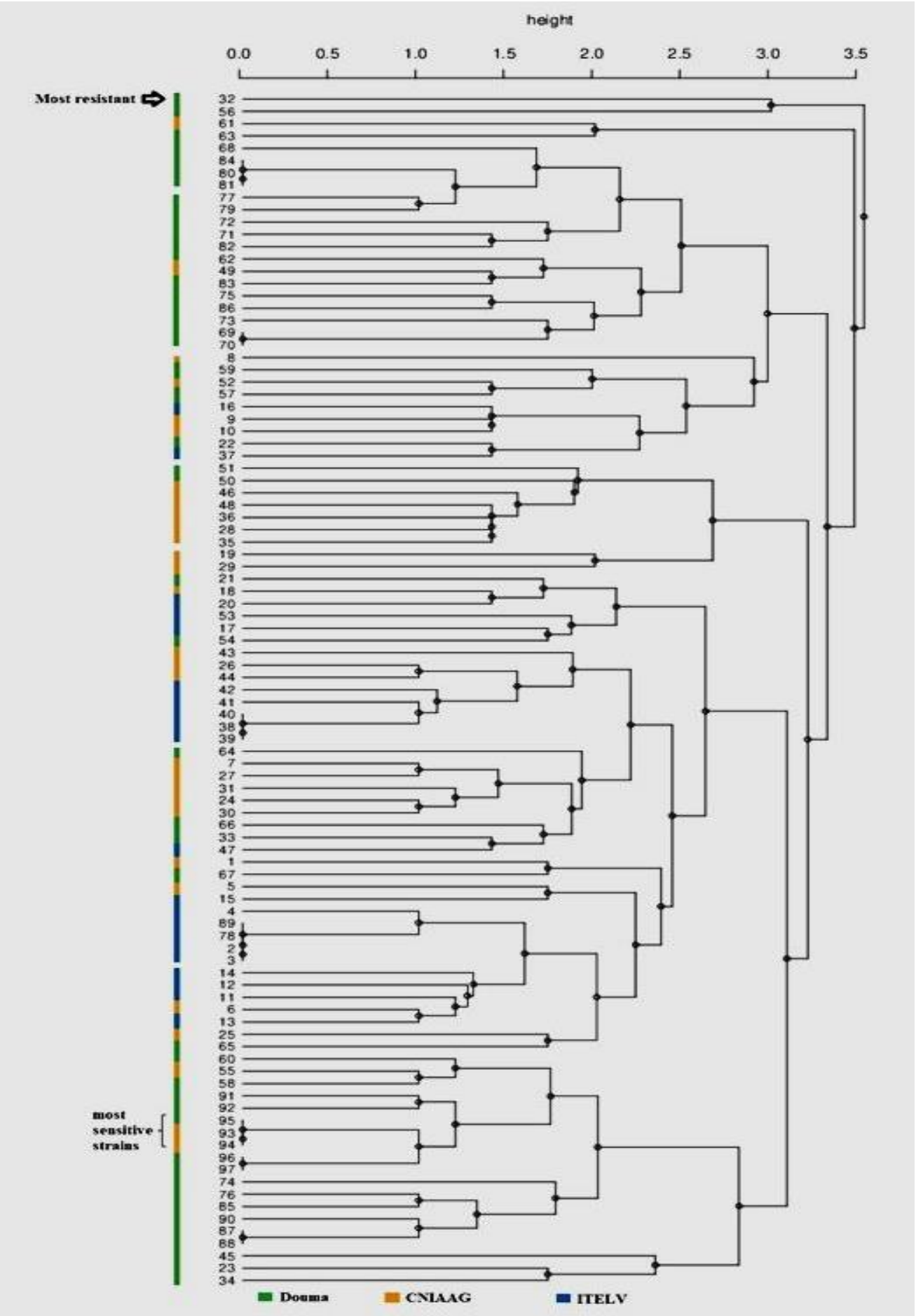


Figure 3. Dendrogram comparing the antibiotic resistance profiles of the *97 Escherichia coli* strains. The *Escherichia coli* isolates were numbered from 1 to 97. The colors indicated the farm where the *Escherichia coli* strain was isolated. The length of the lines is proportional to the distance among the strains (Defays, 1977)

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

	AMP	AMX	AMC	CTX	GEN	SXT	CHL	CST	TET	KAN	NAL	ENR
AMP		0.3	<0.01	<0.01	0.6	0.9	0.7	0.1	0.04	<0.01	0.45	<0.01
AMX			0.37	0.9	0.8	0.37	0.2	0.3	0.33	<0.01	<0.01	<0.01
AMC				<0.01	0.86	0.9	0.83	0.01	0.31	0.2	0.37	0.16
CTX					0.19	0.84	0.72	0.047	0.87	<0.01	0.95	0.11
GEN						<0.01	0.41	0.94	1	0.83	0.49	0.23
SXT							<0.01	1	0.7	1	0.81	1
CHL								1	0.16	1	0.41	1
CST									0.11	1	0.45	0.37
TET										0.55	0.66	0.12
KAN											0.011	<0.01
NAL												<0.01
ENR												

Figure 4. Associations among resistances. An independence chi 2 test has been applied. The p values of the Chi 2 test were indicated. In green ($p \geq 0.05$); there was no association, in orange ($0.05 < p < 0.01$), there was a significant association; and in red ($p < 0.01$), there was a significant association. AMP: Ampicillin, AMX: Amoxicillin, AMC: Amoxicillin/Clavulanate, NAL: Nalidixic acid, CTX: Cefotaxim, GEN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, CHL: Chloramphenicol, CST: Colistin, TET: Tetracycline, KAN: Kanamycin, ENR: Enrofloxacin.

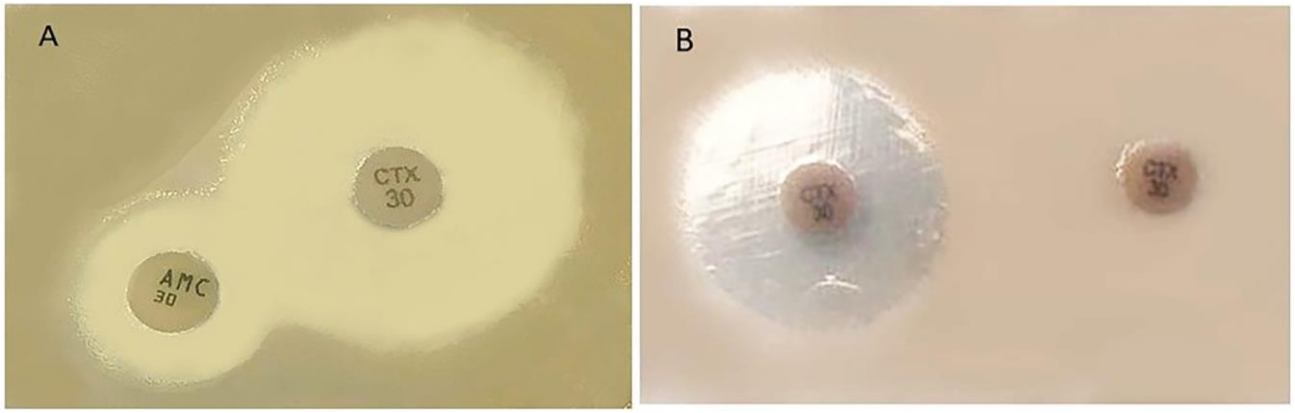


Figure 5. ESBL phenotype identification using synergy and double disk profiles. **A:** Synergy profile between AMC (30 µg) and CTX (30 µg). Due to clavulanate inhibition of ESBL; **B:** Double disk profile. The CTX (30 µg) disk on the left showed a classical inhibition phenotype. The CTX (30 µg) disk on the right showed no inhibition due to the pre-incubation for one hour with an AMC (30 µg) disk.

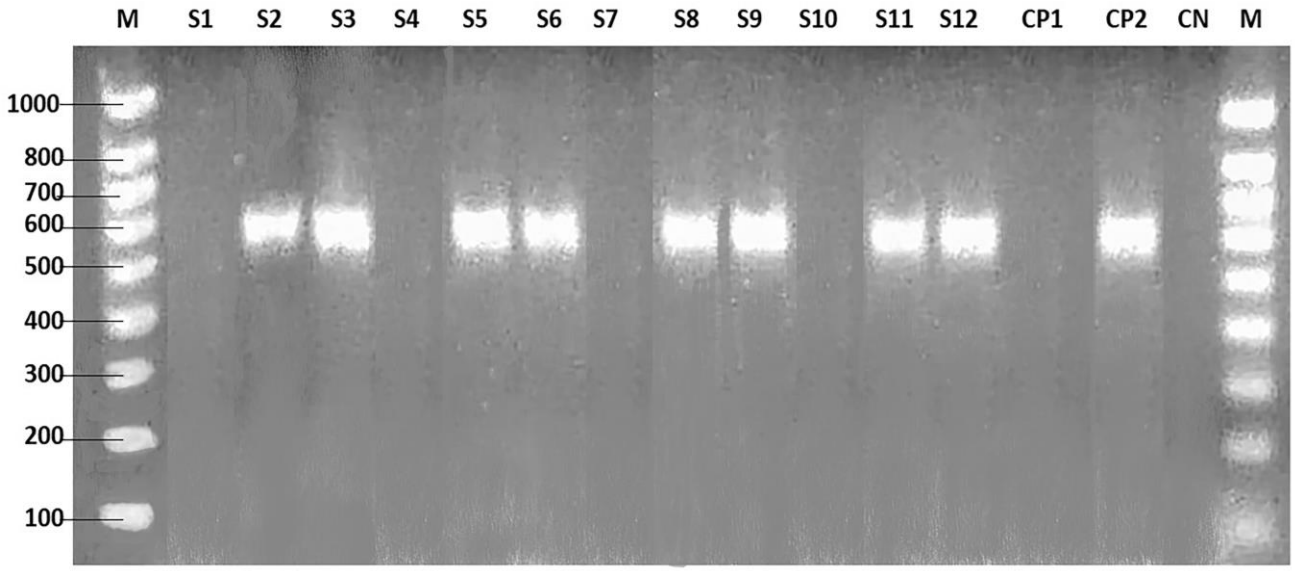


Figure 6. Agarose gel electrophoresis (2%). M: molecular weight ladder (bp). S1, S4, S7, S10: negative samples. S2, S3, S5, S6, S8, S9, S11, S12: positive samples. CP1: CTX-M-1 + strain; CP2: CTX-M-2 strain; CN: Negative PCR control.

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

DISCUSSION

Mastitis is a common inflammation of the cow udder leading to defect in milk production quantitatively and qualitatively leading to economic losses. In the present study, 239 (66.4%) cows presented a positive CMT test which was in accordance with the previous study in Algeria where 79% of the tested cows were CMT + (Asnoune et al., 2012), and with the study on milk quality in West Algeria (Aggad et al., 2009). *Escherichia coli* can harbor and transmit resistance to antibiotics. The virulence and the antibiotic resistance of bacteria are often linked (Cepas and Soto, 2020). *Escherichia coli* is one of the bacteria causing mastitis along with Staphylococci and Streptococci (Burvenich et al., 2003). Given its origin, it is not normal to find this bacterium in milk indicating fecal contamination.

In the current study, the prevalence of *E. coli* in milk samples was 26.94%. When this prevalence was compared with the prevalence of other studies of the literature, the present prevalence is high. For instance, a prevalence of 6.5% was found in Jordan (Ismail and Abutarbush, 2020), 10% in Mexico (Olivares-Pérez et al., 2015), 11.1% in China (Yu et al., 2020), 15.5% in Belgium (Verbeke et al., 2014) and 7% in Egypt (Ameen et al., 2019). Interestingly, the present *E. coli* prevalence in the current study was approximately close to the prevalence (26%) found in another recent study in Algeria (Tahar et al., 2020) or a study in Ethiopia (27.3%) (Haftu et al., 2012), and lower than the prevalence found in Tunisia (31.7%) (Saidani et al., 2018).

A positive association (OR > 1) was observed between the presence of *E. coli* and a CMT+ test. Indeed, *E. coli* is a bacterium often found in mastitis (Burvenich et al., 2003). The hygiene of the farm is very often involved in the contamination of milk by *E. coli*. Heavily soiled litter is a major source of contamination (Magnusson et al., 2007). Decontamination of udders during milking is especially important to eliminate bacterial contaminations as much as possible. Therefore, the presence of *E. coli* in milk is an indicator of the lack of hygiene during milking (Saidani et al., 2018). The generalization of antibiotic therapy has produced spectacular results in the treatment of mastitis. But it has an unfortunate corollary, the selection of Multi-Drug Resistant (MDR) germs. In addition, previous studies have shown that the use of antibiotics for many years has acted as a selection agent of an antibiotic-resistant bacterial population on the farm prior to antibiotic use for a new clinical purpose (Avrain et al., 2003; Boonyasiri et al., 2014).

Food-producing animals harbor bacteria in their intestinal tract include potentially pathogenic bacteria for humans. These bacteria could serve as reservoirs of resistance determinants that could spread throughout the food chain, reducing the effectiveness of antimicrobials used in human and veterinary medicine (Gouvêa et al., 2015; Manyi-Loh et al., 2018).

The obtained results of the current study revealed a wide variation in resistance to the tested antibiotics. The resistance phenotype ranged from 3.1% (SXT) to 75.3% (AMX). It is worth mentioning that most of the *E. coli* strains (90/97, 92.8%) were MDR with a MAR index ranging from 0 to 0.8 with one strain resisting to 10 out of the 12 tested antibiotics. The presence of MDR strains was due to the significant pressure exerted by the extensive use of antibiotics on the farms analyzed. The appearance of MDR strains was favored by different mechanisms, including a resistance mechanism leading to the resistance of more than one antibiotic (efflux pump), the horizontal transfer of resistance by mobile genetic elements such as plasmids or transposons, and the presence of strains with a high mutation rate (Chang et al., 2015).

Recently in Algeria, multidrug-resistant *E. coli* in cows with clinical mastitis in 42 different dairy farms have been investigated (Tahar et al., 2020). The antimicrobial susceptibility testing was performed by the disk diffusion method on 52 *E. coli* strains. The observed resistances were AMX (86.5%), TET (75%), AMC (59.6%), SXT (36.5%), doxycycline (13.5%), and ciprofloxacin (13.5%). Multidrug resistance was observed in 38.4% of isolates (Tahar et al., 2020). In a similar study in Egypt (Ameen et al., 2019), the *E. coli* strains isolated from cow milk were resistant to AM (33%), SXT (23%), and TET (16%) or AMC (6.6%). In Switzerland (Nüesch-Inderbinen et al., 2019), the *E. coli* strains isolated from cow milk were resistant to AM (22%), TET (16%), SXT (9.8%), gentamicin (3.7%), or to AMC (2.4%). The observed differences in the different studies were mostly dependent on the used antibiotics in the farms. Therefore, SXT was the best choice in the present study, although AMC was the most appropriate choice in the Egyptian and Swiss studies.

The study of resistor types of the different strains underlined the existence of clusters in the same farm. Nevertheless, some similar resistance profiles were present in different farms. It is related to the fact that the same antibiotics were used in different farms (Österberg et al., 2016). When the resistance per antibiotic was compared among the farms, significant differences ($p < 0.05$) were observed for the prevalence of the resistance to several antibiotics (AMP, AMC, TET, KAN, and ENR). Therefore, the circulating strains were not the same for the different farms. It is due to the used antibiotics in the farms, and to the fact that the sampling period was not the same for all the farms. When the resistance is considered by the antibiotic family, the results were observed as follows; quinolones, NAL, and ENR (a fluoroquinolone) were tested. In fact, 74.3% and 39.2% of the *E. coli* strains were resistant to nalidixic acid and ENR, respectively. The resistances were significantly associated ($p < 0.01$), which is logical since the mechanisms of resistance were similar (mutations chromosomal genes *gyrA* and *parE*). The high levels of resistance can be related to the large use of ENR by the veterinarians in the sampled farms (personal communication).

For fluoroquinolones, a recent study in Algeria indicated 13.5% of the resistance of *E. coli* strains isolated from cow milk against ciprofloxacin (Tahar et al., 2020). In that study, the sampled region was located 200 km southeast of the current sampling region. The differences can be related to the fact that different antibiotics were used by veterinarians in this region. For example, in this study (Tabar et al; 2020), the most prevalent resistance was resistance to AMX (86.5%), TET (75%), and AMC (59.6%). In Tunisia, in a similar study, 11% of the *E. coli* isolates were resistant to ENR (Saidani et al., 2018).

For sulfonamides, the resistance against SXT in the present study was low (3%) compared to the data of the literature for comparative studies. For example, the resistance rate was 23.3% in Egypt (Ameen et al., 2019) or 17.8% in Tunisia (Burvenich et al., 2003). But the results indicated 25% of intermediate resistance which is concerning.

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

Nevertheless, a low rate of resistance was also found in Canada (5.8%) (Saini et al., 2012). For TET, the resistance rate was 52%. In the same country for a similar survey, the resistance rate was 75% (Tahar et al., 2020). The same high level of resistance rate was observed in Tunisia (46.6%) (Saidani et al., 2018) or Brazil (92%) (Rangel and Marin, 2009). Tetracycline represents one of the oldest molecules used, both in therapy and preventively, generating remarkably high resistance.

Nevertheless, in regions where this antibiotic was used less, lower resistance rates were recorded, for instance, the rates were reported as 2.6% in Canada (Saini et al., 2012), 12% in China (Yu et al., 2020), as well as 16.66% (Ameen et al., 2019) and 27.5% in Egypt (Ombarak et al., 2018). Tetracycline has been used inappropriately in animal feed in many countries, leading to the development of resistance. This has prompted some countries to prohibit their use in animal feed, such as in European countries (OJEU, 2003).

For aminoglycosides, KAN and gentamicin were tested. Following the classification (Saberfar et al., 2008), the results indicated that the resistance rate of the strains found in the current study for KAN goes to group II of middle resistance with a resistance level of 31.9%. The resistance to KAN was significantly different ($p < 0.01$) among the tested farms with a farm presenting 71.4% of resistant *E. coli*. Kanamycin resistance was significantly associated ($p < 0.02$) with the resistance to beta-lactam antibiotics (AMP, AMX, and CTX), and to resistance to quinolones (NAL and ENR). These associations need to be investigated because these antibiotics belong to different families and the resistance mechanisms are different. Moreover, such association was not found for gentamycin.

The high sensitivity of *E. coli* strains to gentamicin was due to the non-use of this antibiotic in veterinary treatments in Algeria, and therefore, no selection of resistant strains. The prevalence of KAN-resistant *E. coli* isolated from cow milk was within the range of 2.8% in China (Yu et al., 2020), 4.1 % in Egypt (Ombarak et al., 2018), and 6% in France (Botrel et al., 2010).

For gentamicin, a rate of 12% was recorded which was close to what has been recorded in Tunisia (19.5%, Saidani et al, 2018), in Egypt (13.3%, Ameen et al, 2019), and In China (12%, Yu et al, 2020). In addition, the present results were extremely low compared to those reported in Brazil (Rangel and Marin, 2009), Lebanon (Barbour et al., 2015), or Iran (Fazel et al., 2019), where the reported rates were 67.9%, 77%, and 49.2%, respectively. For polymixins, colistin (polymyxin E) has been tested. Colistin is a cationic polypeptide antibiotic that interacts with the outer membrane of gram-negative bacteria. Despite colistin once was avoided due to its nephro-and neurotoxicity**,** this drug has now become a last-resort antimicrobial agent for treating life-threatening infections caused by MDR gram-negative bacteria. However, in November 2015, the first plasmid-borne colistin resistance gene *mcr-1*, encoding a phosphoethanolamine transferase, was detected in livestock and raw meat samples as well as humans in China (Liu et al., 2020).

In the current study, 13.4% of the *E. coli* strains were resistant to colistin. Colistin resistance was associated ($p < 0.05$) with CTX resistance. Interestingly, colistin and ESBL resistance are plasmid-borne, and can be co-transferred as previously described in China (Liu et al., 2020). It will be, therefore, interesting to study the plasmid profiles of the isolated strains to identify the genetic support of the colistin resistance.

β-lactamins are classical antibiotics interfering with cell wall synthesis. In the present study, several β-lactamins were tested; AMP, AMX, AMC, and CTX. 75.3% of the *E. coli* strains were resistant to AMX, 57.7% to AM, 51.5% to CTX, and 23.3% to AMC. Therefore, β-lactamin resistance is common in the analyzed farms. The resistance of *E. coli* strains to AMP ranged widely, 2.6% in Canada (Saini et al., 2012), 22% in Switzerland (Nüesch-Inderbinen et al., 2019), 31.7% in China (Yu et al., 2020), 33.3% in Egypt (Ameen et al., 2019), and 59% in Brazil (Rangel and Marin, 2009).

The resistance to AMX was 86.6% in a similar study in another region of Algeria (Tahar et al., 2020), confirming that the *E. coli* strains resistant to AMX are common in cow milk suffering from mastitis in Algeria. Nevertheless, the use of a combination of AMC dropped the resistance rate to 23.7% in the current study and to 59.6% in the other Algerian study (Tahar et al., 2020), which was was confirmed in other studies conducted in Brazil (Rangel and Marin, 2009) and Egypt (Ameen et al., 2019) decreasing from 58% to 14% and 33.3% to 6.7%, respectively.

For CTX, the resistance rate was 4.5% in Egypt (Ombarak et al., 2018) and 18.1% in China (Yu et al., 2020). Therefore, the observed resistance rate in the current study was quite high indicating that *E. coli* strains producing β-lactamases were common in Algeria. This increase is particularly worrying because third-generation cephalosporins have been classified as critically important antibiotics (Collignon et al., 2009). Among the different mechanisms involved in β-lactamins resistance, the production of a β-lactamase, an enzyme hydrolyzing the β-lactam ring of the β-lactamins, is common. The emergence of ESBL and the widespread of this type of resistance is a major concern in animal and human health. Extended Spectrum β-lactamases are the enzymes having the ability to hydrolyze the β-lactam ring of broad-spectrum β-lactams such as oxyimino-cephalosporins, including cefotaxime, ceftriaxone, and ceftazidime. These antibiotics are the so-called "third-generation cephalosporins". Because diverse types of ESBLs genes are usually plasmid-borne, and since the plasmids can be transferred among various species of Gram-negative bacteria, the genes for ESBLs can be acquired by this horizontal transfer mechanism (Kawamura et al., 2017).

In the present study, by using the double-disk synergy test, among 50 *E. coli* strains resistant to CTX, 39 (78%) presented a classical synergy pattern of ESBL strains. Widespread resistance to newer generations of cephalosporins is attributed largely to the spread of CTX-M type Extended-spectrum β-lactamases (ESBLs) in Gram-negative bacteria, especially in *Escherichia coli.* As a result, the rapid global dissemination of the ESBL-producing *E. coli* is an emerging public-health concern (Pitout and Laupland, 2008).

In the current study, by using classical PCR (Tabar et al., 2016), the CTX-M gene has been identified in 27 (69.2%) of the strains. Besides, CTX-M and ESBL can be TEM-derived or SHV-derived (Kawamura et al., 2017), so it is possible that the ESBL *E. coli* strains are of these types. Moreover, the used PCR amplified mainly the CTXM-2 family genes (Figure 7). However, CTX-M-type ESBLs have been roughly divided into four groups based on the sequence

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

similarity in amino acid residues; CTX-M-1 group, CTX-M-2 group, CTX-M-9 group, and CTX-M-8/CTX-M-25 group (Naas et al., 2007; Kawamura et al., 2017). Therefore, it is also possible that the mentioned ESBL strains were from another CTX-M group. For example, CTX-M-15 which is common at this moment is a part of CTX-M-1 group and was the most isolated CTX-M gene in dairy cattle (Afema et al., 2018).

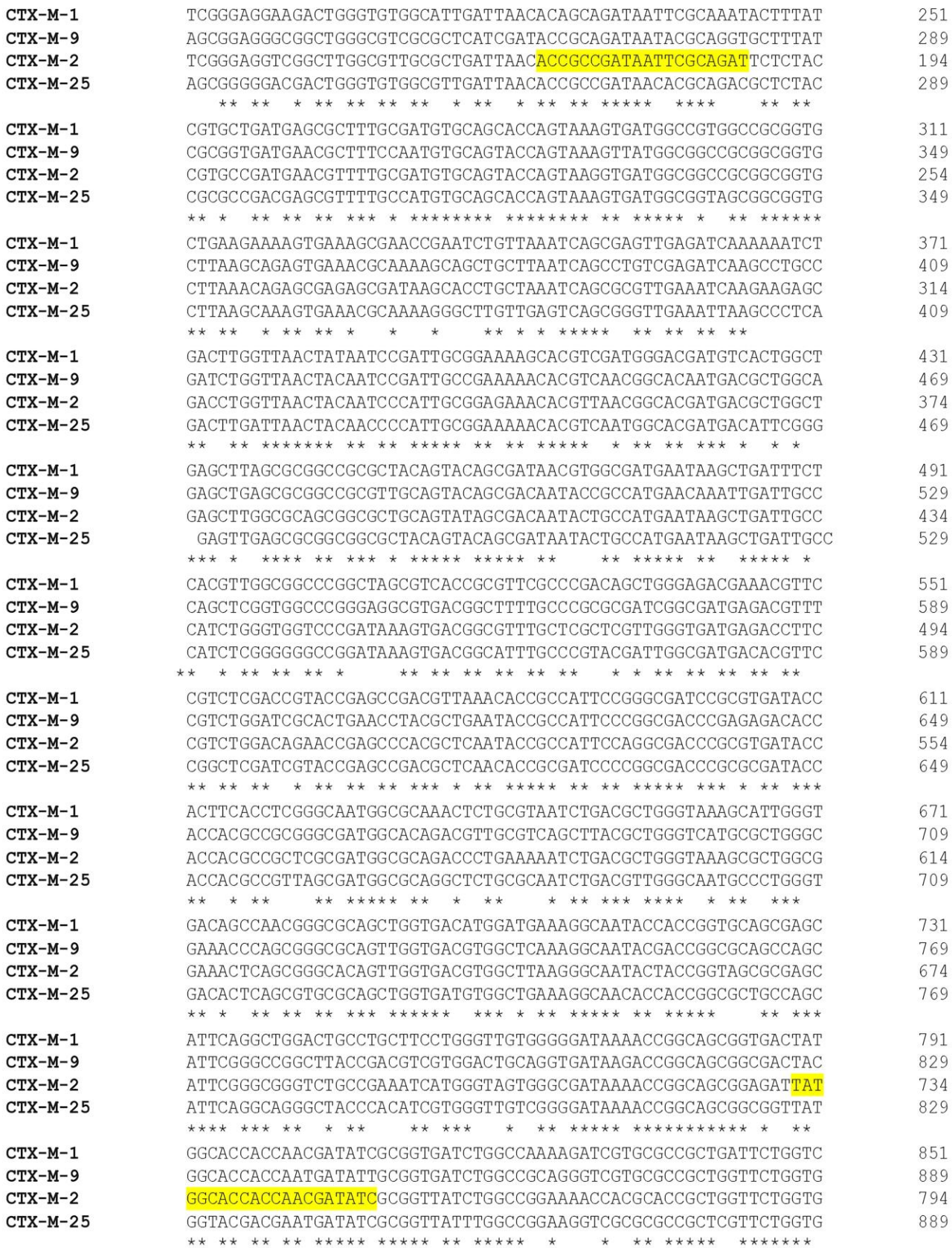


Figure 7. Alignment of the main CT-X-M sequences using ClustalOmega software. CT-X-M-1 (genbank NG_048897.1), CTX-M-2 (genbank NG_048968.1), CTX-M-9 (genbank NG_049043.1), and CTX-M-25 (genbank NG_048974.1). The sequences of the used primers were highlighted. The identities were indicated by a star. The numbers corresponded to the number of the genbank sequences.

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

CONCLUSION

In conclusion, the finidngs of the current study have indicated that cows producing milk are commonly positive to CMT test, and subclinical mastitis is an underestimated problem in Algeria. *Escherichia coli* is frequently isolated in cow milk indicating an improper hygiene level in the farms and an inappropriate disinfection procedure at the milking.

Antibiotic resistance is a major concern in animal and human medicine. The increase of MDR strains in animals and humans leads more and more to therapeutic dead ends. Most of the *E. coli* isolates were found as MDR, and a particular focus was put on ESBL. Of the studied *E. coli*, 40% were ESBL-carriers. Although animals should be cared for, the sparing use of antibiotics is recommended. In any case, the use of antibiotics as a growth promoter should be prohibited.

DECLARATIONS

Author's contributions

Ghallache Loubna and Mohamed-Cherif Abdellah conceived the study design, carried out laboratory work, participated in data analysis and interpretation, drafted the manuscript. Ghallache Loubna and Mohamed-Cherif Abdellah contributed equally to this work. China Bernard conceived the study design, participated in data analysis and interpretation, revised the manuscript. Benfodil Karima conceived the study design, drafted and revised the manuscript. Miroud Kamel revised the manuscript. Ait-Oudhia Khatima conceived the study design, took part in the coordination and management as well as field studies, participated in data analysis and interpretation, and revised the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

Acknowledgments

The authors wish to thank the Algerian Ministry of Higher Education and Scientific Research for their contribution to my Ph.D. training and Dr. Benbouras Mohamed Amin of the high national polytechnic School of Algiers for making up the geographical maps.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Afema JA, Ahmed S, Besser TE, Jones LP, Sischo WM , Davis MA (2018). Molecular Epidemiology of Dairy Cattle-Associated *Escherichia coli* Carrying *bla*$_{CTX-M}$ Genes in Washington State. Applied and Environmental Microbiology, 84(6):e02430-17. DOI : https://www.doi.org/10.1128/AEM.02430-17

Aggad H, Mahouz F, Ahmed Ammar Y,and Kihal M (2009).Evaluation de la qualité hygiénique du lait dans l'ouest algérien. Revue de Médecine Véterinaire, 160:590-595. Available at : https://www.revmedvet.com/artdes-fr.php?id=1769

Ameen F, Reda SA, El-Shatoury SA, Riad EM, Enany ME, Alarfaj AA(2019). Prevalence of antibiotic-resistant mastitis pathogens in dairy cows in Egypt and potential biological control agents produced from plant endophyticactinobacteria Saudi Journal of Biological Sciences, 26:1492–1498. DOI: https://www.doi.org/10.1016/j.sjbs.2019.09.008

Asnoune ZB, Butel MJ, and Ouzrout R (2012).Prévalence des principales bactéries responsables de mammites subcliniques des vaches laitières au nord-est de l'Algérie. Review of breeding and veterinary medicine of tropical countries,65:5-9. DOI: https://www.doi.org/10.19182/remvt.10132

Avrain L, Humbert F, L'Hopsitalier R, Sanders P, Vernozy- Rozand C,Kempf I (2003).Antimicrobial resistance in *Campylobacter* from broilers: Association with production type and antimicrobial use. Veterinary Microbiology, 96:267-276. DOI: https://www.doi.org/10.1016/j.vetmic.2003.07.001

Baazise D (2006). Evaluation de la qualité microbiologique du lait cru de vache dans la région de la Mitidja. Mémoire de magister, Département des sciences vétérinaires, Université de Blida: 198p. Available at : http://di.univ-blida.dz:8080/jspui/bitstream/123456789/475/1/06%20M.MTA.pdf

Barbour EK , Kassabian TJ, Shaib H, Kassaify Z , Iyer A, Azhar E, Harakeh S ,Kumosani T (2015). The significance of *Escherichia coli*-induced mastitis in cows associated with the presence of virulence genes and wide range-resistance to twenty antimicrobials. International Journal of Applied Research in Veterinary Medicine, 13: 51-63. Available at: https://www.cabdirect.org/cabdirect/abstract/20153215249

Boonyasiri A, Tangkoskul T, Seenama C, Saiyarin J, Tiengrim S, Thamlikitkul V(2014). Prevalence of antibiotic resistant bacteria in healthy adults, foods, food animals, and the environment in selected areas in Thailand. Pathogens and Global Health, 108:235-245. DOI: https://www.doi.org/10.1179/2047773214Y.0000000148

Botrel, MA, Haenni M, Morignat E, Sulpice P, Madec JY, Calavas D (2010). Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone- Alpes, France. Foodborne Pathogens and Disease, 7:479-487. DOI: https://www.doi.org/10.1089/fpd.2009.0425

Brown DF, and Kothari D (1975). Comparison of antibiotic discs from different sources. Journal of Clinical Pathology, 28:779-783. DOI: https://www.doi.org/10.1136/jcp.28.10.779

Burvenich C , Merris VV, Mehrzad J, Diez-Fraile A, Duchateau L(2003).Severity of *E. coli* mastitis is mainly determined by cow factors. Veterinary Research, 34:521-564. DOI: https://www.doi.org/10.1051/vetres:2003023

Cepas V, and Soto SM (2020). Relationship between Virulence and Resistance among Gram-Negative Bacteria. Antibiotics (Basel, Switzerland), 9(10): 1-11. DOI: https://www.doi.org/10.3390/antibiotics9100719

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

Chang HH, Cohen T, Grad YH, Hanage WP, O'Brien TF, Lipsitch M (2015). Origin and proliferation of multiple-drug resistance in bacterial pathogens. Microbiol Mol Biol Reviews, 79(1):101-116. DOI: https://www.doi.org/10.1128/MMBR/00039-14

Collignon P, Powers JH, Chiller TM, Aidara-Kane A, Aarestrup FM (2009).World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 49:132-141. DOI: https://www.doi.org/10.1086/599374

Defays D (1977). An efficient algorithm for a complete link method, *The Computer Journal*, 20: 364–366. DOI https://doi.org/10.1093/comjnl/20.4.364

Djahnit N, Chernai S, Catania V, Hamdi B, China B, Cappello S, Quatrini P (2018). Isolation, characterization and determination of biotechnological potential of oil-degrading bacteria from Algerian centre coast. J Appl Microbiol,126(3):780-795. DOI : https://doi.org/10.1111/jam.14185

Société Française de Microbiologie (SFM) Comité de l'antibiogramme (2019). European Committee on Antimicrobial Suscpetibility Testing, recommandations 2019, Paris, 2019. Available at: CASFM2019_V1.0.pdf (sfm-microbiologie.org)

Drieux L, Brossier F, Sougakoff W, and Jarlier V (2008).Phenotypic detection of extended-spectrum beta-lactamase production in Enterobacteriaceae: Review and bench guide. Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 1:90-103. DOI: https://www.doi.org/10.1111/j.1469-0691.2007.01846.x

Fazel F, Jamshidi A, and Khoramian B (2019). Phenotypic and genotypic study on antimicrobial resistance patterns of *E. coli* isolates from bovine mastitis. Microbial Pathogenesis, 132:355-361. DOI : https://www.doi.org/10.1016/j.micpath.2019.05.018

Gouvêa R, Dos Santos FF, and De Aquino MHC (2015). Fluoroquinolones in industrial poultry production, bacterial resistance and food residues: A review. Brazilian Journal of Poultry Science, 17:1-10. DOI: https://www.doi.org/10.1590/1516-635x17011-10

Green MR and Sambrook J (2019). Agarose Gel Electrophoresis. Cold Spring Harb Protoc, (1). DOI: https://www.doi.org/10.1101/pdb.prot100404

Guess MJ, Wilson SB(2002). Introduction to hierarchical clustering. J Clin Neurophysiol, (2):144-51. DOI: https://www.doi.org/10.1097/00004691-200203000-00005

Haftu R, Taddele H, Gugsa G, and Kalayou S (2012). Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia. Tropical Animal Health and Production, 44:1765-1771. DOI: https://www.doi.org/10.1007/s11250-012-0135-z

Halasa T, Nielen M, De Roos AP, Van Hoorne R, de Jong G, Lam TJ, van Werven T, Hogeveen H(2009). Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. J Dairy Sci, 92(2):599-606. DOI: https://www.doi.org/10.3168/jds.2008-1564

Ismail ZB, and Abutarbush SM (2020). Molecular characterization of antimicrobial resistance and virulence genes of *Escherichia coli* isolates from bovine mastitis. Veterinary World, 13:1588-1593. DOI: https://www.doi.org/10.14202/vetworld.2020.1588-1593

Huet, T., Turbelin, C., Esquevin, S., Grisoni, M. L. (2021). BiostaTGV software. Available at: https://biostatgv.sentiweb.fr/#main%20view%20online%2027/06/2021

Kawamura K, Nagano N, Suzuki M, Wachino J, Kimura K, Arakawa Y(2017). ESBL-producing *Escherichia coli* and Its Rapid Rise among Healthy People. Food Safety (Tokyo, Japan), 5:122-150. DOI: https://www.doi.org/10.14252/foodsafetyfscj.2017011

Krumperman PH (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Applied and Environmental Microbiology, 46:165-170. DOI: https://www.doi.org/10.1128/AEM.46.1.165-170.1983

Kohn MA, Senyak J. Sample Size Calculators [website]. UCSF CTSI. 29 April 2021. Available at https://www.sample-size.net/

Leach KA, Green MJ, Breen JE, Huxley JN, Macaulay R, Newton HT, Bradley AJ (2008). Use of domestic detergents in the California mastitis test for high somatic cell counts in milk. The Veterinary Record, 163:566-570. DOI: https://www.doi.org/10.1136/vr.163.19.566

Liu G, Ali T, Gao J, Ur Rahman S, Yu D, Barkema HW, Huo W, Xu S, Shi Y, Kastelic JP, Han B(2020). Co-occurrence of plasmid-mediated colistin resistance (mcr-1) and extended-spectrum β-lactamase encoding genes in *Escherichia coli* from bovine mastitic milk in China. Microbial Drug Resistance, 26:685-696. DOI : https://www.doi.org/10.1089/mdr.2019.0333

Ministère de l'agriculture et du développement rural (MADR) (2003).Rapport National sur les Ressources Génétiques Animale : Algérie. Commission nationale AnGR, 45 pages. Available at: http://www.fao.org/3/a1250e/annexes/CountryReports/Algeria.pdf

Magnusson M, Christiansson A, and Svensson B (2007).*Bacillus cereus* spores during housing of dairy cows: Factors affecting contamination of raw milk. Journal Of Dairy Science, 90:2745-2754. DOI: https://www.doi.org/10.3168/jds.2006-754

Manyi-Loh C, Mamphweli S, Meyer E, and Okoh A (2018). Antibiotic use in agriculture and Its consequential Resistance in environmental sources: potential public health implications. Microbial Drug Resistance (Larchmont, N.Y.), 23:1-48. DOI : https://www.doi.org/10.3390/molecules230407951

Ministère de la santé, de la population et de la réforme hospitalièr (MSPRH) (2014). Algerian network for monitoring bacterial resistance to antibiotics, standardization of antibiotic sensitivity tests at the national level (human and veterinary medicine)178 pages. Available at: www.sante.gov.dz/aarn/index

Naas T, Oxacelay C, and Nordmann P (2007). Identification of CTX-M-type extended-spectrum-beta-lactamase genes using real-time PCR and pyrosequencing. Antimicrobial Agents and Chemotherapy, 51:223-230. DOI: https://www.doi.org/10.1128/AAC.00611-06

Nüesch-Inderbinen M, Käppeli N, Morach M, et al (2019).Molecular types, virulence profiles and antimicrobial resistance of *Escherichia coli* causing bovine mastitis. Veterinary Record Open, 6:000369. DOI: https://www.doi.org/10.1136/vetreco-2019-000369

Official Journal of the European Union (OJEU) (2003). European Union. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Offical Journal of the European Union, 268, 29-43. Available at: https://eur-lex.europa.eu/eli/reg/2003/1831/oj

Okoko IM, Maina N, Kiboi D, and Kagira J (2020).β-lactam resistance in bacteria associated with subclinical mastitis in goats in Thika Subcounty, Kenya. Veterinary World, 13:1448-1456. DOI: https://www.doi.org/10.14202/vetworld.2020.1448-1456

Olivares-Pérez J, Kholif AE, Rojas-Hernández S, et al (2015). Prevalence of bovine subclinical mastitis, its etiology and diagnosis of antibiotic resistance of dairy farms in four municipalities of a tropical region of Mexico. Tropical Animal Health and Production, 47:1497-1504. DOI: https://www.doi.org/10.1007/s11250-015-0890-8

Ombarak RA, Hinenoya A, Elbagory ARM, and Yamasaki S (2018). Prevalence and molecular characterization of antimicrobial resistance in *Escherichia coli*isolated from raw milk and raw milk cheese in Egypt. Journal of Food Protection, 81:226-232. DOI: https://www.doi.org/10.4315/0362-028X.JFP-17-277

Österberg J, Wingstrand A, Nygaard Jensen A, Kerouanton A, Cibin V, Barco L, Denis M, Aabo S, Bengtsson B (2016).Antibiotic Resistance in *Escherichia coli* from Pigs in Organic and Conventional Farming in Four European Countries. PLoS One, 11:0157049. DOI: https://www.doi.org/10.1371/journal.pone.0157049.

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

Peton V, and Le Loir Y (2014). Staphylococcus aureus in veterinary medicine. Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 21:602-615. DOI : https://www.doi.org/10.1016/j.meegid.201

Pilet C, Bourdon JI (1979). Bactériologie médicale et vétérinaire, systématique bactérienne. 2éme édition. Doin .paris. ISBN: 2-7040-0362-9.

Pitout JDD, and Laupland KB (2008). Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infectious Disease, 8:159-166. DOI: https://www.doi.org/10.1016/S1473-3099(08)70041-0.

Rangel P, and Marin JM (2009). Analysis of *Escherichia coli* isolated from bovine mastitic milk. Pesquisa Veterinária Brasileira, 29:363-368. DOI : http://www.dx.doi.org/10.1590/S0100-736X2009000500001

Saberfar E, Pourakbari B, Chabokdavan K, and Dolatshahi FT (2008).Antimicrobial susceptibility of *Escherichia coli* isolated from Iranian broiler chicken flocks, 2005-2006. Journal of Applied Poultry Research, 17:302-304. DOI: https://www.doi.org/10.3382/japr.2007-00102

Saidani M, Messadi L, Soudani A, et al (2018).Epidemiology, Antimicrobial Resistance, and Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae in Clinical Bovine Mastitis in Tunisia. Microbial Drug Resistance (Larchmont, N.Y.), 24:1242-1248. DOI: https://www.doi.org/10.1089/mdr.2018.0049

Saini,V., McClure,J.T, Le´gerD., Keefe, G.P, Scholl, D.T, Morck,D.W, Barkema,H.W (2012). Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. Journal Of Dairy Science, 95:4319-4332. DOI: https://www.doi.org/10.3168/jds.2012-5373

Sharif A, and Muhammad G (2009). Mastitis control in dairy animals. Pakistan Veterinary Journal, 29:145-148. Available at: https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.602.1277&rep=rep1&type=pdf

Schoonjans, F. (2017). Madcalc manual, Easy-to-use statistical software. 17th edition. Medcalc software, Ostend, Belgium. ASIN: B01MZ75LX4. Available at: www.medcalc.org

Tabar MM, Mirkalantari S, and Amoli RI (2016). Detection of ctx-M gene in ESBL-producing *E. coli* strains isolated from urinary tract infection in Semnan, Iran. Electron Physician, 8:2686-2690. DOI: https://www.doi.org/10.19082/2686

Tahar S, Nabil MM, Safia T, Ngaiganam EP, Omar A, Hafidha C, Hanane Z, Rolain JM, Diene SM (2020). Molecular Characterization of Multidrug-Resistant *Escherichia coli* Isolated from Milk of Dairy Cows with Clinical Mastitis in Algeria. Journal of Food Protection, 83:2173-2178. DOI: https://www.doi.org/10.4315/JFP-20-198

ThrusfieldM, and Christley R (2018). Veterinary epidemiology, 4th Edition, Wiley-blackwell editions. Hoboken, NJ, USA, pp. 888.

Available at: https://www.wiley.com/en-us/Veterinary+Epidemiology%2C+4th+Edition-p-9781118280287

Verbeke J, Piepers S, Supré K, and De Vliegher S (2014). Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. Journal Of Dairy Science 97:6926-6934. DOI: https://www.doi.org/10.3168/jds.2014-8173

Wessa P (2021). Free Statistics Software, Office for Research Development and Education, version 1.2.1. Available at: https://www.wessa.net/

Yu ZN, Wang J, Ho H, Wang YT, Huang SN, Han RW (2020). Prevalence and antimicrobial-resistance phenotypes and genotypes of *Escherichia coli* isolated from raw milk samples from mastitis cases in four regions of China. Journal of Global Antimicrobial Resistance, 22:94-101. DOI: https://www.doi.org/10.1016/j.jgar.2019.12.016

To cite this paper: Ghallache L, Mohamed-Cherif A, China B, Mebkhout F, Boilattabi N, Bouchemal A, Rebia A, Ayachi A, Khelef D, Miroud K, and Ait-Oudhia Kh (2021). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019. *World Vet. J.*, 11 (3): 402-415. DOI: https://dx.doi.org/10.54203/scil.2021.wvj52

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 416-421, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*)

Muhammad Hanif[1], Nunuk Dyah Retno Lastuti[1]*, and Rochmah Kurnijasanti[2]

[1]*Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia*
[2]*Department of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia*

*Corresponding Author: Nunuk Dyah Retno Lastuti, Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Campus C UNAIR Jl. Dr. Ir. H Soekarno, Surabaya, East Java, Indonesia 60115

Corresponding author's E-mail: nunukdyah53@gmail.com; ORCID: 0000-0003-0176-1322

ORIGINAL ARTICLE
pii: S232245682100053-11
Received: 20 July 2021
Accepted: 03 September 2021

ABSTRACT

Culex quinquefasciatus mosquitoes are a vector of transmission of several types of diseases, such as filariasis, Japanese encephalitis, and dirofilariasis. Larval control is the key strategy of disease control programs caused by vectors around the world because it can prevent larvae to enter the adult stage. Control of mosquito larvae that are often used is chemically controlled. *Citrus hystrix* is a natural plant and contains chemical compounds that have biological activity, such as flavonoids, carotenoids, and limonoids. The present study aimed to determine the effect of citrus leaf extract (*Citrus hystrix*) on the mortality of the larva *Culex quinquefasciatus*. The method used in the current study was a laboratory experimental study method with the experimental design using a completely randomized design. The research plot carried out was the rearing larvae of *Culex quinquefasciatus*, the manufacture of citrus leaf extract, the manufacture of larvicidal material, and the observation phase after treatment. Extract N-hexane *omplet name hystrix* leaf has high toxicity because it contains essential oils, flavonoids, alkaloids, terpenoids, saponins, and Limonoids. The Optimal concentration of N-hexane extracts of citrus leaves that lead to larval mortality of *Culex quinquefasciatus* in vitro amounted to 93.33% on 4 hours of observation with a concentration of 4000 ppm. Extract N-hexane lime leaf has a larvicidal effect on the larva *Culex quinquefasciatus* in vitro.

Keywords: *Citrus hystrix*, *Culex quiquefasciatus*, Extract, Mosquitoes, Larvicide

INTRODUCTION

Indonesia is a tropical country that has optimal humidity and temperature so as to support insect survival. Mosquitoes are the type of insects that coexist with humans but can harm humans because of their role as destructive organisms and disease vectors (vector-borne diseases; Sumantri and Satar, 2015).

Culex (*Cx.*) *quinquefasciatus* mosquitoes are the vector of transmission of several types of diseases such as filariasis, Japanese encephalitis, and dirofilariasis (Hadi and Koesharto, 2006). Filariasis is an infectious disease caused by microfilarial worms. This disease can cause disability, psychosocial, and decreased patients' productivity. The number of filariasis cases in Indonesia increases annually. Japanese Encephalitis (JE) is a viral disease that causes encephalitis and is a problem of public health in Asia including in Indonesia. Mosquito control can be done at the larvae stage, but in the adult stage that mosquitoes can fly they can transmit the disease widely (Aradilla, 2009). The control of larvae is the key strategy of vector-borne disease control programs caused by vectors around the world because it can prevent larvae to become adults. The larvae stadium has the longest lifespan of about 10-15 days so that the exposure is also longer, adult stage or adult mosquitoes can also fly so that it can spread the disease widely. The use of insecticide as larvacide can be the most commonly used way for people to control the growth of the vector (Manimegalai and Sukanya, 2014). Control of mosquito larva that has been often used is chemical control, namely by using synthetic chemicals. It can suppress vector populations rapidly. However, control in this way when done repeatedly is less effective because it can cause resistance to larvae, death for animal predatory predators and environmental pollution (Yasmin and Fitri, 2013). Biolarvasida of a plant (vegetable) is considered quite potential replacing synthetic insecticide. Plant insecticides are generally more selective than chemical insecticides because they are directly targeted at the target insect species, but also do not pollute the environment because it is easily degraded by nature and its use is quite safe (Aktar et al., 2009). More than 2400 plant types that belong to 255 families are reported to contain pesticide ingredients. Basil, germanium,

To cite this paper: Hanif M, Lastuti NDR, and Kurnijasanti R (2021). Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*). *World Vet. J.,* 11 (3): 416-421. DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

and zodia belong to a group of mosquito repellent plants due to their distinctive aroma. The plant contains compounds, such as methyl eugenol and some microelements, such as Linalool, Terpincol, Eugenol, Sincol, and Geraniol. In addition, there are other types of mosquito repellent including lavender, zodia, rosemary, fragrant lemongrass, and citrus kaffir lime (Kardinan, 2005). *C. hystrix* is a plant that is found easily by people. Plants derived from this genus of *Citrus* have chemical compounds that have biological activities, such as flavonoids, carotenoids, and Limonoids (Nurhaifah and Sukesi, 2015). Limonoid compounds are the teranoriterpen found in the leaves of Citrus lime which is potentially antifeedant to insects, growth regulator, and toxic substances in rice lice, larvacide, antimicrobial, insect repellent, and reproductive inhibitors (Li, 2001). The limonoid compound is an analog of juvenile hormones on insects that serve as a regulator of the growth of larval cuticles (Tunaz and Uygun, 2004). Saponin is also able to become biolarvasida. Saponin compounds in extracts that are eaten by the larvae of *Cx. quinquefasciatus* may irritate the digestive tract of *Cx. Quinquefasciatus*, and damage the cell membrane of the larva. Limonoid, which is an essential oil in orange, can lead to the loss of coordination of the nerve organ of the larva *Cx. Quinquefasciatus* (Pelah et al., 2002). In addition, limonoid has the ability to inhibit the formation of cancer cells, reducing the formation of low-density lipoproteins (LDL) as well as being able to suppress the appetite from insects (Murthy et al., 2021). Therefore, this research is done to find out the benefits of citrus leaves that are thought to have effects on larvae of *Cx. quinquefasciatus*.

MATERIALS AND METHODS

The research was conducted in the entomology laboratory of The Institute of Tropical Disease (ITD), Airlangga University, and the Department of Medicine of Veterinary Faculty of Universitas Airlangga, Surabaya, Indonesia. The material used consists of 420 larvae instar III *Cx. quinquefasciatus* obtained from the laboratory of ITD. Each treatment group used a sample of 20 larvae.

The current study utilized experimental research methods of laboratories with the experimental design using a completely randomized design using simple random sampling. The study was conducted in several phases, namely the rearing phase of the larva of *Cx. quinquefasciatus*, the manufacturing stage of citrus leaf extract of *C. hystrix*, the manufacturing stage of larvacidal material, and the observation phase after treatment. In rearing the larvae of *Cx. quinquefasciatus*, selection was based on the body length (5-6 millimeters, determined by a compound microscope with calibrated eyepiece graticule) and the day after the eggs hatch (four to six days). There was a triangular thickening consisting of many comb-shaped scales, had complete its morphological structure, and clearly, the body could be divided into a part of the head, chest, and abdomen. Prior to the study, the larvae were restored in advance in order to prove that the administration proved optimal. Subsequently, 420 larvae of instar III of *Cx. quinquefasciatus* were divided into seven treatment groups, namely the control group (+) consisting of Temephos 1 ppm + aquadest AD 100 ml solution, the control group (-) consisting of a solution of aquadest 100 ml, treatment 1 entailing 25 mg of citrus leaf extract of kaffir lime + aquadest AD 100 ml, treatment 2 consisting of 50 mg of kaffir lime citrus leaf extract + aquadest AD 100 ml, treatment 3 having 100 mg Citrus leaf extract of kaffir lime + aquadest AD 100 ml, treatment 4 including 200 mg citrus leaf extracts of kaffir lime + aquadest AD 100 ml, treatment 5 consisting of 400 mg of citrus leaf extracts of kaffir lime + aquadest AD 100 ml. The larvacide extract of the kaffir lime citrus leaves was then poured into plastic cups. Then, into the plastic cups that have been contained treatment solution inserted larvae of 20 tails of each glass. Observations were performed every 4 hours for 24 hours to see the dead larvae.

The physical factors measured in the room used during the study included the pH of water, water temperature, and room temperature. Measured water pH ranges from 7.0 to 7.2. The condition is a neutral pH and qualifies for the life of larvae in water. The measured room temperature is 25˚C, which is the temperature suitable for larvae growth.

RESULTS

Result of soaking larvae Instar III of *Cx. quinquefasciatus* in various concentrations of treatment for 24 hours obtained the average value and standard deviation of the number of Instar larva III *Cx. quinquefasciatus* every 4 hours observation that can be seen in Table 1.

Based on Table 1 above, it can be noted that there is a significant difference in each treatment of the percentage of mortality of Instar-III Larva ($p \leq 0.05$). However, the results of a comparison of immersion treatment in solution Temephos with extracts N-hexane citrus leaves of 4000 ppm concentrations do not show a noticeable difference. It is different from the immersion comparison of Temephos solution 1 ppm with the N-hexane extracts of citrus leaves with the concentrations of 2000, 1000, 500, and 250 ppm which show noticeable different results. Extract N-hexane citrus leaves of 4000 ppm concentrations at a 4-hour observation have shown an average death of as much as 19 tails so that it can be known that the value (LC_{90}) of extract n-hexane lime leaf is 4000 ppm. More details can be seen in Figure 1.

Based on Figure 1 of the comparative mortality ratio of Larva *Cx. quinquefasciatus* on the observation every 4 hours for 24 hours obtained the result that the relationship between the concentration of N-hexane extracts of citrus

To cite this paper: Hanif M, Lastuti NDR, and Kurnijasanti R (2021). Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*). *World Vet. J.*, 11 (3): 416-421. DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

leaves with larvae instar-larva III *Cx. quinquefasciatus* have a positive direction, meaning the higher concentrations of citrus leaf extract kaffir lime, the higher the power of the larva of mosquitoes *Cx. quinquefasciatus*. The increased immersion time at the concentrations of P1, P2, P3, and P4 showed an increase in the number of deaths each time of observation, it showed the time of immersion of N-Hexsan extract material affecting the death process of the mosquito larva *Cx. quinquefasciatus*. In negative control does not encounter the death of larvae it indicates there is no reaction of the use of aqueous solutions.

The results in the experiment indicated a significant difference in each treatment. This means that the death of the larvae of instar III Cx. quinquefasciatus was due to the immersion of the larvae in the kaffir lime citrus leaf extract. Insignificant differences in the positive control and the highest concentration of citrus leaf extract kaffir lime resulted from the equation of the effectiveness of citrus leaf extract in the killing of larvae.

Table 1. The results of soaking larvae every four hours

Mortality (%) Error! Bookmark not defined. **Groups**	**Average ± standard deviation**					
	4 hours	**8 hours**	**12 hours**	**16 hours**	**20 hours**	**24 hours**
K (+)	88.33 ± 2.88	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
K (-)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
P1	11 ± 5.77	21.66 ± 5.77	25 ± 5	30 ± 5	38.33 ± 7.63	41.66 ± 7.63
P2	15 ± 5	28.33 ± 7.63	36.66 ± 5.77	38.33 ± 7.63	48.33 ± 10.40	51.66 ± 7.63
P3	30 ± 13.22	45 ± 13.22	56.66 ± 14.43	66.66 ± 10.40	76.66 ± 5.77	78.33 ± 7.63
P4	55 ± 5	63.33 ± 7.63	68.33 ± 10.40	73.33 ± 7.63	80 ± 10	91.66 ± 10.4
P5	93.33 ± 2.88	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

K+: Temephos, K-: Aquadest, P1: 250 ppm, P2: 500 ppm, P3: 1000 ppm, P4: 2000 PPM, and P5: 4000 ppm

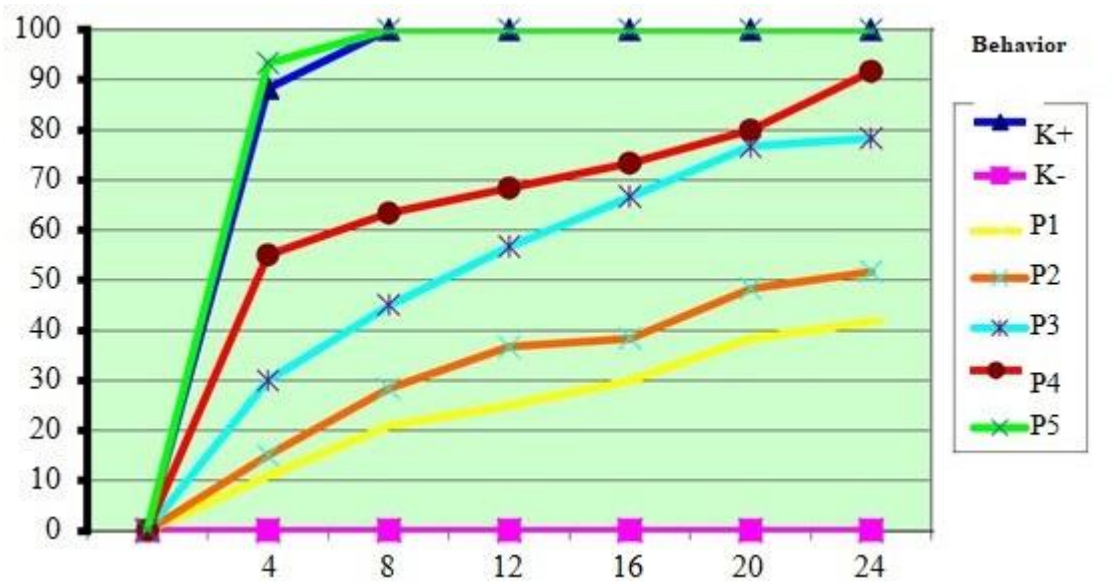


Figure 1. Comparative mortality of Larva *Cx. quinquefasciatus* on observation every 4 hours in a day, K+: Temephos, K-: Aquadest, P1: 250 ppm, P2: 500 ppm, P3: 1000 ppm, P4: 2000 PPM, and P5: 4000 ppm

DISCUSSION

The results of soaking larvae on the negative control (k-) of the larvae mortality rates were 0% for every four hours (Table 1). This means that the larvae were still alive and the addition of aquadest in control treatment did not show any effect on the mortality of larvae. In positive control (k+), the death of Larva instar III *Cx. quinquefasciatus* resulted from the administration of Temephos 1 ppm solution. Temephos is an organophosphate larvicide used to treat water infested with disease-carrying insects. This larvide has a residual power of approximately one month in the water shelter. As with other organophosphates, temephos affects the central nervous system through inhibition of cholinesterase. In larvae, this results in death before reaching the adult stage. The function of the enzyme cholinesterase is to hydrolyze acetylcholine into choline and sour vinegar (acetic acid) so that if the enzyme is inhibited then hydrolyze acetylcholine does not occur so that the muscles will remain contracted for a long time and will occur continuously, and the insect will eventually die (Nugroho, 2011). So as with other organophosphate compounds, Temephos acts as an anticholinesterase (Nugroho,

To cite this paper: Hanif M, Lastuti NDR, and Kurnijasanti R (2021). Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*). *World Vet. J.*, 11 (3): 416-421. DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

2011). Chemical compounds in plants that are potentially biolarvicides, are flavonoids, saponins, tannins, alkaloids, and essential oils (Kristanti et al., 2008). The *C. hystrix* leaf extract has high toxicity of lime leaves (*Citrus aurantifolia*), lime leaves *(c. amblycarapa*), and Balinese citrus leaves (*c. maxima*; Adrianto et al., 2014). The kaffir lime leaves contain essential oils, flavonoids, alkaloids, terpenoids, saponins, and limonoids (Adrianto et al., 2014).

Limonoid compounds are an important natural active component consisting of oxidized triterpenoid components (Khalil et al., 2003). Limonoid causes a bitter taste in orange and has the most potent larvacide effects. The larva cannot be transformed into a pupa because the title of the juvenile hormone in the larvae is not reduced but increases. To be a pupa, larva needed lower amounts of Juvenile hormones. Although the larvae make the skin change to instar IV, the larva will not be able to metamorphosis into the pupa and eventually die (Prijadi et al., 2014). Limonoida can enter into the body of the mosquito larva (*Aedes aegypti)* as a stomach poison. Limonoida goes into digestion through the concentration of the eaten extract concentrations. Insecticides will enter into the digestive organs of insects and are absorbed by the intestinal wall then circulate with blood that will interfere with the metabolism of mosquitoes so that it will lack energy for his life activities that will result in mosquitoes it is seizure and eventually dies (Baskar et al., 2020).

Another substance contained in the leaves of lime that can interfere with insects in digesting food is saponin. Saponin can lower the surface tension of the mucous membrane of the digestive tract larvae. The interaction of the saponin molecules with a layer of larval cuticle will result in damage to the cuticle (Chapagain and Wiesman, 2005). Saponins can also lower the enzyme activity of proteases in the gastrointestinal tract as well as interfere with food absorption (Afidah et al., 2014).

This alkaloid content acts as a stomach poison and contact poison. The alkaloid is a grain of salt so that it can degrade the cell membrane of the digestive tract to enter into and damage cells and can also interfere with the nervous system work of larvae by inhibiting the enzyme work of acetylcholinesterases. Where this enzyme cannot execute its duties in the body mainly forward the delivery of orders to the digestive tract larvae (midgut) so movement cannot be controlled. The occurrence of discoloration in the body of larvae becomes more transparent and the movement of larvae slows down when the touch is stimulated and always bends the body also caused by alkaloid compounds (Ahdiyah and Purwani, 2015).

Tannins are polyphenol compounds that can form complex compounds with proteins. Tannins cannot be digested in the stomach and have a connective power with proteins, carbohydrates, vitamins, and minerals. Tannins can interfere with insects in digesting food because tannins will bind to proteins in the digestive system that required insects for growth so that the digestive process is estimated that become disturbed to the larva *Cx. Quinqufasciatus* (Yunita et al., 2009).

The death of Larva instar III *Cx. quinquefasciatus* not only was resulted from the compounds of limonoid but this can also be caused by various ingredients contained in the extract N-hexane lime leaves such as materials that have been mentioned above which can affect the death of larva *Cx. Quinqufasciatus*. Therefore, The higher the concentration, the higher the content of the active ingredient contained in the extract of N-hexane lime leaves, which can cause a decrease in digestive activity, causing seizures and disrupt the nervous system and suppress the rate of growth of larvae (Minarni et al., 2013). The content of essential oils that are the secondary metabolite of extracts that are dissolved with aquadest will cause an oil layer to be formed due to differences in polarity. This coating will cover the water surface and siphon. The presence of oil will prevent larvae from taking oxygen, even the coating of oil will stick to the siphon will prevent the diffusion of oxygen towards the tissues, so that oxygen transport is interrupted. The nature of this essential oil can be respiratory poison (Hedayat, 2008).

After soaking the extract of N-hexane citrus leaf, the following kaffir lime signs were observed. In the first 15 minutes, the larvae *Cx. quinquefasciatus* showed a rapidly rising movement, convulsive movements, such as forming the U, weakened body movements, at 2 hours of observation time at concentrations of 4000 ppm and 2000 ppm has begun to show the situation where the larvae are unable to move, stiff, and subsequently die. The results of soaking larvae every 4 hours were observed. Larvacida contact poison can kill enough larva when exposed to external body parts. Then larvae show the sign of ascending movement rapidly (telescopic movement).

The results of the research on the concentration of the extract of N-hexane lime leaves at all treatments except the negative control (k-) were the death of larvae. The mortality rate of larvae at a concentration of 4000 ppm was 93.33%. This concentration is the best concentration of N-hexane extracts of citrus leaves as larvacide. The difference between the numbers of larvae deaths is caused by the concentration of each treatment. This indicates that the higher the concentration and duration of observations of N-hexane citrus leaves of the purut, the higher the active content and the larvasidal power to kill the larva of quinquefasciutus mosquitoes.

When compared, concentration 4000 ppm extract N-hexane citrus leaves are equally effective with a solution Temephos 1 ppm in killing the larva instar III *Cx. quinquefasciatus* is the same as indicating the death of 100%. But for the efficiency of the solution temephos is higher in killing the larvae *Cx. Quinqufasciatus* then extract N-hexane citrus leaves of kaffir lime with a concentration of 4000 ppm because it only takes 1 ppm (mg/l) of Temephos solution, compared to 4000 ppm (mg/l) extract N-hexane lime leaves to kill 100% larvae for 8 hours in a 24-hour time.

To cite this paper: Hanif M, Lastuti NDR, and Kurnijasanti R (2021). Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*). *World Vet. J.*, 11 (3): 416-421. DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

CONCLUSIONS

The findings of this study have confirmed that extracts of N-hexane lime leaves contain active substances that can be larvicidal. Therefore, the results larvacides are considered good when the test material shows the value of mortality between 90-100% of test larvae. In conclusion extract of N-hexane kaffir lime orange leaves have a larvicidal effect on the larva *Cx. quinquefasciatus* in vitro. The optimal concentration of N-hexane extracts of citrus leaves that cause the larval mortality of *Cx. quinquefasciatus* in vitro amounted to 93.33% on 4 hours of observation with a concentration of 4000 ppm.

DECLARATIONS

Competing interests

Authors declare no competing interests.

Authors' contribution

The authors have participated in this study equally.

Ethical consideration

Ethical issues (Including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Adrianto H, Yotopranoto S and Hamidah (2014). Efektivitas ekstrak daun jeruk purut (Citrus hystrix), jeruk limau (Citrus amblycarpa), dan jeruk Bali (Citrus maxima) terhadap larva Aedes aegypti. Aspirator, 6(1): 1-6. DOI: https://www.doi.org/10.22435/ASPIRATOR.V6I1.3516.1-6

Afidah R, Yuliani and Haryono T (2014). Pengaruh kombinasi filtrat umbi gadung, daun sirsak, dan herba anting-anting terhadap mortalitas larva ordo lepidoptera. LenteraBio, 3(1): 45-49. available at: http://ejournal.unesa.ac.id/index.php/lenterabio

Ahdiyah I and Purwani KI (2015). Pengaruh ekstrak daun mangkokan (Nothopanax scutellarium) sebagai larvasida nyamuk Culex sp. Jurnal sains dan Seni ITS, 4(2): 32-36. DOI: https://www.doi.org/10.12962/j23373520.v4i2.10804

Aktar MW, Sengupta D, and Chowdhury A (2009). Impact of pesticides use in agriculture: their benefits and hazards. Interdiscip Toxicol, 2(1): 1-12. DOI: https://www.doi.org/10.2478/v10102-009-0001-7

Aradilla AS (2009). uji efektivitas larvasida ekstrak ethanol daun mimba (Azadirachta indica) tehadap larva Aedes aegypti. Medical faculty, pp. 1-47. DOI: https://www.doi.org/10.1002/bjs.1800560511

Baskar K, Chinnasamy R, Pandy K, Venkatesan M, Sebastian PJ, Subban M, Thomas A, Kweka EJ, and Devarajan N (2020). Larvicidal and histopathology effect of endophytic fungal extracts of Aspergillus tamarii against Aedes aegypti and Culex quinquefasciatus. Heliyon, 6(10): 1-11. DOI: https://www.doi.org/10.1016/j.heliyon.2020.e05331

Chapagain B and Wiesman Z (2005). Larvicidal effects of aqueous extracts of Balanites aegyptiaca (desert date) against the larvae of Culex pipiens mosquitoes. African journal of Biotechnology, 4(11): 1351-1354. Available at: https://www.ajol.info/index.php/ajb/article/view/71379/60329

Hadi UK and Koesharto FX (2006). Nyamuk dalam buku hama permukiman Indonesia, pengenalan, biologi dan pengendalian, UKPHP FKH IPB, Bogor.

Hedayat KM (2008). Essential oil diffusion for the treatment of persistent oxygen dependence in a three-year-old child with restrictive lung disease with respiratory syncytial virus pneumonia. Explore, 4(4): 264-276. DOI: https://www.doi.org/10.1016/j.explore.2008.04.005

Kardinan A (2005). Tanaman pengusir dan pembasmi nyamuk. Jakarta: Agro Media Pustaka, pp. 2–5. Available at: https://agromedia.net/katalog/tanaman-pengusir-pembasmi-nyamuk

Khalil AT, Maatooq GT and El Sayed KA (2003). Limonoids from Citrus reticulata. Zeitschrift für Naturforschung C. Verlag der Zeitschrift für Naturforschung, 58(3–4): 165–170. Available at: https://pubmed.ncbi.nlm.nih.gov/12710721/

Kristanti et al (2008) Buku Ajar Fitokimia, Surabaya: Jurusan Kimia Laboratorium Kimia Organik FMIPA Universitas Airlangga, pp. 49–149. Available at: https://onesearch.id/Record/IOS1.INLIS000000000294108

Li J (2001). Brief Introduction to Citrus Limonoids, TAMU-College Station, TAMUK-Citrus Center. Available at: https://aggie-horticulture.tamu.edu/syllabi/422/ppt/LI.ppt

To cite this paper: Hanif M, Lastuti NDR, and Kurnijasanti R (2021). Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*). *World Vet. J.*, 11 (3): 416-421. DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

Manimegalai K and Sukanya S (2014). Biology of the filarial vector, Culex quinquefasciatus (Diptera:Culicidae). International Journal of Current Microbiology and Applied Sciences. 3(4): 718-724. Available at: https://www.ijcmas.com/vol-3-4/K.Manimegalai%20and%20S.Sukanya.pdf

Minarni E, Armansyah T and Hanafiah M (2013). Daya larvasida ekstrak etil asetat daun kemuning (Murraya paniculata (L) jack) terhadap larva nyamuk Aedes aegypti. Jurnal Medika Veterinaria, 7(1): 27-29. Available at: http://jurnal.unsyiah.ac.id/JMV/article/view/2915

Murthy KNC, Jayaprakasha GK, Safe S, and Patil BS (2021). Citrus limonoids induce apoptosis and inhibit the proliferation of pancreatic cancer cells. Food and Function, 12(3): 1111-1120. DOI: https://www.doi.org/10.1039/d0fo02740e

Nugroho AD (2011). Kematian Larva Aedes aegypti Setelah Pemberian Abate Di bandingkan Dengan Pemberian Serbuk Serai. Jurnal Kesehatan Masyarakat, 7(1): 91-96. Available at: https://journal.unnes.ac.id/nju/index.php/kemas/article/view/2802/2858

Nurhaifah D and Sukesi TW (2015). Efektivitas Air Perasan Kulit Jeruk Manis sebagai Larvasida Nyamuk Aedes aegypti. Kesmas: National Public Health Journal, 9(3): 156-207. DOI: https://www.doi.org/10.21109/kesmas.v9i3.566

Pelah D, Abramovich Z, Markus A, and Wiesman Z (2002). The use of commercial saponin from Quillaja saponaria bark as a natural larvicidal agent against Aedes aegypti and Culex pipiens. Journal of Ethnopharmacology. 81(3): 407-9. DOI: https://www.doi.org/10.1016/s0378-8741(02)00138-1

Prijadi DK, Wahongan GJP, and Bernadus JBB (2014). Effectiveness Test Citrus Leaf Extract (Citrus aurantifolia) on Inhibits the Growth of Aedes spp Larvae. Jurnal e-Biomedik, 2(1): 1-7. Available at: https://media.neliti.com/media/publications/68248-ID-uji-efektifitas-ekstrak-daun-jeruk-nipis.pdf

Sumantri A and Satar YP (2015). Uji Efikasi Ekstrak Biji Srikaya (Annona squamosa L) Sebagai Bioininteksida Dalam Upaya Integrated Vector Management Terhadap Aedes aegypti. pp. 1-123. Available at: https://docplayer.info/58394667-Uji-efikasi-ekstrak-biji-srikaya-annona-squamosal-l-sebagai-bioinsektisida-dalam-upaya-integrated-vector-management-terhadap-aedes-aegypti.html

Tunaz H, and Uygun N (2004). Insect growth regulators for insect. Turkish Journal of Agriculture and Forestry, 28(6): 377-387. Available at: https://journals.tubitak.gov.tr/agriculture/issues/tar-04-28-6/tar-28-6-1-0309-5.pdf

Yasmin Y and Fitri L (2013). Perubahan morfologi larva nyamuk akibat pemberian larvasida bakteri kitinolitik. Jurnal Entomologi Indonesia, 10(1): 18-23. DOI: https://www.doi.org/10.5994/jei.10.1.18

Yunita EA, Suparpti NH and Hidayat JW (2009). Pengaruh ekstrak daun teklan (Eupatorium riparium) terhadap mortalitas dan perkembangan larva Aedes aegypti. Department of Biology, MIPA UNDIP, 11(1): 11–17. Available at: https://core.ac.uk/download/pdf/11703242.pdf

To cite this paper: Hanif M, Lastuti NDR, and Kurnijasanti R (2021). Effect of Larvicidal Extract N-Hexane Lime Leaves (*Citrus hystrix*) on Larva Instar III Mosquito (*Culex quinquefasciatus*). *World Vet. J.*, 11 (3): 416-421. DOI: https://dx.doi.org/10.54203/scil.2021.wvj53

2021, Scienceline Publication
World's Veterinary Journal
World Vet J,11(3): 422-430, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance

Haiam Abd Elsalam Sayed*, Mahmoud MetwalyEl-Maghraby, and Mohamed Mahmoud Elbadawy

Animal Production Research Institute, Ministry of Agriculture, Dokki, Giza-12618, Egypt

*Corresponding author's Email: haes1979@yahoo.com; ORCID: 0000-0002-4746-5134

ORIGINAL ARTICLE
pii: S232245682100054-11
Received: 23 June 2021
Accepted: 12 August 2021

ABSTRACT

The aim of the present study was to evaluate the effect of replacing concentrate feed (CFM) mixture with different levels of treated lemon pulp (TLP) on milk production and composition, nutrients digestibility, and blood parameters of Zaribi goats. A total of 24 Zaribi female goats at the end of the pregnant period were divided into three groups of eight according to live weight. Concentrate feed mixture and clover hay at a rate of 50:50 were offered twice a day. Treatedlemon pulp replaced CFM at rates 0%, 25%, and 50 % in diets of R1, R2, and R3 treatment groups, respectively. The feeding trial lasted 90 days. Dry matter digestibility and Nitrogen free extract digestibility were significantly raised by the increased level of TLP in groups R2 (72.37% and 70.36%) and R3 (72.28% and 70.30%), compared to (70.99% and 68.51%) in R1, respectively. The same trend was observed for organic matter digestibility (OMD), crude protein digestibility (CPD), crude fiber digestibility (CFD), and ether extract digestibility (EED) with R3, compared with either R1 or R2. However, there were insignificant differences between R1 and R2 in terms of OMD, CPD, and EED but CFD was significantly higher in R2, compared to R1. There was a significant increase in the milk yield by the increased level of TLP in the diet and for R2 (1448.4g/h/d) and R3(1558.7g/h/d), while it was reported as 1377.6 g/h/d in the control group. Feeding dams on the R3 diet had a significant effect on improving total antioxidant capacity by 63.5%, compared with control. The results of the present study indicated that the replacement of CFM by TLP decreased the costs of feed and increase milk production for the replacement level by 50% (R3). Moreover, no adverse effects were noticed on nutrient digestibility and blood parameters of the investigated samples.

Keywords: Blood parameters, Lemon pulp, Milk production, *Saccharomyces cerevisiae*

INTRODUCTION

Traditional feedstuffs are very expensive so the use of agro-industrial by-products in animals' diets has been introduced as the best way to decrease the cost of the feed. In Egypt, there is a shortage of animal feeds which had a negative effect on increasing animal production. Ruminant animals can depend on by-product feedstuffs (BPF) in their diets because the rumen microorganisms can use BPF which contains high levels of crude fiber and low protein content to cover their requirements for growth, reproduction, and production.

Adding citrus by-products to ruminants' diet is suitable because the rumen microorganism has the ability to ferment high fiber feeds (Grasser et al.,1995). The citrus residues of the juice industry (oranges, tangerines, lemons, or grapefruits) are called the citrus pulp. The citrus pulp can be used in animal feeding either fresh or after ensilage or dehydration. The nutritional value of citrus pulp is high owing to its high content of readily fermentable carbohydrates and contains a variety of energy substrates for ruminal microbes (Habeeb et al., 2017). The high content of easily fermentable carbohydrates and different energy sources found in the citrus pulp which are available for ruminal microorganisms can increase its nutritional value (Habeeb et al., 2017).

This important bioprocess is characterized by microbial growth in the insoluble substrate, in the presence of small amounts of free fluid, using the nutrients and residual elements present in these residues (Pinto et al., 2005). The microbial growth in this insoluble substrate, which contains small amounts of free fluid, depends on residual elements (Pinto et al., 2005). Micro-fungi solid substrate fermentation of this pulp could increase the protein content, antioxidant activity, and digestibility of the pulp. In fact, the citrus pulp is an energy source for rumen micro-organisms.

Protein enrichment of these by-products by microorganisms improves the nutritional value of the forage palm for ruminants (Vendruscolo et al., 2009). Residues of fruits and vegetables have been good substrate for protein enrichment and bioconversion into value adds products as enzymes and other metabolites. Using *Saccharomyces cerevisiae* (*S. Cerevisiae*) and fungi to bioconvert these agro-industrial by-products in raising their protein content. Fermented pineapple wastes by *S. cerevisiae* for 48 hours caused an increase in protein from 6.4% to 16.1% (Roberta et al., 2007).

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

Improving protein content by processing wet citrus pulp increases their efficiency in livestock nutrition. Some studies indicated that processing citrus pulp with microorganisms increases the crude protein contents as a result of converting lignocelluloses and easily-digestible materials into energy, protein, and CO2 by their extracellular enzymes (Scerra et al., 1999). Using pectin, digestible fiber, and soluble carbohydrates, which are found in citrus pulp residues, as a source of energy are easily and extensively degraded instead of starch led to producing acetic acid, which does not cause a pH drop like lactic acid and result in acidosis. Long rumination period and high fiber content cause large quantities of saliva buffering rumen ph. Lemon pulp is considered as a safer feed than cereals for animals fed low-roughage and high-concentrate diets in high-yielding dairy cows (Crawshaw, 2004). So, the use of agro-industrial by-products in ruminant diets might be an alternative to replace food energy and protein concentrates, increasing the efficiency and reducing the economic costs of the production system and consequently, making it more competitive. Also, the citrus family (Citrus lemon) contains an antioxidant component. The antioxidant activity of phenolic compounds such as flavonoids and phenols in lemons can improve in reducing the risk of cardiovascular disease (Ghasemi et al., 2009).

The objective of this study was to evaluate the impact of partial replacing of CFM by lemon pulp fermented with *S. Cerevisiae* on chemical composition, digestion coefficients, blood parameters, and milk production performance of the Zaribi goats.

MATERIALS AND METHODS

This experiment was carried out at Sakha, Animal Production Research Station of Animal Production Research Institute, Agriculture Research Center during the summer season of 2020.

Ethical approval

This study was approved by the Scientific Committee of Animal Production Research Institute (protocol no: 1-2-2- 924).

Preparing lemon pulp

Lemon pulps were collected from the Elmarowa factory in Sixth of October City, Egypt, and sun-dried for 7 days, then, the dried lemon pulp was mixed with water at 1:2 ratio (pulp: water). Then, temperature, humidity, and acidity of lemon pulp were adjusted to be suitable for yeast growth, whereas the optimal humidity and pH for yeast growth are 85% and 5.6, respectively, according to Dadvar et al. (2015), so, bicarbonate was added by 6.4% to the media to adjust the pH. Samples of prepared lemon pulp were put in polyethylene bags and inoculated with4% yeast and incubated at 35°C for 24 hours. After the incubation period, the samples were sun-dried for 4 days, then, kept for chemical analysis. Feed ingredients, *S. cerevisiae* powder, Concentrate Feed Mixture (CFM), Clover Hay (CH), and feces were analyzed for proximate analysis according to AOAC (1995) whereas Nitrogen Free Extract (NFE) was calculated by difference.

Table 1. Chemical composition of untreated lemon pulp and treated lemon pulp on dry matter basis

Items (%)	DM	OM	CF	CP	EE	NFE	ASH
ULP	90.98	91.22	13.59	8.90	5.03	63.70	8.78
TLP	89.80	87.68	10.86	13.92	3.46	59.44	12.32
Relative change	-1.3	- 3.9	-20.1	+56.4	- 31.2	- 6.69	+40.3

DM: Dry matter, OM: Organic matter, CF: Crud fiber, CP: Crud protein, EE: Ether extract, NFE: Nitrogen free extract, ASH: Ash, ULP: Untreated lemon pulp, TLP: Treated lemon pulp

Experimental diets and animals

A total of 24 Zaribi female goats at the end of the pregnant period were divided into three groups of eight according to live weight for 90 days trial. Goats in each group were fed CFM consisting of 35% decorticated cottonseed cake, 25% corn grain, 30% wheat bran, 5% molasses, 2% limestone, 1.5% salt, and mineral, and 1.5%vitamins mixture, clover hay, CH, and treated lemon pulp (TLP). Feed was offered twice a day at 8 am and 4 pm and the remaining amounts from the previous day were measured. Water is offered freely all day round. The CFM is adjusted biweekly according to the body weight changes and milk production. Daily feed intakes, milk production, were recorded and feed efficiency (g feed/g milk) was calculated. Kids weighed biweekly before morning feeding. According to live body weight and milk production goats were fed the experimental diets as 50 % CFM and 50% clover hay.

The efficiency of dams was measured by litter weight at birth, which was calculated by total kids birth weight per dam. Litter weight at weaning was measured by total kids weight at weaning per dam, and total litter weight gain was estimated by total kids birth weight minus total kids weight at weaning per dam. Finally, the average daily gain was

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

calculated by the differences between total kids birthweight per dam and total kids weight at weaning divided on suckling period. On the other hand, the first group (control, R1) received experimental diets while, (R2) and (R3) experimental groups received the experimental diets by replacing 25% and 50% of CFM with treated lemon pulp, respectively. The chemical composition of raw materials and different experimental diets are presented in Table 2.

Table 2. Chemical composition of concentrate feed mixture, clover hay, treated lemon pulp, and experimental diets on a dry matter basis.

Parameters (%)	DM	OM	CF	CP	EE	NFE	ASH
Tested materials							
CH	89.93	87.59	25.22	12.80	2.57	47.00	12.41
CFM	91.53	89.97	11.62	14.02	2.96	61.37	10.03
TLP	90.98	87.68	13.59	13.92	3.46	56.71	12.32
Experimental diets							
R1(control)	90.73	88.78	18.42	13.41	2.77	54.19	11.22
R2	90.66	88.50	18.67	13.40	2.83	53.6	11.50
R3	90.60	88.21	18.91	13.39	2.89	53.02	11.79

DM: Dry matter, OM: Organic matter, CF: Crud fiber, CP: Crud protein, EE: Ether extract, NFE: Nitrogen free extract, ASH: Ash, TLP: Treraterd lemon pulp, CFM: Concentrate feed mixture, CH: Clover Hay, R1: Control diet (50%concentrate feed mixture+ 50% clover hay), R2:37.5% concentrate feed mixture+ 50% clover hay+12.5 %treated lemon pulp, R3:25% concentrate feed mixture +50% clover hay+25 % treated lemon pulp.

Digestibility trials

Three animals from each group were used in digestibility trials using the acid insoluble ash (AIA) technique as an internal marker according to Van-Keulen and Young (1977) to determine the nutrients digestibility and feeding values of the experimental diets. Fecal samples of nearly 100 g were taken from the rectum twice daily at 8 a.m and 6 p.m for 3 days collection period. Representative samples of feed and feces from the whole collection period were prepared for proximate analysis according to AOAC (1995).

Blood and milk sample analyses

The blood samples were taken at the end of the termination of the experiment from the jugular vein in dry clean glasses tubes. Blood samples were collected into sterile blood tubes and immediately placed on ice before centrifuging to obtain serum. The serum was immediately frozen for subsequent biochemical analysis. Blood parameters, including total protein, urea-N, triglycerides, cholesterol, high-density lipoprotein, and low-density lipoprotein, were measured using enzymatic procedures and commercial kits. Daily milk yield was recorded and milk samples were collected at first, middle, and end of the experiment for analysis of milk composition using Milko Scan (model 130 series, type 10900 FOSS electric, Denmark). Body weight changes were individually determined biweekly before morning feeding. The growth of kids was also measured biweekly.

Statistical analysis

Analysis of variance (one-way, ANOVA) was performed to compare between different groups. Statistical analysis was carried out using SAS (2001) and Duncan's multiple range test (Duncan, 1955) was used to separate the means when the main effect was statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The obtained results of Table 1 indicated that treating lemon pulp with yeast caused a decrease in dry matter, organic matter, ether extract, and nitrogen-free extract by 1.3%, 3.9%, 31.2%, and 6.69%, respectively. These decreases might be due to the internal metabolism and respiration of yeast, some amounts of carbon in lemon pulp are lost as carbon dioxide. Moreover, crude fiber content was a decrease by 20.1% after treatment and this may be due to the possible secretion of some extracellular enzymes, such as amylase and cellulose into the substrates which break the starch and other polysaccharides into simpler sugars easily metabolized by yeast as a source of carbon (Dadvaret al., 2015). However, there was an increase in crude protein (CP) and ash content with that treatment by 56.4% and 40.3%, respectively. These results were in agreement with the findings of Ezekiel et al. (2010) that there was an increase in ash content when cassava peels were fermented with *Trichoderma viride*. There was an increase in CP content since sugar could be an appropriate substrate for single-cell protein leading to the considerable final protein content of bioprocesses product *S. cerevisiae.* These results were in agreement with the findings of Roberta et al. (2007) who treated pineapple waste with *S. cerevisiae* leading to a significant increase in crude protein content of pineapple waste after processing. This increase can be attributed to high residual sugar as an appropriate substrate for single-cell protein leading to the considerable final

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

protein content of bioprocesses product *S. cerevisiae*, which did not produce fungal toxins excreted by some filamentous fungi, such as *aspergillus*. Generally, yeasts have been shown to convert starchy materials, such as sweet potatoes and cassava peels, to protein. The increase in the ash content may not be a product of fermentation (Oboh, 2006).

Data in Table 2 represents the chemical composition of feedstuff and the similarity in chemical composition among the experimental diets used in the current experiment. The chemical composition of clover hay and concentrate feed mixture is in agreement with the findings of Sayed and Ghobashy (2018).

Nutrients digestibility and feeding values

Data in Table 3 showed that there was a significant increase in dry matter digestibility and NFE digestibility ($p < 0.05$) when TLP feed in R2 or R3 was reported as (72.37 and 70.36%) and (72.28 and 70.30%), respectively, compared to (70.99 and 68.51%) for R1. The same trend was observed for organic matter digestibility (OMD), crude protein digestibility (CPD), crude fiber digestibility (CFD), and ether extract digestibility (EED) with R3, compared to either R1 or R2. However, there were insignificant differences between R1 and R2 in OMD, CPD, and EED ($p > 0.05$) but CFD was significantly higher in R2 ($p < 0.05$), compared with R1.

These results might be due to the highly digestible NFE and high content of nonstructural carbohydrates of TLP diets, compared with control (Habeeb et al., 2017), or might be improving the rumen digestion environment with the TLP as mentioned by McDonald et al. (2011). This is due to yeast and its enzymes improving the rumen condition for fermentation. Miron et al. (2002) indicated that replacing 11% dry citrus pulp with corn seeds resulted in better conditions for rumen microorganisms and improved the digestion of crude protein. Moreover, treated lemon pulp with *S. cerevisiae* increased the digestibility of crude protein leading to an increased crude protein in the rumen for micro-organism utilization. Additionally, yeast caused an increase in fibrolytic bacteriain rumen (Habeeb et al., 2017). This positive effect for TLP might be due to replacing starchy concentrates with feeds rich in easily degradable cell walls had generally been associated with a more favorable rumen condition for cellulolytic bacteria (Arthington et al., 2002).

The results of nutrient digestibility were reflected on the nutritive value of the experimental diets where R3 recorded the highest significant total digestible nutrient (TDN) followed by R2 then R1 as 62.15%, 60.98%, and 59.35%, respectively ($p < 0.05$). However, there was an insignificant difference among groups regarding digestible crude protein (DCP) value ($p > 0.05$). These results agreed with the finding of Kim et al. (2007) that citrus pulp supplementation might improve TDN and DCP.

Table 3. Digestion coefficients and nutritive values of the experimental Zaribi goats' diet

Parameters	Treatments			
	R1	R2	R3	SEM
Digestibility coefficients (%)				
DM	70.99[b]	72.37[a]	72.28[a]	0.35
OM	69.19[b]	69.25[b]	70.76[a]	0.42
CP	69.44[b]	69.77[b]	73.90[a]	0.76
CF	58.57[c]	62.76[b]	67.13[a]	0.37
EE	76.60[b]	77.60[b]	79.13[a]	0.44
NFE	68.51[b]	70.36[a]	70.30[a]	0.37
Feeding values on DM basis (%)				
TDN	59.35[c]	60.98[b]	62.15[a]	0.44
DCP	9.31	9.35	9.90	0.055

[a,b,c] Means different superscripts in the same row are significantly different ($p < 0.05$). DM: Dry matter, OM: Organic matter, CF: Crud fiber, CP: Crud protein, EE: Ether extract, NFE: Nitrogen free extract, ASH: Ash, TDN: Total digestible nutrient, DCP: Digestible crude protein, R1: Control diet (50% concentrate feed mixture + 50% clover hay), R2: 37.5% concentrate feed mixture + 50% clover hay + 12.5 %treated lemon pulp, R3: 25% concentrate feed mixture + 50% clover hay + 25% treated lemon pulp, SEM: Standard error mean.

Milk yield and composition

According to Table 4, milk yield significantly increased ($p < 0.05$) by increasing the level of TLP in the diet as 1448.4 and 1558.7 g/h/d for R2 and R3, compared to 1377.6 g/h/d for R1. This increase might be due to the high TDN content in these diets, compared with the control diets (Schingoethe et al., 2004). Citrus pulp (dehydrated or ensiled) in diets for lactating ewes give conflicting results whereas some authors found no effect (Volanis et al., 2004), others reported a positive effect on daily milk yield (Piquer et al., 2011). Milk composition of T3 showed a significant increase in fat yield by 6.40%, compared with control, this agrees with the finding of (Volanis et al., 2004) who found a significant increase in milk fat percentage in ewes fed ensiled citrus pulp attributed their results to the easily digestible fiber in citrus pulp silage, which modified condition in the rumen and improved microbial activity, which leads to fiber degradation and subsequent higher production of acetic acid (Piquer et al., 2009), and promotes milk fat synthesis in the mammary glands.

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

Opposite results were seen by Piquer et al. (2011) when studied the effect of adding the citrus pulp to the diet of dairy ewes on the composition of milk. The results obtained by these authors showed that the use of 30% citrus pulp in the diet led to a reduction in milk fat content, while protein content was increased for ewes with 10% of citrus in the diets. The protein content of T3 had the highest value compared with control, this may result from supplementation of ruminant diets with citrus pulp may improve N utilization (Kim et al., 2007; Piquer et al., 2009), reducing the ruminal NH3-N escape, and consequently, the milk urea level, which several studies support this hypothesis in dairy ewes (Scatassa et al., 2006). Highly degradable carbohydrates led to the higher digestibility of the LP diets (Piquer et al., 2009), increased level of nitrogen absorption, and therefore, increased level of nitrogen excreted in milk. All treatments showed a significant increase in lactose and solid not fat, compared with control by increasing the level of TLP into the diets.

As can be seen in Table 5, there was a significant decrease between treatments in glucose concentration in the blood of goats fed LP this disagrees with the findings of Ali et al. (2016). Furthermore, blood serum cholesterol and TG levels decreased ($p < 0.05$) in R2 and R3, compared with control, which disagrees with the findings of a study by Alnaimyet al. (2017) reporting that blood cholesterol concentration increased in cows fed with a diet containing dried citrus pulp. This decrease in cholesterol content might result from the prevention of cholesterol formation by Trippine in lemon pulp (Takarada et al., 2002).

Table 4. Effect of different experimental of Zaribi goats' diet on milk yield and its composition

Parameters	Treatments			
	R1	R2	R3	SEM
Daily milk yield (g/h/d)	1377.6[c]	1448.4[b]	1558.7[a]	
Daily 4%-FCM (g/h/d)	1243.3[c]	1313.7[b]	1359.9[a]	2.48
Milk composition				
Fat (%)	3.35[a]	3.38[a]	3.15[b]	0.14
Fat yield (g)	46.14[b]	48.95[a]	49.09[a]	0.75
Protein (%)	2.45	2.59	2.77	0.37
Protein yield (g)	33.75[c]	37.5[b]	43.17[a]	1.08
Lactose (%)	3.94	3.45	3.44	0.54
Lactose yield (g)	54.27[c]	49.96[b]	53.61[a]	1.44
Total solids (%)	10.5[a]	10.31[b]	10.29[b]	0.78
Total solids yield (g)	144.78 [c]	149.33[b]	160.39 [a]	1.84
Solids not fat (%)	7.16	6.93	7.14	0.74
Solids not fat yield (g)	98.63 [b]	100.37[b]	111.29 [a]	1.71
Ash (%)	0.76	0.88	0.93	0.14

[a,b,c] Means different superscripts in the same row are significantly different ($p < 0.05$). R1: Control diet (50% concentrate feed mixture + 50% clover hay), R2: 37.5% concentrate feed mixture + 50% clover hay + 12.5% treated lemon pulp, R3: 25% concentrate feed mixture + 50% Clover hay + 25% treated lemon pulp, SEM: Standard error mean, Fat corrected milk (4%) for goats calculated according to gains equation: 4% FCM = 0.4 milk yield + 15 fat yield

Table 5. Biochemical parameters in blood serum of Zaribi goats as affected by feeding experimental diet

Parameters	Treatments			
	R1	R2	R3	SEM
Tp (g/dl)	6.45[c]	7.33[b]	7.84[a]	0.92
Alb (g/dl)	3.94	3.97	4.17	2.38
Glo (g/dl)	2.51	3.36	3.67	3.77
Ur (mg/dl)	6.24	6.44	6.78	2.55
Cr mg/dl	1.14	1.12	1.18	0.17
Glu (mg/dl)	94.47[a]	80.15[b]	72.55[c]	0.58
ALT (U/L)	15.74[a]	14.33[b]	14.77[b]	0.13
AST (U/L)	26.79[a]	24.16[b]	22.48[b]	0.91
Cho (mg/dl)	141.19[a]	124.22[b]	108.45[c]	2.96
HDL	77.72[a]	71.67[b]	65.43[c]	0.57
LDL	44.13[a]	35.03[b]	26.55[c]	0.27
TAC	0.52[b]	0.58[ab]	0.85[a]	0.24
TG (mg/dl)	96.71[a]	87.56[b]	82.35[c]	1.68

[a,b,c] Means different superscripts in the same row are significantly different ($p < 0.05$), TP: Total protein; Alb: Albumin, Glo: Globulin; Glu: Glucose, Cr: Creatinine, Ur: Urea-N Cho: Cholesterol, TG: Triglycerides, ALT: Alanine transaminase, AST: Aspartic transaminase, TAC: Total antioxidant capacity, SEM: Standard error mean, R1: Control diet (50% concentrate feed mixture + 50% clover hay), R2: 37.5% concentrate feed mixture + 50% clover hay + 12.5% treated lemon pulp, R3: 25% concentrate feed mixture + 50% Clover hay + 25% treated lemon pulp

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

Data showed that there were no significant ($p < 0.05$) differences among groups in blood concentrations of albumin, globulin, urea, and creatinine. However, feed TLP in R2 and R3 significantly decreased blood concentration of glucose, ALT, AST, HDL and LDL ($p < 0.05$), compared with control. Feeding dams on diets contained TLP improved total antioxidant capacity insignificantly for R2 ($p > 0.05$) and significantly for R3 ($p < 0.05$) by 11.5% and 63.5%, respectively, compared with control. These results agreed with the findings of Ali et al. (2016), while lemon increased the concentrations of high-density lipoprotein (HDL) increased, but the concentration of cholesterol and triglycerides declined. The increase in total antioxidant capacity with R2 and R3 might be due to the fact that lemon had a strong antioxidant activity of its essential oil on the removal of free radicals; this may be related to phenolic and flavonoids compounds (Ghasemi et al., 2009).

According to Table 5, blood total protein significantly increased linearly with the increasing TLP ($p < 0.05$). These results are in agreement with those of Oni et al. (2008) that total plasma protein increased linearly with the increasing levels of citrus pulp. Table 6 shows the development of the body weight of kids and goats throughout the experimental period. Diets (R3) had a higher birth weight of kids, followed by those born from goats fed R2 diets, and less birth weight was obtained for goats in the control group (R1). This indicated that the diets improve the weight of the kids because the goat fed these diets at the last period of pregnancy.

Goats showed an increase in body weight after the parturition period in R3 followed by R2 indicating that an increase in digestion coefficient of TLP caused an increase in body weight for those groups. At the end of the experiment, kids in the group fed R3 showed the highest body weight and average daily gain, compared to the control (R1). This may be the result of an increase in milk yield so the kids had the best values of 280.3 and 226.3g/day for R3 and R2, respectively. Bueno et al. (2002) reported that different levels of orange pulp on Saanen kids' diets led to an increase in ADG, and kids fed a diet containing 42.3% orange pulp had the highest value. This finding is in agreement with that of Castollo-Gonzalez (2014) who concluded that the ruminal microflora improved production. The reason is that ruminal microorganisms are very important for ruminant animals, so, it is necessary to increase information about microbial ecosystems to improve the rumination process. Therefore, using additives in ruminant diet improves the efficient nutrients digestibility and reduces the production cost.

Data in Table 8 indicates the prices of experimental materials and average daily feed intake. The results revealed that daily milk yield increased by 1558.7g/ dam/day for R3 which might result from replacing 50% pf CFM with TLP. This led to an increase in nutrient digestion, TDN, and modification of rumen fermentation which causes high improvement in milk yield. When comparing the total feed cost (LE/dam/day), it was found that R3 had the lowest price being 5.20 while the highest value was found in R1 being 5.63 LE. However, there was a decrease in feed cost/kg milk by 18.38% and 7.10% for R3 and R2, compared with control, respectively. This represented that treatment LP with yeast could decrease the cost of production.

According to Table 7, feeding TLP in R2 and R3 respectively improved feed efficiency of DMI/FCM being 1.06 and 1.02 as well as DCPI/ FCM being 0.099 and 0.1 compared to 1.10 and 0.103 for R1.

Table 6. Effect of feeding different experimental diets on dams and their offspring performance

Parameters	Experimental diets			SEM
	R1	R2	R3	
Dams performance				
No. of dam kidded	8	8	8	-
Initial weight at Late-pregnancy (kg)	34.22	33.88	34.15	3.75
Body weight at parturition (kg)	28.34	31.17	32.38	2.41
Body weight at 1st month before parturition (kg)	22.49^{c}	24.88^{b}	26.33^{a}	0.17
Body weight two months before parturition (kg)	26.12	28.13	31.04	3.44
Body weight three months before parturition (kg)	29.71	31.85	32.79	2.18
Offspring performance				
Total number of kids	14	15	17	-
Litter size/ dam at birth	1.75	1.87	2.12	-
Birth weight (kg)	2.63	2.48	2.35	0.47
weaning weight (kg)	13.44	13.37	14.25	2.48
Total weight gain (kg)	10.81	10.89	11.90	-
Average daily gain (g/ day)	120.11^{b}	121.00^{b}	132.22^{a}	2.86
Relative improve (%)	100	100.7	110.1	-
Dam production				
Litter weight at birth (kg)	4.60	4.63	4.98	2.46
Litter weight at weaning (kg)	23.52^{b}	25.00^{b}	30.21^{a}	1.67
Total litter weight gain (kg)	18.92	20.37	25.23	-
Average daily gain (g/day)	210.2	226.3	280.3	-

^{a,b} Means different superscripts in the same row are significantly different ($p < 0.05$). SEM: Standard error mean, NO: Number, R1: Control diet (50%concentrate feed mixture + 50% clover hay), R2: 37.5% concentrate feed mixture + 50% clover hay + 12.5 %treated lemon pulp, R3: 25% concentrate feed mixture + 50% clover hay + 25 % treated lemon pulp

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

Table 7. Feed intake, milk yield, feed conversion, and feed efficiency of milk production of dams during the suckling period (90 days)

Parameters	Experimental diets			SEM
	R1	R2	R3	
Total DM intake (g/ dam/day)	1370	1390	1380	-
TDNI (g/dam/day)	813	848	858	-
DCPI (g / dam /day)	128	130	137	-
Daily 4%FCM (g/ dam/day)	1243.3[c]	1313.7[b]	1359.9[a]	2.48
Feed conversion ratio				
DM intake, g/g (4% FCM) milk	1.10	1.06	1.02	-
TDNI, g/g (4% FCM) milk	0.65	0.65	0.63	-
DCPI, g/g (4% FCM) milk	0.103	0.099	0.100	-

[a,b,c] Means different superscripts in the same row are significantly different ($p < 0.05$). SEM: Standard Erro Mean, FCM: Fat corrected Milk, TDN: Total digestible nutrient, DCP: Digestible crude protein, TDNI: Total digestible nutrient intake, DCPI: Digestible crud protein intake

Table 8. Feed intake and economical evaluation of dams during the suckling period of 90 days

Parameters	Experimental diets		
	R1	R2	R3
Average daily feed intake, g/dam/day (feed)			
CFM	712.5	550	347
Clover hay (CH)	818.75	830	848
Treated lemon pulp (TLP)	-	180	365
Daily milk yield (g/ dam/day)	1377.6 [c]	1448.4[b]	1558.7[a]
Economical evaluation			
Average total feed cost (LE/dam/day)			
CFM	3.34	2.58	1.63
Clover hay (BH)	2.29	2.32	2.37
Treated lemon pulp (TLP)	-	0.59	1.20
Total feed cost (LE/dam/day)	5.63	5.49	5.20
Price of average daily milk (LE/dam/day)	8.26	8.69	9.35
Feed cost (L.E) / milk (kg)	4.08	3.79	3.33

[a,b,c] Means different superscripts in the same row are significantly different ($p < 0.05$). Prices of concentrate feed mixture (CFM), Treated Lemon Pulp (TLP), and Clover Hay (CH) were reported as 4700 L.E./ton, 3300 L.E./ton, and 2800 L.E./ton, respectively. Based on the market price in 2020 and 6 LE/kg raw milk. R1: Control diet (50% concentrate feed mixture + 50% clover hay), R2: 37.5% concentrate feed mixture + 50% clover hay + 12.5 %ttreated lemon pulp, R3: 25% concentrate feed mixture + 50% clover hay + 25 % treated lemon pulp, Total daily feed cost (L.E.): Cost of CFM + TLP + clover hay, SEM: Standard error mean.

CONCLUSION

It could be concluded that replacement of concentrate feed mixture by lemon pulp treated by *Saccharomyces cerevisiae* in dairy goats diets can decrease the costs of feed and increase milk production by 50% without any adverse effect on milk yield, its composition, and animals health with high economic efficiency.

DECLARATION

Authors' contributions

Dr. Haiam A. Sayeddesigned the study, performed the chemical analysis and wrote the manuscript. Dr.Mouhmed M. Elmaghraby and Dr. Mohamed M. Elbadwy carried out the practical part of the experiment, collected samples from animals, and reviewed the manuscript. All authors read and approved the final manuscript

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

To Dr. Helmey M. Yacout (professor of animal nutrition) for helping us to finish this work.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Ali M, Reza S, and Azadeh B (2016). Evaluation of antioxidant activity of citrus lemon L. essential oil and its effect on some blood parameters in Moghani sheep. Nutrition Science, 14(12): 108-113. DOI: https://www.doi.org/10.7537/marsnsj141216.17

Alnaimy A, Gad AE, Mustafa MM, Atta MA, and Basuony HA (2017). Using of citrus by-products in farm animals feeding. Open Access Journal of Science, 1(3): 58-67. DOI: https://www.doi.org/10.15406/oajs.2017.01.00014

Arthington JD, Kunkle WE, and Martin AM (2002). Citrus pulp for cattle. VeterinnaryClinics of North America Food Animal Protection, 18(2): 317-326. DOI: https://www.doi.org/10.1016/s0749-0720(02)00023-3

Association of Official Analytical Chemists (AOAC) (1995). Official methods of analysis. 16th Ed. Washington DC, USA. Available at: https://www.worldcat.org/title/official-methods-of-analysis-of-aoac-international/oclc/421897987

Bueno MS, Ferrari JE, Bianchini D, Leinz FF, and Rodrigues CF (2002).Effect of replacing corn with dehydrated citrus pulp in diets of growing kids. Small Ruminant Research, 46: 179-185. DOI: https://www.doi.org/10.1016/S0921-4488(02)00184-0

Castollo-Gonzalez AR, Burrola-Barraza ME, Domingues-ViverosJ, and Chavez-Martineez A (2014). Rumen microorganism and fermentation.ArchvosMedicinaVeterinaria, 46: 349-361. DOI: http://www.dx.doi.org/10.4067/S0301-732X2014000300003

Crawshaw R (2004). Co-product feeds: Animal feeds from the food and drinks industries, Nothingham University Press. Available at: http://www.amazon.com/Co-Product-Feeds-Animal-Drinks-Industries/dp/1897676352

Dadvar P, Dayani O, Mehdipour M, and Morovat M (2015). Determination of physical characteristics, chemical composition and digestion coefficients of treated lemon pulp with *Saccharomyces cerevisiae* in goat diet. Journal of Animal Physiology and Animal Nutrition, 99:107-113. DOI: https://www.doi.org/10.1111/jpn.12204

Duncan DB (1955). Multiple ranges and multiple F test. Biometrics, 11:1-20. DOI: http://www.dx.doi.org/10.2307/3001478

Ezekiel OO, Aworh OC, Blaschek HP, and Ezeji TC (2010). Protein enrichment ofcassava peel by submerged fermentation with *Trichodermaviride* (ATCC 36316). African Journal of Biotechnology, 9(2): 187-194. Available at: https://www.ajol.info/index.php/ajb/article/view/77788

GhasemiK, Ghasemi Y, and Ebrahimzadeh MA (2009). Antioxidant activity, phenol and flavonoid contents of 13 Citrus species peels and tissues. Pakistan Journal of Pharmaceutical Science, 22(3): 277-281. Available at: https://pubmed.ncbi.nlm.nih.gov/19553174/

Grasser LA, Fadel JG, Garnett I, and De Peters EJ (1995). Quantity and economic importance of nine selected by-products used in California dairy rations. Journal of Dairy Science, 78: 962-971. Available at: https://www.doi.org/10.3168/jds.S0022-0302(95)76711-X

Habeeb AA, Gad AE, Mostafa MM, Atta AA, and Basuny AM (2017). Using of citrus by- products in farm animal feeding. Themed section: Science and Technolog, 3:2395-6011. Available at:https://www.academia.edu/34606849

Kim SC, Adesogan AT, and Arthington JD (2007). Optimizing nitrogen utilization in growing steers fed forage diets supplemented with dried citrus pulp. Journal of Animal Science, 85: 2548-2555. DOI: https://www.doi.org/10.2527/jas.2007-0059

McDonald P, Edwards RA, Greenhalgh JF, Morgan CA,Sinclair LA, and Wilkinson RG (2011). Animal nutrition. 7th ed. Pearson, Harlow, UK: Prentice Hall/Pearson, p. 692. Available at:https://www.bookdepository.com/Animal-Nutrition-Peter-McDonald/9781408204238

Miron J, Yosef E, Ben- GhedaliD, Chase L, Bauman D, and Solomon R (2002). Digestibility by dairy cows of monosaccharaides constituents in total mixed rations containing citrus pulp. Journal of Dairy Science, 85: 89-94. DOI: https://www.doi.org/10.3168/jds.S0022-0302(02)74056-3

Oboh G (2006). Nutrient enrichment of cassava peels using *Saccharomycescerevisae* and *Lactobacillus spp*. solid media fermentation techniques. Electronic Journal of Biotechnology, 9:46-49. DOI: https://www.doi.org/10.2225/vol9-issue1-fulltext-1

Oni AO, Onwuka CF, Oduguwa OO, Onifade OS, and Arigbede OM (2008). Utilization of citrus pulp-based diets and *Enterolobiumcyclocarpum*(JACQ. GRISEB) foliage by West African dwarf goats. Livestock Science, 30: 184-91. DOI: https://www.doi.org/10.1016/j.livsci.2007.12.010

Pinto GA, Brito ES, Andrade AM, Fraga SL, and Teixeira RB (2005). Fermentação em estado sólido: Uma alternativa para o aproveitamento e valorização de resíduos agroindustriais tropicais. Comunicadotécnico Online, 102(1): 1-5. Available at: https://www.infoteca.cnptia.embrapa.br/bitstream/doc/426390/1/Ct102.pdf

Piquer O,Ródenas L, Casado C, Blas E, and Pascual JJ (2009). Whole citrus fruits as an alternative to wheat grain or citrus pulp in sheep diet: Effect on the evolution of ruminal parameters. Small Ruminant Research, 83: 14-21. DOI: https://www.doi.org/10.1016/j.smallrumres.2008.11.009

PiquerO, Ródenas L, Blasé, and Pascual JJ (2011). Whole citrus fruits in total mixed ration for Mediterranean milking ewes. Milk Production and Composition, pp. 251-258. Available at:https://riunet.upv.es/bitstream/handle/10251/147521

Roberta C, Margarida M, and Gorete M (2007). Protein enrichment of pineapple waste with *saccharomyces cervisiae* by solid state bioprocessing. Journal of Scientific and Industrial Research, 66: 259-262. Available at: http://nopr.niscair.res.in/handle/123456789/1239

Sayed HA, and Ghobashy H (2018). Effect of processing orange pulp with *Saccharomyces cerevisiae* yeast on growth performance, nutrients digestibility, and blood parameters of Barki lambs. Journal of Sheep and Goat Science, 13:33-45. DOI: https://www.doi.org/10.21608/EJSGS.2018.26301

Scatassa ML, TodaroM, Bruno S, and Giaccone P (2006). Le polpe fresche di limone nella dieta delle pecore da latte: Effetti sulla qualità del latte di massa (Fresh lemoncitrus pulp in dairy ewes feeding: Effects on tank milk quality). In: Proc. LX Congresso SIS Vetriney, Terrasini (PA), Italypp, pp. 515-516. Available at: https://refhub.elsevier.com/S0377-8401(16)30677-0/sbref0195

Scerra V, CaridiA, Foti F, and Sinatra MC (1999). Influence of dairy *penicillium spp*. on nutrient content of citrus fruit peel. Animal Feed Science and Technology, 78: 169-176. DOI: https://www.doi.org/10.1016/S0377-8401(98)00264-8

Schingoethe DJ, Linke KN, Kalscheur KF Hippen AR, Rennich DR, and Yoon I (2004). Feed efficiency of mid-lactation dairy cows fed yeast culture during summer. Journal of Dairy Science, 87: 4178-4181. DOI: https://www.doi.org/10.3168/jds.S0022-0302(04)73561-4

Statistical Analysis System (SAS) (2001). SAS user's guide statistics SAS Institute Inc. Editors, Cary, NC. Available at: https://support.sas.com/documentation/onlinedoc/91pdf/sasdoc_91/stat_ug_7313.pdf

Takarada K, Kimizuka R, Takahashi N, Honma K, Okuda K, and Kato T (2002). A comparison of the antibacterial efficacies of essential oils against oral pathogens. Oral Microbiology Immunology, 19: 61-64. DOI: https://www.doi.org/10.1046/j.0902-0055.2003.00111.x

Van-Keulen J, and Young BA (1977).Evaluation of acid-insoluble ash as neutral marker in ruminant digestibility studies. Journal of Animal Science, 44: 282-287. DOI: https://www.doi.org/10.3168/jds.2012-6442

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

Vendruscolo F, Silva C, Esposito E, and Ninow JL (2009). Protein enrichment of apple pomace and use infeed for Nile Tilapia. Applied Biochemistry and Biotechnology, 152: 74-87. DOI: http://www.dx.doi.org/10.1007/s12010-008-8259-3

VolanisM, Zoiopoulos P, and Tzerakis K (2004). Effects of feeding ensiled sliced oranges to lactating dairy sheep. Small Ruminant Research, 53: 15-21. DOI: http://www.dx.doi.org/10.1016/j.smallrumres.2003.07.011

To cite this paper:Sayed HAE, El-Maghraby MM, and Elbadawy MM (2021). Effect of Processed Lemon Pulp with *Saccharomyces cerevisiae* in Zaribi Goats Diet on Milk Production, Nutrients Digestibility, Blood Parameters, and Reproductive Performance. *World Vet. J.*, 11 (3): 422-430. DOI: https://dx.doi.org/10.54203/scil.2021.wvj54

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 431-438, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish

Pramaziyah Fuaidah, Juni Triastuti*, and Heru Pramono

Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya-60115, Indonesia
*Corresponding author's Email: juni.triastuti@fpk.unair.ac.id ; ORCID: 0000-0003-4378-1946

SHORT COMMUNICATION
pii: S232245682100055-11
Received: 26 July 2021
Accepted: 06 September 2021

ABSTRACT

Scad fish (*Decapterus kurroides*) is the most productive fish species. To provide the added value, fishermen can process scad fish into various forms of processed products, such as preserved fish, which extend the shelf life. In order to extend the shelf life of canned fish, natural preservatives could be used that contain antimicrobial agents, such as bay leaves. The present study aimed to determine the effects of bay leaf extract in different concentrations on the shelf life of preserved scad fish. In the present study, the completely randomized design was used as an experimental research method. The treatments given differed in terms of the concentration of the bay leaf extract. The groups were treated as the scad fish without bay leaf extract (A), scad fish soaked in 6% of bay leaf extract (B), scad fish soaked in 7% of bay leaf extract (C), scad fish soaked in 8% of bay leaf extract (D), and scad fish soaked in 9% of bay leaf extract (E). The analyzed parameters included the total plate count, pH, and water level test using Analysis of Variance (ANOVA). The supportive parameters observed included an organoleptic test. The results indicated that the lowest total bacterial count from the beginning to the end of the experiment (18 hours) occurred on treatment C (7% of bay leaf extract addition), which was 1.54 × 103 to 5.85 × 106. Regarding the water level test from the beginning to the end of the experiment, treatment A (control) was not significantly different from other treatments. The difference in the concentration of bay leaf extract on scad fish effectively inhibited the growth of bacterial colonies. In conclusion, adding bay leaf extract to scad fish could inhibit bacteria for up to 12 hours. Treatment C (7% of bay leaf extract) gave the best results since this concentration level could inhibit the bacteria on scad fish.

Keywords: Bay leaf, Preservation, Scad fish, Shelf life

INTRODUCTION

Scad fish (*Decapterus kurroides*) is the most productive fish species, accounting for 52% of the total catch of approximately 2323365 tons per year. In addition, scad fish comes at a relatively low price. To achieve added value, fishermen process scad fish into various forms of processed products, such as *petis* (fish paste) and preserved fish. Preserved fish is one of the most famous marine products in Indonesia due to a combination of boiling and salting. The resulting product is a preserved fish with low salt content. The traditional preservation process of boiling the fish in the salt solution is called *pindang cue* (Triarso, 2012; Junianingsih, 2015).

Pindang cue uses scad fish, which can be found in traditional markets and are commonly placed in *paso* or *naya* (small woven tray) without any packaging. Thus, microbial contamination occurs during the sales process. The shelf life of scad fish is approximately 1-2 days, and safety is not guaranteed. Scad fish contain high water (60%) and protein (27%), which are suitable for the microorganisms' growth. The maximum limit for the microorganism in food products is 5.0 × 105 cells/gram (Ariyani, and Yennie 2008).

Preservation is an appropriate way to extend the shelf life of scad fish. Fish preservation is a process that is aimed at developing high-quality processed fish products (in terms of color, odor, texture), offering a wide range of food and varietal products on the market, increasing the economic value of the product, as well as enhancing the shelf life of fish. Since these preserved scad fish had a limited shelf life and are prone to deterioration, fish should be stored longer to provide benefits to traders while increasing consumers' appeal. Conservation also is used to inhibit the activities of enzymes and microorganisms (Herawati, 2008).

Preservation with hazardous chemicals, such as formalin, is mostly used because the price is lower than safe preservation. The use of formalin has been constantly increasing. Thus, natural preservations are required to inhibit the growth of spoilage bacteria (Hastuti, 2010; Nafisyah, 2015). Herbs and spices native to Indonesia contain active antimicrobial substances that potentially can be used as natural preservatives. Bay leaf is one of the natural ingredients

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.,* 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

that can be used as a preservative, and also has many benefits to be utilized in cooking as a seasoning because of its distinctive aroma and odor, as well as its low price and easy procurement (Purwani and Muwakhidah, 2008).

Bay leaf is an ingredient that can be used as a fish preservative since it contains flavonoid, terpenoid, and tannin, which have antibacterial properties. Flavonoids inhibit bacterial growth by forming complex compounds with extracellular proteins through hydrogen bonds, that damage the cell membrane. Tannin inhibits bacterial growth by denaturating bacterial cell proteins in the cell membrane. The terpenoid inhibition mechanism for bacterial growth is to react with porin (transmembrane protein) on the external membrane of the bacterial cell wall. Bay leaf indicated good antibacterial activity against *Staphylococcus aureus* (Roslizawaty, 2013; Nurwijayanti, 2016). Based on the fact that bay leaf indicated sufficient antibacterial activity against Staphylococcus aureus, the present study was conducted to investigate the effects of bay leaf extract with different concentrations on the shelf life of scad fish.

MATERIALS AND METHODS

The current research used an experimental research method and the experiment design of a completely randomized design. The following is the bay leaf treatments on scad fish, scad fish without bay leaf extract treatment (A), scad fish soaked in 6% of bay leaf extract (B), scad fish soaked in 7% of bay leaf extract (C), scad fish soaked in 8% of bay leaf extract (D), and scad fish soaked in 9% of bay leaf extract (E).

The variables included in the present study were independent, dependent, and control variables. The independent variable was the bay leaf extract. The dependent variables of the study were the Total Plate Count (TPC), organoleptic value, pH, and water level of scad fish. The control variables of the research were bay leaves, preserved scad fish, and bay leaves boiled at 90°C for 30 minutes.

The research procedure was divided into two stages of preparation and implementation. The preparatory stage consisted of fish processing, sterilization of tools and materials, bay leaf extraction, and preparation of Plate Count Agar (PCA) media. The implementation stage included the treatment of scad fish, marked with the letter A, B, C, D, and E. Then, the treatment was performed by mixing bay leaf extracts with the preserved scad fish. In treatment A, the scad fish were not soaked in the bay leaf extract. In treatments B, C, D, and E, the scad fish were soaked in bay leaf extract with concentrations of 6%, 7%, 8%, and 9%, respectively, for 30 minutes. Furthermore, the soaked scad fish were then placed into the oven for 30 minutes at a temperature of 50°C. Then, the fish were transferred to clean containers and stored at room temperature. Afterward, the total number of bacteria, the pH value, water content, and the organoleptic value were observed before storage and after 3, 6, 9, 12, 15, and 18 hours of storage.

The research parameters were the main parameters and supporting parameters. The main observed parameters entailed the TPC test, pH, and water level. The supporting parameters observed included the organoleptic tests. The research result data were analyzed in the terms of TPC, pH, and water level using Analysis of Variance (ANOVA) to determine the difference in the different concentrations of bay leaf extracts on the shelf life of scad fish. Duncan's Multiple Range Test was then performed to determine the differences between treatments. The data analysis for organoleptic tests used descriptive methods. The distinction between the two approaches to sensory evaluation of fish products was made to first emphasize that both of these approaches were necessary for the evaluation of this commodity. Secondly, these two aspects of sensory evaluation of fish products were not mutually exclusive but rather acted as a mutual information resource. Sensory assessment of fish products would continue to be essential in quality testing, even as with time, useful chemical tests are developed which are as efficient and cost-effective as sensory testing. In product grading, standard chemical and physical tests can only measure what is already known. The sensory assessor can measure both the known attributes of the product and assess the presence of new attributes, especially in the area of measurement of 'taint' in fish products. The factor of sensory testing is part of the definition of taint in a product ['fish that is rancid or has an abnormal odor or flavor (DFO, 1978). However, chemical testing can accurately assess the level of a particular compound in a sample, it cannot by itself, indicate what this number means relative to the final quality of the product and the action that needs to be taken as to whether the product will continue to be used as a food, or whether it has deteriorated to a point where it is no longer usable. The definition of the 'cut-off' point for taint will always be based on a human definition of this level for the particular culture in which the product is being used (York and Sereda, 2014).

RESULTS

Total bacterial count

The highest mean total bacterial count from the beginning of the experiment until the end of the experiment (18 hours) occurred in treatment A (control), which was 1.57 ×103 to 8.55 ×106. Meanwhile, the mean value of the lowest total bacterial count occurred from the beginning to the end in treatment C (7% of bay leaf extract), which was 1.54×103 to 5.85×106 (Table 1).

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

Water level test

The water level test utilized a moisture analyzer. The average value of the water content found in the redtail scad at the beginning of the experiment and the end of the experiment of each treatment decreased during storage at room temperature until the end of the study. The results of the ANOVA test showed that the mean value of the water level at the beginning and in hour 18 of the experiment in treatment A (control) did not significantly differ ($p > 0.05$) from the other treatments (Table 2).

PH level test

The results of the ANOVA test showed that the mean value of the water level at hour 12 in group D and hour 15 in group C, and E was significantly different ($p < 0.05$) from the other treatments (Table 3).

Table 1. Total bacterial count (CFU/gram) of Staphylococcus aureus and bacterias in Scad fish meat

Observation Time (hour)	Treatment (Average ± SD)				
	A (0%)	B (6%)	C (7%)	D (8%)	E (9%)
1	$1.57\times10^{3a} \pm 0.11$	$1.52\times10^{3a} \pm 0.15$	$1.54\times10^{3a} \pm 0.17$	$1.82\times10^{3a} \pm 0.12$	$1.43\times10^{3a} \pm 0.14$
3	$8.25\times10^{3a} \pm 0.04$	$6.85\times10^{3ab} \pm 0.05$	$5.1\times10^{3c} \pm 0.07$	$5.95\times10^{3bc} \pm 0.04$	$6.45\times10^{3b} \pm 0.07$
6	$2.58\times10^{4a} \pm 0.04$	$2.22\times10^{4ab} \pm 0.13$	$1.72\times10^{4b} \pm 0.07$	$2.22\times10^{4ab} \pm 0.12$	$2.11\times10^{4ab} \pm 0.11$
9	$6.60\times10^{4a} \pm 0.04$	$6.65\times10^{4a} \pm 0.03$	$5.11\times10^{4b} \pm 0.03$	$6.50\times10^{4a} \pm 0.05$	$5.94\times10^{4a} \pm 0.02$
12	$2.78\times10^{5a} \pm 0.02$	$2.23\times10^{5b} \pm 0.10$	$1.47\times10^{5c} \pm 0.02$	$1.87\times10^{5b} \pm 0.03$	$2.18\times10^{5b} \pm 0.08$
15	$2.35\times10^{6a} \pm 0.08$	$2.12\times10^{6a} \pm 0.07$	$1.45\times10^{6b} \pm 0.05$	$1.94\times10^{6a} \pm 0.06$	$2.08\times10^{6a} \pm 0.08$
18	$8.55\times10^{6a} \pm 0.04$	$7.17\times10^{6ab} \pm 0.04$	$5.85\times10^{6b} \pm 0.06$	$6.1\times10^{6b} \pm 0.08$	$6.57\times10^{6b} \pm 0.07$

The different superscript letters in the same column shows a significant difference ($p < 0.05$). CFU: Colony-forming unit, SD: Standard deviation.

Table 2. Water level test of scad fish meat

Treatment (%)	The water level at the beginning of the study (%)	The water level at the end of the study (%)
A (0%)	$45.97^{a} \pm 1.34$	$34.74^{a} \pm 6.87$
B (6%)	$48.6^{a} \pm 4.49$	$34.133^{a} \pm 8.88$
C (7%)	$47.41^{a} \pm 2.44$	$33.78^{a} \pm 11.02$
D (8%)	$43.08^{a} \pm 5.89$	$37.81^{a} \pm 9.25$
E (9%)	$45.94^{a} \pm 5.42$	$36.51^{a} \pm 7.62$

Remarks: The notation indicated by different superscript letters in the same column shows a significant difference ($p < 0.05$).

Table 3. PH Level of scad fish meat

Treatment / Observation time	A (0%)	B (6%)	C (7%)	D (8%)	E (9%)
1	$7.05^{a} \pm 0.40$	$6.99^{a} \pm 0.42$	$6.78^{a} \pm 0.24$	$6.78^{a} \pm 0.21$	$6.73^{a} \pm 0.09$
3	$6.89^{a} \pm 0.57$	$6.88^{a} \pm 0.40$	$6.73^{a} \pm 0.12$	$6.7^{a} \pm 0.23$	$6.6^{a} \pm 0.11$
6	$6.92^{a} \pm 0.48$	$6.95^{a} \pm 0.34$	$6.75^{a} \pm 0.10$	$6.7^{a} \pm 0.21$	$6.7^{a} \pm 0.08$
9	$6.85^{a} \pm 0.35$	$6.7^{a} \pm 0.18$	$6.65^{a} \pm 0.24$	$6.53^{a} \pm 0.17$	$6.53^{a} \pm 0.05$
12	$6.78^{a} \pm 0.28$	$6.7^{ab} \pm 0.00$	$6.63^{ab} \pm 0.22$	$6.5^{b} \pm 0.08$	$6.53^{ab} \pm 0.05$
15	$6.73^{a} \pm 0.09$	$6.55^{ab} \pm 0.10$	$6.5^{b} \pm 0.14$	$6.6^{ab} \pm 0.11$	$6.5^{b} \pm 0.18$
18	$6.45^{a} \pm 0.13$	$6.45^{a} \pm 0.24$	$6.28^{a} \pm 0.17$	$6.3^{a} \pm 0.00$	$6.3^{a} \pm 0.23$

Remarks: The notation indicated by different superscript letters in the same column shows a significant difference ($p < 0.05$). A: Scad fish without bay leaf extract treatment, B: Scad fish soaked in 6% of bay leaf extract, C: Scad fish soaked in 7% of bay leaf extract, D: Scad fish soaked in 8% of bay leaf extract, E: Scad fish soaked in 9% of bay leaf extract.

Organoleptic test

The organoleptic tests on preserved scad fish included five parameters, including visual, aroma, odor, texture, and mucus parameters that create an individual experience via the senses including taste, sight, smell, and touch. The minimum acceptable score was 6. The results of the organoleptic tests were provided at the beginning of the experiment and after 18 hours of preservation time.

Visual parameter

The results of the visual parameters at the beginning of the trial indicated the preserved scad fish in treatment A, B, C, D, and E experienced organoleptic values until the end of the storage period (18 hours). The average visual score of the beginning of the experiment in treatments A, B, C, D, and E ranged from 7.39 to 8.35. Furthermore, the average visual score at the 18 hours in treatments A, B, C, D, and E ranged from 5.48 to 6.97 (Figure 1).

Aroma parameter

The results of the aroma parameter of scad fish at the beginning of the experiment up to hour 18 of all treatments A, B, C, D, and E indicated a decrease in the organoleptic score. The average odor score at the beginning of the

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

experiment in all treatments was between 7.33 and 8.31. Furthermore, the average aroma value in all treatments at hour 18 ranged from 5.8 to 6.89 (Figure 2).

Odor parameter

The results of the odor parameter of the scad fish at the beginning of the experiment in all treatments indicated a decrease of the organoleptic values after 18 hours of storage period. The average odor value in all treatments at the beginning of the experiment was between 7.28 and 8.03 (Figure 3). The odor parameter results at the 18 hours were not assessed because the total value of the bacteria exceeded the limit of microorganisms.

Texture parameter

The results of the texture parameter at the beginning of the experiment at all treatments indicated a decrease in the organoleptic values by the end of 18 hours of the storage period. The average texture value at the beginning of the experiment was between 7.18 and 8.3 for all treatments. Furthermore, the average texture value was between 6.27 and 6.92 for all treatments after 18 hours (Figure 4).

Mucus parameter

The results of the mucus parameter at the beginning of the experiment for all treatments indicated a decrease in the organoleptic values by the end of 18 hours of the storage period. The average mucus value at the beginning of the experiment for all treatments was between 7.18 and 8.3. Furthermore, the average mucus value was between 6.13 and 6.87 for all treatments after 18 hours (Figure 5).

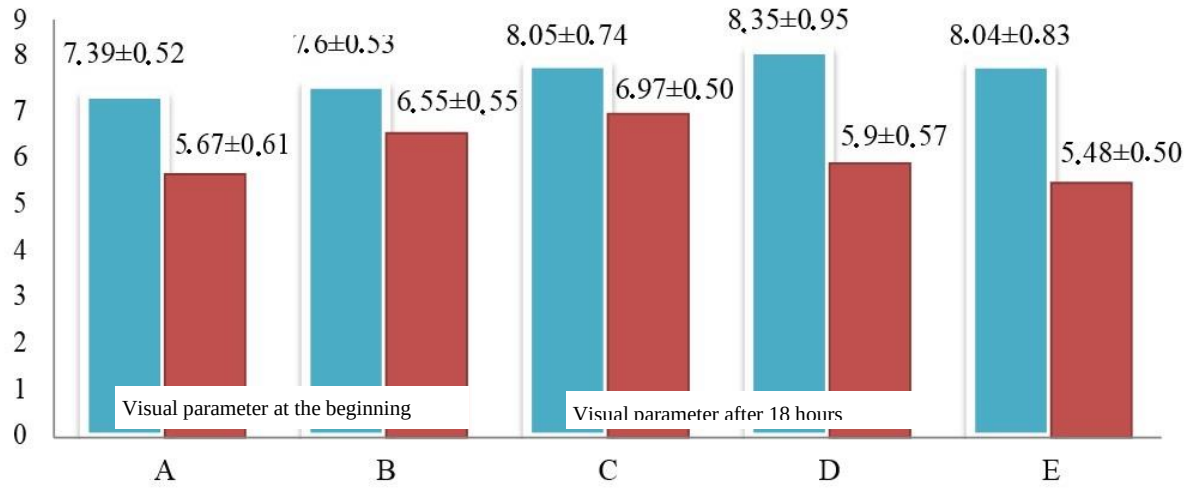


Figure 1. The results of the visual parameters of scad fish at the beginning of the experiment and after 18 hours of the storage period.

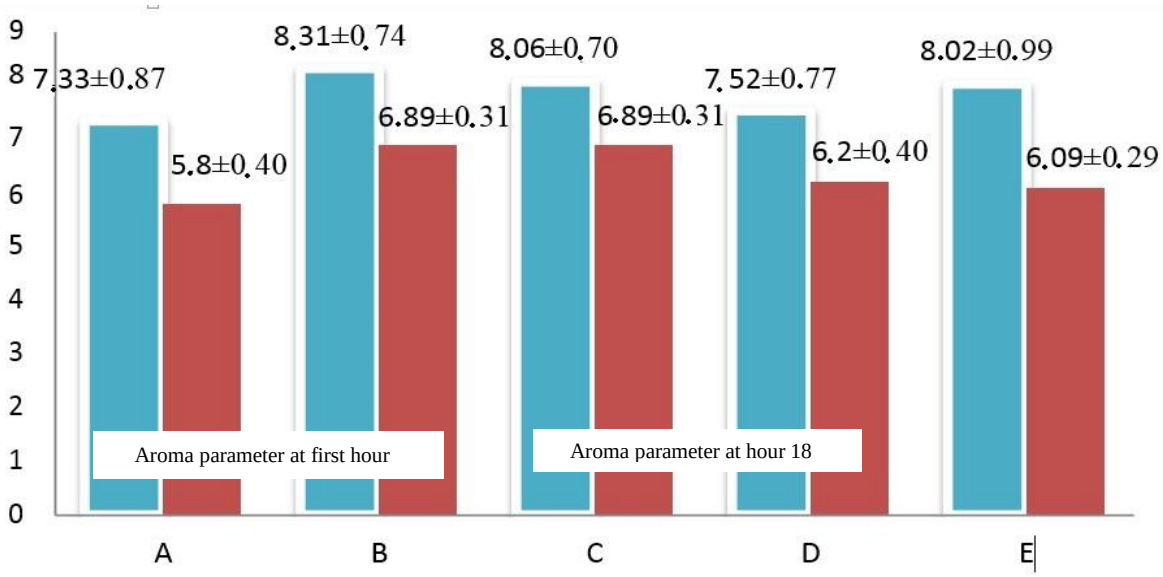


Figure 2. The results of the aroma parameters of scad fish at the beginning of the experiment and after 18 hours of storage period.

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

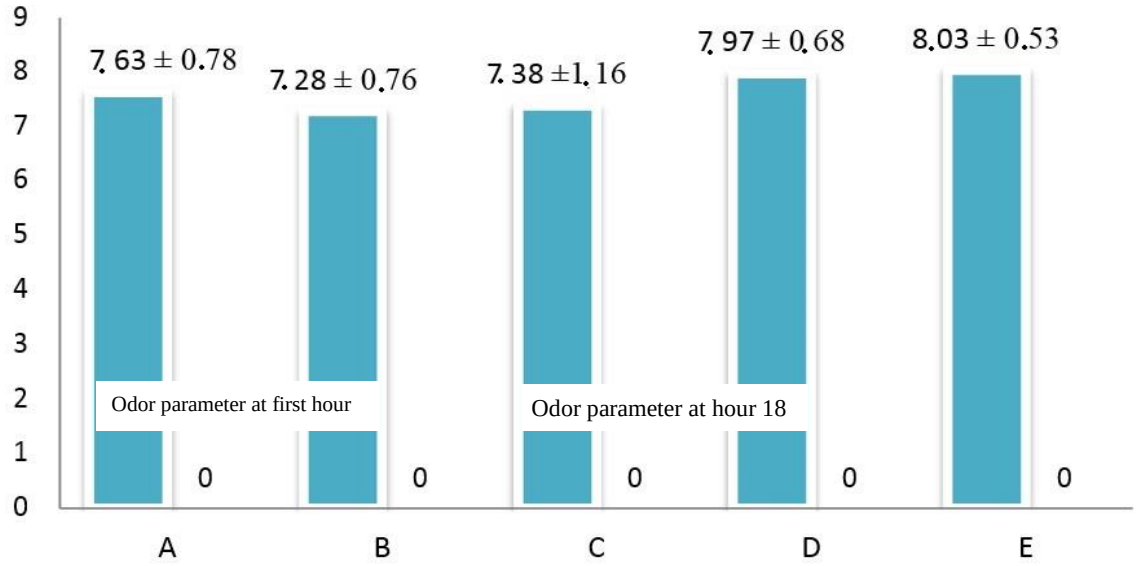


Figure 3. The results of the odor parameters of the scad fish at the beginning of the experiment and after 18 hours of the storage period.

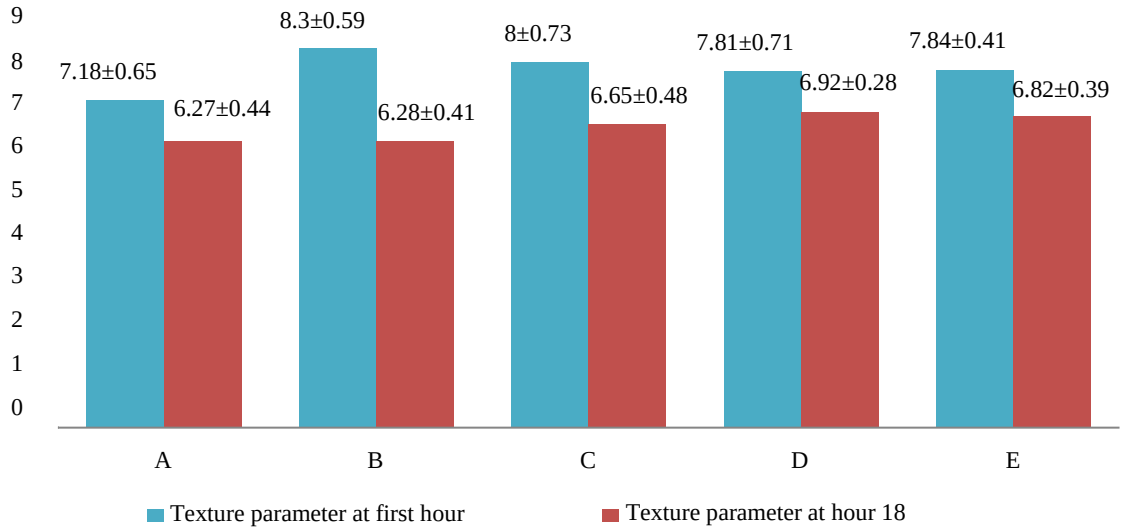


Figure 4. The results of the texture parameters of scad fish at the beginning of the experiment and after 18 hours of the storage period.

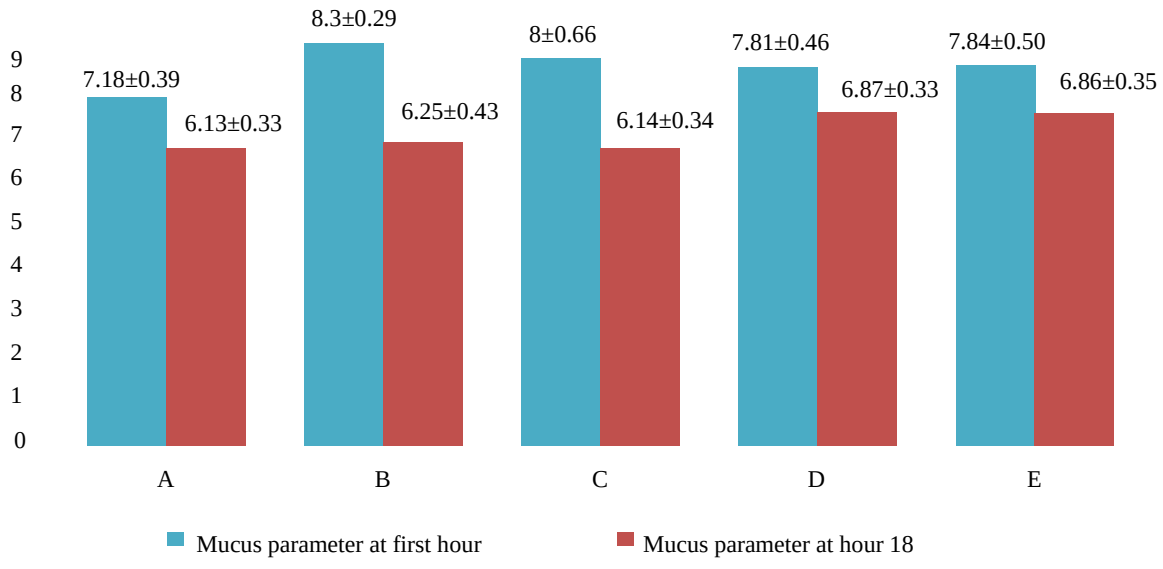


Figure 5. Mucus parameter of scad fish at the beginning of the experiment and after 18 hours of the storage period.

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

DISCUSSION

Shelf life is the period in which the quality of fresh food is impaired and becomes inedible. In the present research, the shelf life was determined using the TPC of the microbiological tests, chemical tests (water level and pH), and sensory tests (organoleptic test). The food product quality is influenced by the microbial content of the product as it determines consumption safety. The shelf life of food is determined by the total bacterial count in the product using a method called the TPC. The results of total bacteria calculation on the scad fish in all treatments increased constantly from the beginning of the experiment up to after 18 hours since the storage time could increase the total bacteria count. The test results of bay leaf extract on scad fish from the beginning up to 12 hours indicated that the number of microorganisms was still at the maximum growth limit of 5×105 Colony-Forming Unit (CFU)/gram (Buckle, 1987; Herawati, 2008).

Various factors that influenced microbial growth in the food products included intrinsic factors such as acidity (pH), water activity, nutrient content, biological structure, and antimicrobial content, while the extrinsic factors included storage temperature, humidity, type and amount of gases in the environment, and preparation and processing errors. All the treatments indicated that the bay leaf extract could inhibit the bacteria for up to 12 hours. The best treatment was obtained in treatment C (7% of bay leaf extract) because it had the lowest bacterial count among all treatments. The current result is in line with the previous study conducted by Buckle (1987) and Sahputra (2015) which suggested that a combination between 7% of bay leaf extract treatment and 8% of salt can inhibit bacteria in the milkfish (Buckle, 1987; Sahputra, 2015).

Bay leaf extract contained antibacterial compounds with high tannin, terpenoid, and flavonoid contents. These compounds could inhibit the growth of microbes. Flavonoid compounds have an important role in the inhibition of bacteria by forming complex compounds with extracellular proteins through hydrogen bonds that degrade bacterial cell membranes. Hydrogen and flavonoid bonds cause unstable cell wall structure and cell membrane and cellular deterioration. Tannin compounds play a role in shrinking cell walls and membranes to interfere with cell permeability. The impaired cell permeability means that the cell cannot perform living activities. Thus, it could inhibit bacterial growth and kill the bacteria. In addition, terpenoid compounds act as antibacterial compounds on the outer membrane of terpenoid cell walls by reacting with porin (transmembrane protein) to form strong polymer bonds. The polymer bonds caused the porin to deteriorate as a connecting gateway to reduce the permeability of the bacterial cell walls; and that caused bacterial cells to lack in the nutrient, whereby the bacterial growth is inhibited (Roslizawaty, 2013).

The water level is a factor that affects the shelf life of processed food and determines the freshness of food against microbes. The high water content causes bacteria, mold, and yeast to multiply easily. So there will be changes in the food. When stored in-room temperature, water level changes would be caused by the free water formed as a result of protein denaturation by microorganisms (Nirmala, 2016). Based on the results of the present study, the water level decreased from the beginning to the end of the storage process. This condition was caused by the evaporation of most of the water as the storage process was only stored at room temperature. The low water content led to a decrease in bacterial activity. However, based on the current research, the water level and the TPC indicated the opposite results, where the water level decreased, while the TPC increased. This phenomenon was caused by the still good water level, which allowed the bacteria to grow well. More than 30% of the water content in the food could affect bacterial growth. Elevated microbes could damage the hydrophilic protein, resulting in a reduced ability to hold water when the water level finally decreased (Siskos, 2007).

The pH value is an indicator that is measured to chemically determine the freshness level of fishery products. The optimal pH for bacterial growth is 6.5-7.5. The pH value of scad fish decreased from the beginning up to 18 hours. The pH change was caused by several factors, such as poor storage and storage temperature. The pH values obtained indicated that the scad fish marinated in bay leaf extract had an acidic pH value. The bay leaf boiling time affected the pH value of the leaf extract because the longer the leaves were boiled, the more organic acids from the leaves were extracted, resulting in a decrease in the pH value of the bay leaf. The pH value is closely related to the growth of microbes in fishery products. Fresh fish has a neutral pH, making it an ideal medium for bacterial growth (Junior, 2010). The organoleptic test on the scad fish, with and without adding bay leaf extract, included the visual, aroma, odor, texture, and mucus parameters. The result showed that at the starting hour, the quality of the fish was still acceptable by the panelists according to the Indonesia Nasional Standard with a minimum organoleptic value of 6. At the starting hour, the appearance of the scad fish received an average value in the range of 7.39 to 8.35. Meanwhile, the results of the organoleptic test at the 18 hours of storage suggested that the visual score of the scad fish decreased from 5.48 to 6.97. The results also indicated that there was an increasing bacterial count by hour 18. The appearance change was caused by the growth of microbe that could damage the products as the microbe were highly destructive to food.

The odor of the scad fish averaged between 7.33 to 8.31 at the beginning of the experiment. The odor value indicated that the fish was still fresh and smelled good. The fish smelled fresh since the bay leaves contained terpenoid compounds containing essential oils, which produced a distinctive but less sharp aroma. After 18 hours, the average result of the odor value decreased and was between 5.8 and 6.89. Therefore, the aroma parameter of scad fish at the

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

beginning of the experiment up to hour 18 of all treatments A, B, C, D, and E indicated a decrease in the organoleptic score. Treatment A gave the lowest score, reaching 5.8 at hour 18. The lowest odor score parameter on treatment A was also supported by the result indicating the high bacterial count found in the fish, which was 8.55×106. A decrease in the organoleptic matter could occur because the room temperature could accelerate the disintegration process during the storage of the fish. These bacteria could carry out a perfect metabolism that produced a bad odor.

Initially, the scad fish began to smell an average value of between 7.28 to 8.03, indicating that the fish was tasty, savory, or less savory. Meanwhile, after 18 hours, the odor test was not carried out by the organoleptic test due to the high TPC level of enzyme in the scad fish. In addition, the texture of the scad was given an average value in the range of 7.18 to 8.3, indicating that the fish were solid and elastic or less elastic. This texture was caused by the salt that was added in the process of making preserved scad fish since salt could improve the texture of fish. The salt used in the process of making scad fish reduced the water contents in the fish, which made the texture more solid and firm. Meanwhile, after 18 hours, the texture of scad fish decreased from 6.27 to 6.92. Treatment A had the lowest score, 6.27 at the 18 hours. The texture parameter of treatment A was also indicated by the high bacterial count found in the fish, which was 8.55 × 106, compared to the other treatments. The high bacterial count was caused by microorganism activity affecting the texture changes. The protein denaturation produced a simple protein, and also decreased the protein capacity to hold water so that the texture of fish became less solid and firm.

At the starting hour, the mucus of the scad fish obtained an average score from 7.18 to 8.3, indicating that the fish had thin and more neutral mucus and was odorless. Meanwhile, at 18 hours, the mucus content of scad fish fell from 6.13 to 6.87. The mucus parameter of the treatment is considered treatment with the lowest value of 6.13. The mucus parameter of treatment A was also indicated by the high bacterial count found in the fish, reaching 8.55×106, compared to other treatments. Mucous could be caused by bacteria. The mucus found in the fish's skin could be used as a parameter to determine the freshness level of the scad fish by focusing on the clearness and thickness of the mucus. The mucus changes were caused by the increasing number of spoilage bacteria activity on the fish after 18 hours (Lougovois and Kyrana, 2005).

CONCLUSION

Adding bay leaf extracts to preserved scad fish indicated that the total bacterial count was lesser than the Indonesian National Standard (SNI), of 5×105 CFU/gram, which could inhibit bacterial development by 12 hours. The best result was with treatment C (7% of bay leaf extract addition), as the concentration at this percentage could inhibit bacteria in preserved scad fish.

DECLARATIONS

Authors' contribution

Fuaidah designed the study, Triastuti collected the data and Pramono wrote the manuscript. Finally, all of the authors approved the final draft of the manuscript for submission.

Competing interests

The authors have not declared any conflict of interest.

Acknowledgments

Thanks to the authors and all the staff at the Faculty of Fisheries and Marine for their supports and facilities.

REFERENCES

Ariyani F, and Yennie Y (2008). Preservation of boiled salted scad mackerel (Decapterus russelli) using chitosan. Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan 3 (2): 139-146. DOI: http://dx.doi.org/10.15578/jpbkp.v3i2.16

Buckle KA, Edwards RA, Fleet GH, Wooton M, and Purnomo H (1987). Ilmu Pangan. Terjemahan: H. Purnomo dan Adiono. Jakarta Penerbit Univ Indones. Available at: https://opac.perpusnas.go.id/DetailOpac.aspx?id=508531

Hastuti S (2010). Analisis kualitatif dan kuantitatif formaldehid pada ikan asin di Madura [Formaldehyde qualitative and quantitative analysis on salted fish in madura]. Agrointek, 4(2): 132–7. DOI: https://www.doi.org/10.21107/agrointek.v4i2.1366

Herawati H (2008). Penentuan umur simpan pada produk pangan [Determination of shelf life in food products]. Jurnal Litbang Pertanian, 27(4): 124–30. Available at: http://203.190.37.42/publikasi/p3274082.pdf

Junianingsih I (2015). Quality test of pindang cue-besek Decapterus sp. In Jangkar Village District Situbondo. Samakia: Jurnal Ilmu Perikanan, 6(2): 91–8. DOI: https://www.doi.org/10.5281/jsapi.v6i2.289

Junior MRM, Leite AV, and Dragano NRV (2010). Supercritical fluid extraction and stabilization of phenolic compounds from natural sources–review (supercritical extraction and stabilization of phenolic compounds). The Open Chemical Engineering Journal, 4: 51-60. DOI: https://www.doi.org/10.2174/1874123101004010051

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

Lougovois, V. P., & Kyrana, V. R. (2005). Freshness quality and spoilage of chill-stored fish. In Food policy, Control and Research, Nova Science Publishers, Inc. pp. 35-86. https://www.academia.edu/27627359/Freshness_Quality_and_Spoilage_of_Chill-Stored_Fish

Nafisyah AL, Tjahjaningsih W, Kusdarwati R, Abdillah AA (2015). Pengaruh Alga Merah (Kappaphycus alvarezii) terhadap Mutu Ikan Kembung (Rastrelliger sp.) [Effect of Red Algae (Kappaphycus alvarezii) on The Quality of Mackerel (Rastrelliger sp.)]. Jurnal Ilmiah Perikanan dan Kelautan, 7(1): 87–94. DOI: http://www.dx.doi.org/10.20473/jipk.v7i1.11240

Nirmala D, Masithah ED, and Purwanto DA (2016). Kitosan Sebagai Alternatif Bahan Pengawet Kamaboko Ikan Kurisi (Nemipterus nematophorus) pada Penyimpanan Suhu Dingin [Chitosan as an Alternative Preservative Material for Curry Fish (Nemipterus nematophorus) in Cold Storage]. Jurnal Ilmiah Perikanan dan Kelautan, 8(2): 109-125. http://journal.unair.ac.id/download-fullpapers-jipk7df6c50aa02full.pdf

Nurwijayanti, Hasdianah, and Suhita BM (2016). Rekayasa Daun Salam untuk Pengawetan Ikan dalam Upaya Menghindari Penggunaan Efek Formalin terhadap Kesehatan Tubuh [Bay Leaf Engineering for Fish Preservation in an Effort to Avoid the Use of Formalin Effects on Body Health]. STIKes Surya Mitra Husada Kediri, 1: 120–128. Available at: https://docplayer.info/46728577-Rekayasa-daun-salam-untuk-pengawetan-ikan-dalam-upaya-menghindari-penggunaan-efek-formalin-terhadap-kesehatan-tubuh-stikes-surya-mitra-husada-kediri.html

Purwani E, and Muwakhidah (2008). Efek Berbagai Pengawet Alami sebagai Pengganti Formalin Terhadap Sifat Organoleptik dan Masa Simpan Daging dan Ikan [Effects of Various Natural Preservatives as Substitutes for Formalin on Organoleptic Properties and Shelf Life of Meat and Fish]. Jurnal Penelitian Sains and Teknologi, 9(1):1-14. Available at: https://publikasiilmiah.ums.ac.id/bitstream/handle/11617/414/1.%20ENI%20PURWANI%202.pdf?sequence=1&isAllowed=y

Roslizawaty, Ramadani NY, Fakhrurrazi, and Herrialfian (2013). Antibacterial Activity of Ethanol's Extract and Stew of Ant Plant (Myrmecodia sp.) Against Bakteria Escherichia coli . Jurnal Medika Veterinaria, 7(2): 91-94. http://jurnal.unsyiah.ac.id/JMV/article/download/2938/2788

Sahputra NW (2015). Daya Simpan Ikan Bandeng Yang Diawetkan Menggunakan Pengawet Alami Kombinasi Daun Salam (Eugenia polyantha, Weight.) [Shelf Life of Milkfish Preserved Using Natural Preservatives Combination of Bay Leaves (Eugenia polyantha, Weight.)] Dan Garam. Universitas Muhammadiyah Surakarta, 1: 1-11. Available at: http://eprints.ums.ac.id/35371/1/Naskah%20Publikasi.pdf

Siskos I, Zotos A, Melidou S, and Tsikritzi R (2007). The effect of liquid smoking of fillets of trout (Salmo gairdnerii) on sensory, microbiological and chemical changes during chilled storage. Food Chemistry, 101(2): 458–464. DOI: https://www.doi.org/10.1016/j.foodchem.2006.02.002

Triarso I (2012). Potential and opportunities for developing capture fisheries business in the north coast of Central Java. Saintek Perikanan: Indonesian Journal of Fisheries Science and Technology, 8(1): 65-73. DOI: https://www.doi.org/10.14710/ijfst.8.1.65-73

York RK, and Sereda LM (1994). Sensory assessment of quality in fish and seafoods. In: Shahidi F., Botta J.R. (eds) Seafoods: Chemistry, Processing Technology and Quality. Springer, Boston, MA. pp 233-262. https://www.doi.org/10.1007/978-1-4615-2181-5_13

To cite this paper: Fuaidah P, Triastuti J, and Pramono H (2021). The Effects of Different Concentrations of Bay Leaf Extract on Shelf Life of Preserved Scad Fish. *World Vet. J.*, 11 (3): 431-438. DOI: https://dx.doi.org/10.54203/scil.2021.wvj55

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 439-447, September 25, 2021
ISSN 2322-4568

Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria

Zahra Berrama[1, 2*], Samir Souames[1, 2], Rachid Merati[2, 3], Hakima Mefti Korteby[4], Manel S. Chirane[1], Nadia Negab[1, 5], Kenza Hettab[1, 5], Hayet Idris[1, 5], Nawel Morzouglal[5], and Soraya Temim[1, 2]

[1]*Ecole Nationale Supérieure Vétérinaire, Alger, Algérie*
[2]*Laboratoire de recherche Santé et Production Animale, ENSV, Algérie*
[3]*Animal Hygiene and Pathology Laboratory, Ibn Khaldoun University, Tiaret, Algeria*
[4]*Saad Dahlah University, Blida, Algeria*
[5]*Department of Reproductive Biology and Physiology, USTHB, Algiers, Algeria*
*Corresponding author's Email: z.berrama@ensv.dz; ORCID: 0000-0001-6422-3594

ORIGINAL ARTICLE
pii: S232245682100056-11
Received: 24 July 2021
Accepted: 11 September 2021

ABSTRACT

Egg quality traits in quail breeders depend on various factors which may influence embryo survival during incubation, affecting the chicks' production and quality. The current study aimed to determine the effect of the laying period on the external and internal quality of the egg, the chemical composition of albumen, yolk, and eggshell as well as the reproductive parameters of quail breeders. A total of 450 quails (*Coturnix japonica*) obtained from the same hatchery, were reared in a battery cage with a sex ratio of 1 male to 3 females. All the quails were subjected to standard breeding conditions and fed a balanced laying diet. A total of 960 eggs were collected at two different periods of the laying cycle, the beginning period of the laying (BP indicating 10 weeks of quail age) and the peak period of laying (PP showing 20 weeks of quail age). At each laying period, 30 eggs were used to analyze the various parameters of egg quality, and 450 eggs were randomly selected to assess the reproductive performances. Overall, no differences in the external quality of eggs, such as egg length, egg width, and the egg shape index, were recorded between the two laying periods. Apart from albumen weight that tended to be higher at the peak laying period, the shell and the yolk weights were not affected by the period of laying. Additionally, Japanese quail tend to deposit similar proportions of shell, albumen, and yolk at the two periods of laying. Likewise, the rate of dry matter of the three egg components, shell mineral concentrations, and yolk fat concentrations did not show any noticeable variation with the laying period. The most significant effect of the laying period was related to the potential reduction in the total protein content of the albumen and the yolk of eggs laid at the peak period of laying. Finally, the laying period did not significantly affect the fertility and hatchability rate of the incubated quail eggs but slightly improved the embryonic mortality rate during the peak laying phase.

Keywords: Albumen, *Coturnix japonica*, Eggshell, Fertility, Quail

INTRODUCTION

To fight against food insecurity that increasingly threatens the growing world population, identifying alternative food resources is a key intervention to improve and ensure dietary protein. The integration of game birds, such as geese, guinea fowl, pheasant, and quail in FAO food security programs, has been recommended, especially for developing countries (Geldenhuys et al., 2013). As in several African countries, Algeria must be oriented towards the diversification of its animal protein resources due to its demographic growth, which may reach 74 million in 2050, and change consumers' eating habits, which is becoming more and more demanding. The development of quail farming could make it possible to improve the population's consumption of animal proteins (eggs and meat) at a lower cost (Kaci, 2015).

Hatching egg quality is a matter of great importance in the production of living and healthy chicks. Indeed, the physical and chemical properties of an egg play an essential role in the proper development of embryos by protecting them and ensuring their nutritional needs (Bai et al., 2016; D'Alba et al., 2016; Chen et al., 2019) and even serve as a food source during the first days of the chick's life (Abanikannda, 2007). However, as Zita et al. (2013) mentioned, several factors affect the reproductive performances and egg quality of Japanese quails. Some are directly linked to the animal (genetics, age of breeders, laying cycle), and others depend on the quails' rearing conditions, such as nutrition, rearing system, light, and temperature (Yambayamba and Chileshe, 2019, Ratriyanto et al., 2020), the sex ratio (Ipek et al., 2004) as well as the storage and incubation conditions of the eggs (Kuurman et al., 2002).

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.,* 11 (3): 439-447.

The quail egg-laying period ranges from 8 to 12 months for breeders (Chelmonska et al., 2008) and could continue to 14 months for laying hens. This reproductive cycle is characterized by a beginning period of laying that lasts from the time when quails start laying (6 weeks) (Daikwo et al., 2014) to the time when quails lay maximum eggs, namely the peak laying period (17-22 weeks of age, Sauveur, 1988). The start of laying is a very critical period for female breeders. Their transfer from the grower house to the breeders' house could lead to stressful conditions for females, which results in poor laying start-up, and consequently low productivity and economic losses. Furthermore, it is well known that the weight of a bird determines its sexual maturity. Thereby, the variability of body weight within female breeders causes flock heterogeneity which may result in unsynchronized sexual maturity (delayed ovary development) and consequently failure in the mating process and performance.

Indeed, in the hen species, the beginning-laying phase of the breeders is characterized by high variability in egg quality, such as weight and egg fertility, most often attributed to the lack of uniformity of the flock (Pedroso et al., 2005).

The effects of breeder age were observed on the eggs' external and internal characteristics (Zita et al., 2013; Nasri et al., 2020), the eggs' fertility (Majhi et al., 2016), and embryonic development (Pokhrel et al., 2018). Several studies have revealed that during a laying period the weight of the eggs increases with the age of the breeders (Nasri et al., 2020). On the other hand, the weight and thickness of the shell decrease (Travel et al., 2010), and the proportion of yolk increases at the detriment of the albumen and shell proportions (Suarez et al., 1997). The beginning-laying period usually leads to the production of many small eggs. However, the component proportions of the hatching egg are affected by egg size. At the same breeders' age, smaller eggs contain more proportion of yolk than larger eggs (Vieira and Moran, 1998). It is well known that the egg yolk provides the nutrients for the developing embryo. However, the larger eggs, with a smaller proportion of yolk provide less nutrient content which could negatively affect embryo development and, consequently, incubation performance by increasing early and late embryo mortality. Moreover, the eggshell quality greatly influences the viability of the embryo and the hatchability of the eggs (Portugal et al., 2014; Ergun and Yamak, 2017).

Few studies have reported the effect of the laying period on Japanese quail reproductive parameters and quail egg quality so far. The objective of this study was to evaluate the effects of two laying periods of the breeders' quail, the beginning-laying period (BP) and the period of maximum production called the peak laying period (PP) on the external and internal quality of the hatching eggs as well as the variation of reproductive parameters.

MATERIALS AND METHODS

Ethical approval

The study was approved by the scientific council of the Superior National Veterinary School of Algeria with certificate reference 255/FDCS/2021.

Quails (*Coturnix japonica*) and protocol design

The current study was conducted at the farm of the technical institute of breeding, department of monogastric, Ministry of agriculture and rural development, BABA ALI, Algeria. A total of 960 eggs were obtained from 450 Japanese quail breeders, housed in a five-story battery cage. Each story was divided into 3 cages of 30 quails each, with a sex ratio of 3 females to 1 male. All the quails were subjected to the same standard breeding conditions and fed the same layer diet containing 2700 kcal.Kg-1 of metabolic energy, 19.01% of crude protein, 4% of calcium, and 0.7% phosphorous (ITELV, 1988).

All the eggs were collected at two different periods of the laying cycle, namely BP (at week 5 of egg production, representing 10 weeks of quail age) and PP (week 10 of egg production, representing 20 weeks of quail age). At each laying phase, 30 eggs were used to assess egg quality traits, and 450 eggs were randomly selected from eggs laid during one week to analyze the reproductive and hatching performances. For both laying phases (BP and PP), each egg was given individual identification and weighed by an electronic precision scale with 0.01g accuracy. The length and width egg measurements were recorded by a caliper, and the egg shape index was calculated (SI = width/length ×100; Smai et al., 2018). At the end of the egg collection, the eggs for external and internal quality determination were transferred to a feed analysis laboratory of the Superior National Veterinary School. However, those used for reproductive traits assessment were set in a vertical incubator (La Nationale, France. Serial Number:4391) at a temperature of 37.7°C, relative humidity of 60 %, and automatic egg-turning every 8 hours.

Measurements of egg quality

In the first step, each egg was broken. Afterward, eggshell, albumen, and yolk were carefully separated and weighed by a scale with 0.0001 g accuracy to determine their fresh weights and then expressed them as a percentage of whole egg weight (Zita et al., 2013). The dry matter (DM) of the three separated structures was determined by drying the

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

samples at 80°C in a ventilated oven until constant weight (3 days). The chemical composition of each part of the quail egg was determined according to the methods of AOAC (1990), namely, eggshell mineral matter, yolk and albumin crude proteins, and yolk fat matter. Thus, the mineral matter of each dehydrated eggshell was determined by gradual incineration (550°C) in a muffle furnace until all organic material was destroyed. The dry ash was weighed and then expressed as a DM percentage. The crude proteins of the albumen and yolk were determined using the Kjeldahl method by measuring the total nitrogen content and then using a factor of 6.25 to convert total nitrogen into crude proteins (CP = total nitrogen × 6.25). The total fat of the yolk was determined by the Soxhlet method using petroleum ether as a solvent. The extraction process lasted for 18 hours. After completion of the extraction, the solvent was evaporated, and the mass of lipid remaining was dried in an oven, weighed, and then expressed as a DM percentage.

Reproductive performances determination

On day 14 of incubation, the eggs were removed from the incubator and candled to detect infertile eggs. On day 15 of incubation, the eggs were transferred to a hatcher where the temperature and relative humidity were maintained as 36.7°C and 80%, respectively. After hatching, the unhatched eggs were broken to investigate fertility that was expressed as the fertile eggs/ incubated eggs×100. The hatchability of fertile eggs was determined as a number of chicks hatched/fertile eggs ×100. The hatchability of incubated eggs was calculated as the number of chicks hatched /number of incubated eggs ×100. Embryo mortality was obtained as the proportion of dead embryos out of the number of fertile eggs (Hegab and Hanafy, 2019).

Statistical analysis

Data were descriptively presented as means and subjected to one-factor variance analysis (ANOVA) performed with the Statview software (Abacus Concepts, 1996, Inc., Berkeley, CA94704-1014, USA). Version 4.57.0.0. Student-Newman-Keuls test was used to detect the differences. The statistical significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Egg external traits

The external qualities of Japanese quail breeders' eggs laid at BP and PP are presented in Table 1. The results indicated that the laying period of Japanese quails did not affect the weight of the eggs ($p > 0.05$). Also, no variation in the eggs' length, width, and shape index was associated with periods of the laying cycle ($p > 0.05$). These findings are consistent with those obtained by some previous research (Moula et al., 2014; Smaï et al., 2018). In an experiment by Smaï et al. (2018), no difference in the egg weight of Japanese quails was observed between the two periods of laying (10.2 vs 11.1 g, $p > 0.05$ at BP and PP, respectively). In contrast, other authors have reported different egg weights of Japanese quail in various phases of the reproductive cycle. Some studies revealed an increase in egg weight during the breeding cycle of Japanese quails (Nazligul et al., 2001). Zita et al. (2013) have reported that the egg weight increased in quails by 18 % ($p < 0.05$) from the beginning phase of laying (week 9 of age) to the age of 21 representing the same peak laying phase in the current study. Moreover, it has been found that the egg shape index changes with the breeders' age (Molnar, 2016; Rakib et al., 2016; Kraus et al., 2020). In the present study, the shape index of eggs laid by the quail breeders remained unchanged on average over the two experimented periods of laying (77% vs 76% for BP vs PP, respectively). These results are consistent with those reported by Zita et al. (2013) and Moula et al. (2014) but did not agree with those of Orhan et al. (2001), who recorded a significant decrease in the form index of Japanese quail eggs by their age. Investigations in breeder hens and Peking ducks indicated a higher egg form index towards the end of the breeding season rather than the beginning (Romanoff and Romanoff, 1949; Kokoszynski et al., 2007). It is often acknowledged that at the beginning of production, the smaller eggs have a rather spherical shape, which tends to lengthen gradually during the laying period showing an ellipsoidal form for larger eggs (Gonzalez et al., 1982). This change in egg shape is thought to result from a weakening of the muscle tone of the shell gland in older hens (Travel et al., 2010).

Table 1. The effects of beginning and peak of laying periods on the external quality of eggs in Japanese quail breeders

Trait \ Laying period	BP	PP	SEM	p-value*
Weight (g)	10.72	11.18	0.20	0.12
Length (cm)	2.95	2.98	0.03	0.43
Width(cm)	2.28	2.28	0.02	0.89
Shape Index (%)	77.53	76.82	0.53	0.35

BP: Beginning laying period, PP: Peak laying period. SEM: Standard Error of the Mean. *Significant differences ($p \leq 0.05$).

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

Egg components

The effects of the laying period (BP and PP) of Japanese quail breeders on the shell, albumen, and yolk weights, as well as their proportions in relation to the weight of whole quail eggs, are presented in Table 2.The laying periods (BP and PP) did not affect the weight of the different egg components, namely shell, albumen, and yolk ($p > 0.05$). Moreover, the current results have revealed that the proportions of the different quail egg components, such as eggshell, albumen, and yolk, do not change significantly with variations in the laying period ($p > 0.05$). Eggs tended to have the same proportions of shell, albumen, and yolk at BP and PP periods. In contrast, Zita et al. (2013) recorded an increase in the weight of all three egg components over the variation in quail laying periods. Similarly, Moula et al. (2014) reported an impact of the laying period on the weights of the two nutritive components of the Japanese quail egg, namely albumen, and yolk. An increase of +3 and +4 % was recorded for quail eggs laid at 20 weeks of age for albumen and yolk, respectively. However, the same authors did not reveal any effect of the laying period on eggshell weight, whereas Curtis et al. (1986) reported that the percentage of shell in hens' eggs decreased throughout the production periods. During the laying period spanning from the beginning to the peak, albumen proportion increased from 61.10% to 62.10%, however, the yolk proportion decreased from 30.97% to 29.75% during the same period (Nowaczewski et al., 2010). Hegab and Hanafy (2019) attribute this variation in weight and proportion of the different egg constituents with the laying period to the increase in weight, length, and width of the egg with the age of the breeders. Similarly, Cook et al. (2005) reported a tendency in yolk proportion to be lower in large eggs than smaller ones.

As the egg size increases, the relative amount of yolk decreases. Flock age and egg size are major factors that determine the albumen and yolk content in eggs (Nangsuay et al., 2011). Furthermore, Nasri et al. (2020) reported that as breeders become older, ovulation intervals increase. This results in equal quantities of yolk synthesized by the liver being deposited in fewer follicles causing higher yolk weights (Zakaria et al., 1983).

The effect of the laying period (BP and PP) of Japanese quail breeders on the shell, albumen, and yolk dry matter is presented in Figure 1. The effect of the laying period of Japanese quail breeders on mineral eggshell content, total protein albumen and yolk contents, and total yolk lipids is presented in Figure 2. No effect of laying period was noted for the rate of dry matter of the shell, the albumen, and the yolk of Japanese quail eggs ($p > 0.05$). These results contrasted with the finding of Ulmer-Franco et al. (2009), who reported that dry shell percentage was greater in eggs laid by younger commercial broiler breeder hens than those laid by older hens. Similarly, Cook et al. (2005) and Nasri et al. (2020) found that whites from eggs produced by older hens had lower solids content than those obtained from younger hens. However, the latter authors showed that contrary to albumen DM, yolk DM percentage increased with breeder age.

The eggshell is the major source of minerals during the latter stages of embryonic development (Ono and Wakasugi, 1984; Solomon, 2010). Thus, this component should be considered when setting eggs for incubation to achieve successful hatching. In the current study, the laying period of Japanese quail breeders did not affect mineral deposition in the eggshell, the mineral matter percentage remained constant throughout the two periods of laying. These results are in agreement with the findings of Crosara et al. (2019), who reported that the contents of mineral eggshells are the same throughout the broiler breeders' life. Proteins are present primarily in egg albumen and egg yolk, while lipids are almost exclusively in the yolk (Nangsuay et al., 2011). In the present study, the crude protein concentrations in each of the albumen and yolk of quail eggs were influenced by the laying period. Both the yolk and albumen of eggs laid at the BP of laying contained +28 % and +36 % more crude proteins, respectively, than those of eggs laid at the PP of laying ($p < 0.05$). However, the laying period did not affect the concentration of the total lipid of the breeder quail egg yolk. Thus, a similar lipids deposition was recorded at the two periods of egg production. These results were consistent with those reported by Curtis et al. (1986), who noted a significant period effect on the percentage of albumen protein with a gradual decline over the production period and those that reported no effect of flock age on the lipids contents of hen egg yolk (Cook et al., 2005). In contrast, no variation in the yolk protein contents of eggs was observed to be associated with hens' ages (Cook et al., 2005).

Table 2. The effects of beginning and peak of laying periods on the shell, albumen and yolk weights, and their proportions in relation to the weight of whole eggs in Japanese quail breeders

Egg components	Trait	Laying period		SEM	p-value*
		BP	PP		
Albumen	Weight (g)	5.33	5.74	0.16	0.07
	Weight proportion (%)	49.57	51.17	0.87	0.20
Yolk	Weight (g)	3.47	3.54	0.08	0.55
	Weight proportion (%)	32.39	31.60	0.48	0.26
Shell	Weight (g)	1.52	1.58	0.15	0.75
	Weight proportion (%)	14.25	14.33	1.49	0.97

BP: Beginning laying period, PP: Peak laying period. SEM: Standard Error of the Mean. * Significant differences ($p \leq 0.05$).

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

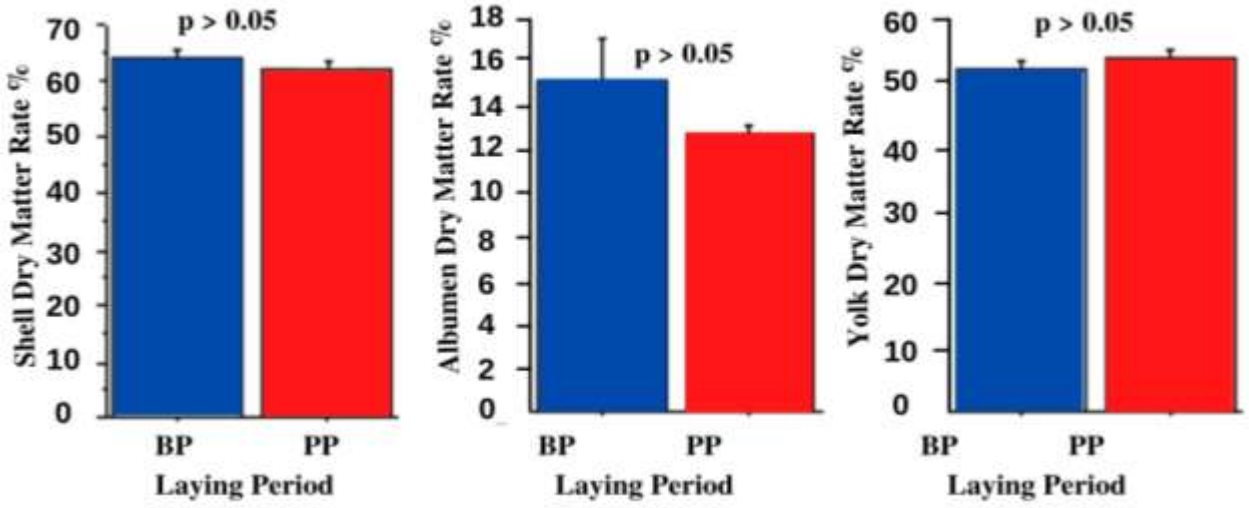


Figure 1. The effects of beginning and peak of laying periods on shell, albumen and yolk dry mater in Japanese quail breeders. BP: beginning laying period, PP: peak laying period, significant differences ($p \leq 0.05$).

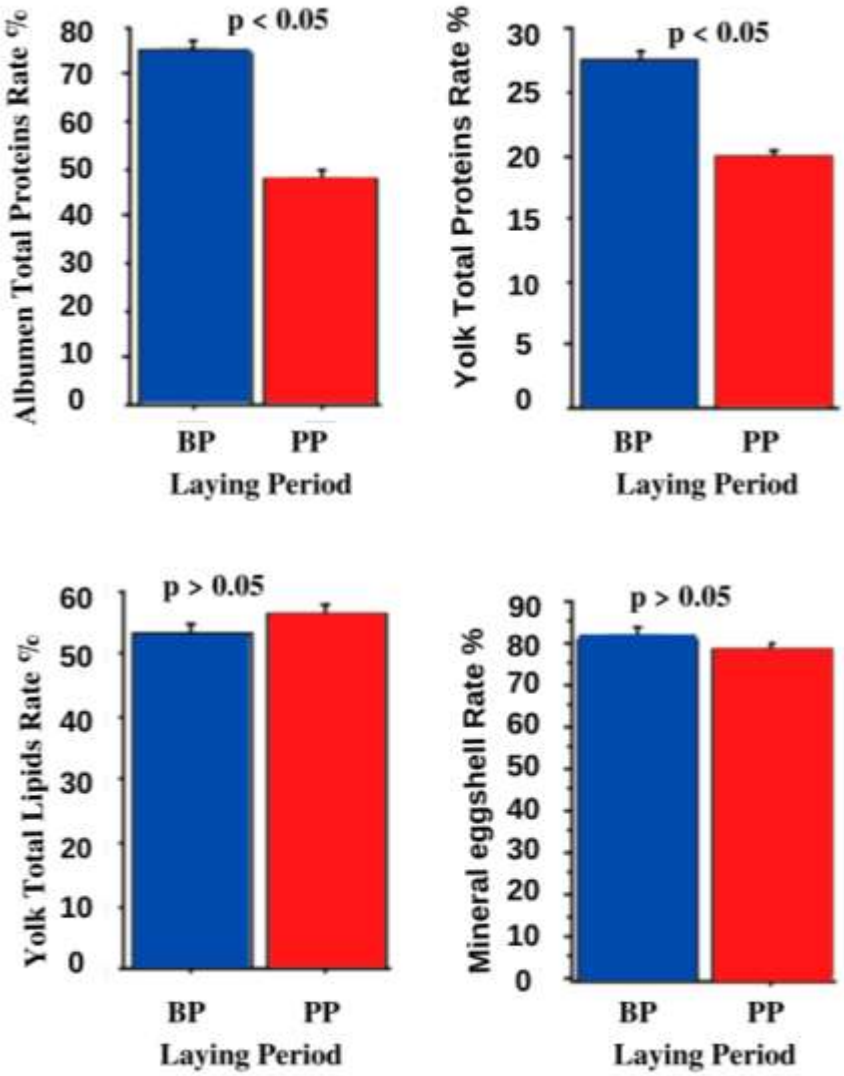


Figure 2. The effects of beginning and peak of laying periods on mineral eggshell content, total protein albumen and yolk contents and total yolk lipids in Japanese quail breeders. BP: beginning laying period, PP: peak laying period, significant differences ($p \leq 0.05$).

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

Reproduction parameters: Fertility, hatchability, and embryo mortality

The effect of the laying period (BP and PP) of Japanese quail breeders on the fertility, hatchability, and embryo mortality of the incubated and fertile eggs is shown in Table 3. The current results indicated that the laying periods did not affect the fertility of the incubated eggs ($p > 0.05$). These results agreed with the findings of Ipek et al. (2004) regarding Japanese quail and those of Santos et al. (2015) concerning European quail. According to these authors, the laying period had no effect on egg fertility throughout the reproductive life of European quails. In contrast, Ipek et al. (2004) have shown that the fertility of eggs in Japanese quails was lower at ages of 7-10 weeks than those of 15-18 weeks. This inconsistency in results may be due to a difference in the experimental protocol, such as the applied male-female ratio. In poultry, the male is one of the main factors limiting the fertility of flocks (Farooq et al., 2018). In fact, any delayed growth and sexual maturity in males affect sexual behaviors in young flocks, often caused by nutritional factors or inappropriate light stimulation (Brillard, 2003). Previous studies have explained the improvement of fertility rate at the peak of laying of quails by the acquiring of a greater number of mature ovarian follicles (Bagh et al., 2016). The laying period of the Japanese quail breeders did not significantly affect the hatchability of the incubated and fertile eggs ($p > 0.05$). However, statistical analysis revealed that the numerical values of hatchability rates tended to be slightly higher, with + 3.5 % ($p = 0.14$) for incubated eggs and +3 % ($p = 0.06$) for fertile eggs during the peak laying period, compared to the beginning laying ones. Similarly, previous studies have reported that the laying period, which is closely related to the age of the breeders, has no effect on the hatching capacity of incubated eggs (Ipek et al., 2004; Smaï et al., 2018). Likewise, Smaï et al. (2018) did not detect any significant variation in the hatchability of incubated eggs during the breeding season of domestic quails. These authors recorded a numerical improvement in the hatching rate of the incubated eggs of +7 % ($p > 0.05$) between the period of the beginning of laying (8-12 weeks of age) and the period when the maximum eggs are laid (16th and 26th weeks of age). However, the recorded hatchability rate values remain lower (70% vs. 75%, respectively, BP and PP), compared to the results of the current study. These low rates could be explained by the sex ratio applied (1/2) by these researchers, which probably affected the fertility of the eggs and, consequently, their hatchability rate. Some contradictory findings were also reported. Elibol and Brake (2006) recorded better hatchability of incubated eggs in laying hens at the early-laying period, compared to the other periods of the reproductive cycle. Seker et al. (2004) reported a higher hatchability rate of fertile eggs at the peak of laying (93.33%) than at the beginning of laying (81.53 %). The variation in the hatching rate of fertile eggs during a reproductive cycle is most often attributed to a variation in the quality of the eggs during that cycle (Sahan et al., 2014; Boleli et al., 2016). In the current study, the laying period did not affect the embryo mortality rate of the incubated eggs ($p > 0.05$). A similar result was reported by Santos et al. (2015) in European quail. However, a significant decrease of -40 % in the mortality rate for the fertile eggs during the PP of laying is recorded ($p = 0.05$).

However, Majhi et al. (2016) and Amiar et al. (2017) noted a gradual decrease in embryo mortality of incubated eggs from the beginning to the peak period of laying. In the same trend, Seker et al. (2004) recorded an improvement in the embryonic mortality rates of fertile eggs over the two periods of the reproductive cycle. This is in contrast with Wilson et al. (2003), who suggested an opposite effect of quail age on embryonic mortality due to the poor positioning of the embryos before hatching. It should be noted that in poultry, several physiological parameters change with flock age (O'Sullivan et al 1992). The most noticeable change is the increasing egg weight (North and Bell, 1991). Indeed, Hegab and Hanafy (2019) reported that the embryonic mortality rate is lower for heavy eggs, compared to lighter eggs, which is explained by the richness of heavy eggs in sufficient nutrient reserves, compared to lighter eggs.

Table 3. The effects of beginning and peak of laying periods on the fertility, hatchability, and embryo mortality of the incubated and fertile eggs in Japanese quail breeders

Trait	Parameters	Laying period		SEM	p value*
		BP	PP		
Fertility (%)	Incubated egg	95.11	94.89	1.03	0.88
Hatchability (%)	Incubated egg	86.89	90.00	1.50	0.14
	Fertile egg	91.36	94.61	1.22	0.06
Mortality (%)	Incubated egg	12.67	9.33	1.47	0.11
	Fertile egg	8.18	4.92	1.18	0.05

BP: Beginning laying period, PP: Peak laying period. SEM: Standard Error of the Mean. *Significant differences ($p \leq 0.05$).

CONCLUSION

In conclusion, the laying period shows stability in egg external quality, their overall composition, and in the most of chemical components of albumen, yolk, and shell. Nevertheless, the peak of laying has a large negative impact on the protein rate of albumen and yolk but without affecting the threshold egg quality needed for the successful development

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

of the embryo. Eggs laid by quail breeders at the beginning and the peak periods of laying were of equal fertility and hatchability performances. However, the low proteins could affect negatively chicks' quality at hatch and their growth performances. Further research must be conducted to draw more clear conclusions, determining the effect of quality of eggs on growth performances of chicks hatched from eggs of different periods of laying cycle and examining the possible interaction effects of the laying period with other factors such as egg storage duration.

DECLARATIONS

Authors' contribution

Z Berrama and H Mefti-Korteby designed the experiment, Z Berrama, S Souames, and R Merati curated the data. Laboratory analyses were done by MS Chirane, N Negab, H Idris, K Hettab, and Z berrama. Z Berrama wrote the manuscript. S Temim, S Souames, and N Morzouglal revised the manuscript. All authors confirmed the manuscript's final draft and data analysis.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

REFERENCES

Abanikannda OTF, Olutogun O, Leigh AO and Ajayi LA (2007). Statistical modelling of egg weight and egg dimension in commercial layers. International Journal of Poultry Science, 6: 59-63. DOI: https://www.doi.org/10.3923/ijps.2007.59.63

Amiar WH (2017). Study of the effect of the use of a quail-type feed on the reproduction performance of the Japanese quail Coturnica japonica. Agronomic Master thesis pp.1-38. University of Abdelhamid Ibn Badis Mostaganem. Available at: http://e-biblio.univ-mosta.dz/handle/123456789/12401

AOAC (1990). Official methods of analysis of the Association of Official Analytical Chemists. 15th Edition, Arlington, VA, (USA) pp. 1028-1039. Available at:https://law.resource.org/pub/us/cfr/ibr/002/aoac.methods.1.1990.pdf

Bagh J, Panigrahi B, Panda N, Pradhan CR, Mallik BK, Majhi B and Rout SS (2016). Body weight, egg production, and egg quality traits of gray, brown, and white varieties of Japanese quail (*Coturnix coturnix japonica*) in coastal climatic condition of Odisha. Veterinary World, 9: 832-836. DOI: https://www.doi.org/10.14202/vetworld.2016.832-836

Bai JY, Pang YZ, Zhang X and Li YX (2016).Study on the morphological development of quail embryos. Revista Brasileira de Ciência Avícola, 18: 91-93. DOI: https://doi.org/10.1590/1806-9061-2015-0177

Boleli IC, Morita VS, Matos JJB, Thimotheo M and Almeida VR (2016). Poultry Egg Incubation: Integrating and Optimizing Production Efficiency. Brazilian Journal of Poultry Science, 18(2): 1-16. DOI: https://www.doi.org/10.1590/1806-9061-2016-0292

Brillard JP (2003). Practical aspects of fertility in poultry. World's Poultry Science Journal, 59: 441-446. DOI: https://doi.org/10.1079/WPS20030027

Chelmonska B, Jerysz A , Lukaszewicz E, Kowalczyk A and Malecki I (2008).Semen collection from Japanese quail using a teaser female. Turkish Journal Veterinary and Animal Science, 32: 19-24. Available at: https://journals.tubitak.gov.tr/veterinary/issue.htm?id=757

Chen X, Li X, He Z, Hou Z, Xu G, Yang N and Zheng J (2019).Comparative study of eggshell antibacterial effectivity in precocial and altricial birds using Escherichia coli, PLoS ONE, 14(7): e0220054. DOI: https://www.doi.org/10.1371/journal.pone.0220054

Cook MI, Beissinger SR,Toranzos GA and Arendt WJ (2005).Incubation reduces microbial growth on eggshells and the opportunity for trans-eggshell infection. Ecology Letter, 8: 532-537. DOI: https://www.doi.org/10.1111/j.1461-0248.2005.00748.x.

Crosara FSG, Pereira VJ, Lellis CG, Barra KC, Santos SKA, Souza LCGM, Morais TA, Litz FH, Limao VA, Braga PFS and Fernandes EA (2019). Is the Eggshell Quality Influenced by the Egg Weight or the Breeder Age? Brazilian Journal of Poultry, 21: 1-8. DOI: http://www.dx.doi.org/10.1590/1806-9061-2018-0896

Curtis PA, Gardner F A and Mellor D B (1986). A comparison of selected quality and compositional characteristics of brown and white shell eggs. III. Composition and nutritional characteristics. Poultry Science, 65: 501-507. DOI: https://www.doi.org/10.3382/ps.0650501

Daikwo SI, Dim NI and Momoh MO (2014). Genetic parameters of some Egg Production traits in Japanese quail in a tropical Environment. OSR-JAVS. 7: 39-42. DOI: https://www.doi.org/10.9790/2380-07933942

D'Alba L, Maia R, Hauber ME and Shawkey MD (2016). The evolution of eggshell cuticle in relation to nesting ecology. Proceedings Royal Society B, 283:2832016068720160687 DOI: https://wwwdoi.org/10.1098/rspb.2016.0687

Elibol O and Brake J (2006). Effect of flock age, cessation of egg, and turning frequency through the second week of incubation on hatchability of broiler hatching eggs. Poultry Science, 85: 1498-1501. DOI: https://www.doi.org/10.1093/ps/85.8.1498.

Ergun OF and Yamak US (2017). The effect of eggshell thickness on hatchability of quail eggs. Veterinary World, 10: 1114-1117. DOI: https://www.doi.org/10.14202/vetworld.2017.1114-1117

Farooq U, Malecki IA, Misbah M and Graeme BM (2018). Correlation between objective semen analysis and fertility in Japanese quail. Theriogenology, 4: 12. DOI: https://www.doi.org/10.1016/j.theriogenology.2018.04.012

Geldenhuys G, Hoffman LC and Muller N (2013). Aspects of the nutritional value of cooked Egyptian goose (*Alopochen aegyptiacus*) meat compared with other well-known fowl species. Poultry Science, 92: 3050-3059. DOI: https://www.doi.org/10.3382/ps.2013-03342

Gonzalez M, Roca P, Sainz F and Alemany M (1982). A comparison between egg surface areas and volumes of several avian species. Comparative Biochemistry and Physiology Part D, 73: 301–302. DOI: https://doi.org/10.1016/0300-9629(82)90074-3

Hegab IM and Hanafy AM (2019). Effect of Egg Weight on External and Internal Qualities, Physiological and Hatching Success of Japanese Quail Eggs (*Coturnix coturnix japonica*). Brazilian Journal of Poultry Science, 21: 1-8. DOI: https://www.doi.org/10.1590/1806-9061-2018-0777

Institut Technique des Elevages (ITELV) (1988). Guide d'élevage de la caille et du pigeon. Institut Technique des Elevage, BABA ALI, ALGERIA pp. 28. Available at: http://www.itelv.dz/index.php/telechargements/send/54-aviculture/237-guide-d-elevage-de-la-caille-et-du-pigeon-itpe-1988.html

Ipek A, Sahan U and Yilmaz B (2004). The effect of live weight, male to female ratio and breeder age on reproduction performance in Japanese quails (*Coturnix coturnix japonica*). South African Journal of Animal Science, 34: 130-134. Available at: https://www.sasas.co.za/journals/the-effect-of-live-weight-male-to-female-ratio-and-breeder-age-on-reproduction-performance-in-japanese-quails-coturnix-coturni/

Kaci A (2015). La filière avicole algérienne à l'ère de la libéralisation économique. Cahiers Agricultures, 24: 151-160. DOI: https://www.doi.org/10.1684/agr.2015.0751

Kokoszynski D, Bernacki Z and Korytkowska H (2007). Eggshell and egg content traits in peking duck eggs from the P44 reserve flock raised in Poland. Journal Central European. Agriculture, 8: 9-16. DOI: https://jcea.agr.hr/en/issues/article/398

Kraus A, Zita L, Krunt O, Volek Z, Tyller M and Anderle V (2020). Comparison of basic internal and external egg quality traits of brown and white egg-laying hens in relationship to their age. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 68: 49-56. DOI: https://www.doi.org/10.11118/actaun202068010049

Kuurman WW, Bailey BA, Koops WJ and Grossman M (2002). Influence of storage days on the distribution for time of embryonic mortality during incubation. Poultry Science, 81: 1-8. DOI: https://www.doi.org/10.1093/ps/81.1.1

Majhi B, Panigrahi B, Panda N, Mallik BK, Pradhan CR, Bagh J and Chichilichi B (2016). Effect of age and mating ratio on fertility and hatchability of Japanese quail (*Coturnix coturnix japonica*) in the coastal climate of Odisha. Indian Journal of Poultry Science, 51: 187-91. DOI: https://www.doi.org/10.5958/0974-8180.2016.00030.1

Molnar A, Maertens L, Ampe B, Buys J, Kempen I, Zoons J and Delezie E (2016). Changes in egg quality traits during the last phase of production: is there potential for an extended laying cycle? British Poultry Science, 57(6): 842-847. DOI: https://www.doi.org/10.1080/00071668.2016.1209738

Moula N, Philippe FX, Ait Kaki A, Touazi L, Antoine-Moussiaux N and Leroy P (2014). Ponte et qualité d'œufs de cailles élevées en conditions semi intensives dans l'est algérien. Archivos de Zootecnia, 63: 693-966. DOI: http://www.dx.doi.org/10.4321/S0004-05922014000400013

Nangsuay A, Ruangpanit Y, Meijerhof R and Attamankune S (2011). Yolk absorption and embryo development of small and large eggs originating from young and old breeder hens. Poultry Science, 90: 2648-2655. DOI: https://www.doi.org/10.3382/ps.2011-01415

Nasri H, Van Den Brand H, Najjar T and Bouzouaia M (2020). Egg storage and breeder age impact on egg quality and embryo development. Journal of Animal Physiology and Animal Nutrition, 104: 254-68. DOI: https://www.doi.org/10.1111/jpn.13240

Nazligul A, Turkyilmaz K and Bardakcioglu HE (2001). A study on some production traits and egg quality characteristics of Japanese quail. Turkish Journal Veterinary and Animal Science, 25: 1007-1013. Available at: https://journals.tubitak.gov.tr/veterinary/abstract.htm?id=5119

North MO and Bell DD (1991). Commercial chicken production manual. 4th ed. Van Nostrand Reinhold, pp.710. Available at: https://scholar.google.com/scholar_lookup?title=Commercial%20Chicken%20Production%20Manual&publication_year=1990&author=M.O.%20North&author=D.B.%20Bell

Nowaczewski S, Kontecka H, Rosiński A, Koberling S and Koronowski P (2010) Egg quality of Japanese quail depends on layer age and storage time. Folia Biologica (Krakow), 58: 201-207. DOI: https://www.doi.org/10.3409/fb58_3-4.201-207.

O'Sullivan NP, Dunningtone A and Siegel PB (1991). Relationships Among Age of Dam, Egg Components, Embryo Lipid Transfer, and Hatchability of Broiler Breeder Eggs. Poultry Science, 70: 2180-2185. DOI: https://www.doi.org/10.3382/ps.0702180

Ono T and Wakasugi N (1984). Mineral content of quail embryos cultured in mineral-rich and mineral-free conditions. Poultry Science, 63: 159-166. DOI: https://www.doi.org/10.3382/ps.0630159.

Orhan H, Erensaying C and Aktan S (2001). Determining egg quality characteristics of Japanese quails (*Coturnix Coturnix Japonica*) at different ages. Journal of Animal Production, 42: 44-49. Available at: https://dergipark.org.tr/en/download/article-file/85202

Pedroso AA, Andrade MA, Café MB, Leandro NS, Menten JF and Stringhini JH (2005). Fertility and hatchability of eggs laid in the pullet-to-breeder transition period and in the initial production period. Animal Reproduction Science, 90: 355-364. DOI: https://www.doi.org/10.1016/j.anireprosci.2005.03.001

Pokhrel N, Ben-Tal Cohen E, Genin O, Ruzal M, Sela-Donenfeld D and Cinnamon YE (2018). Effects of storage conditions on hatchability, embryonic survival and cytoarchitectural properties in broiler from young and old flocks. Poultry Science, 97: 1429-1440. DOI: https://www.doi.org/10.3382/ps/pex393

Portugal SJ, Maurer G, Thomas GH, Hauber ME, Grim T and Cassey P (2014). Nesting behaviour influences species-specific gas exchange across avian eggshells. Journal of Experimental Biology, 217: 3326-3332. DOI: https://www.doi.org/10.1242/jeb.103291

Rakib TM, Akter L, Barua SR, Nur EA, Erfan R, Islam MS Faruk AA, Faruk M, and Miazi OF (2016). Effects of age, rearing system and their interaction on phenotypic characteristics in hisex brown laying hens. Scientific Journal of Veterinary Advances, 5: 87–96. DOI: https://www.doi.org/10.14196/SJVA.V5I5.2174

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

Ratriyanto A, Firmand F, Purwanti H, and Murjoko M (2020). Nutrient digestibility, performance, and egg quality traits of quails raised in different stocking densities and ascorbic acid supplementation in a hot, tropical environment. Turkish Journal Veterinary and Animal Science (2020) 44: 350-357. DOI: https://www.doi.org/10.3906/vet-1909-1

Romanoff AL and Romanoff AJ (1949). The Avian Egg. Ed. John Wiley & Sons, (NY), (USA) pp.918. Available at: https://www.cabdirect.org/cabdirect/abstract/19490101699

Şahan U, Ipek A and Sozcu A (2014). Yolk sac fatty acid composition, yolk absorption, embryo development, and chick quality during incubation in eggs from young and old broiler breeders. Poultry Science, 93: 2069-2077. DOI: https://www.doi.org/10.3382/ps.2013-03850.

Santos TC, Murakami AE, Oliveira CAL, Moraes GV, Stefanello C, Carneiro TV, Feitosa CCG and Kaneko IN (2015). Influence of European Quail Breeders Age on Egg Quality, Incubation, Fertility and Progeny Performance. Brazilian Journal of Poultry Science, 17: 49-56. DOI: https://www.doi.org/10.1590/1516-635x170149-56

Sauveur B (1988). Reproduction des volailles et production d'œufs. 1ère édition. (Inra) Paris pp.472. Available at: https://hal.inrae.fr/hal-02858486

Seker I, Kul S and Bayraktar M (2004). Effects of parental age and hatchability egg weight of japanese quails on hatchability and chick weight. International Journal Poultry Scencei, 3: 259-265. DOI: https://www.doi.org/10.3923/ijps.2004.259.265

Smaï A, Saadi-Idouhar H, Zenia S, Haddadj F, Ameziane S, Koulougli S, Milla A, Marniche F and Doumandji S (2018). Effects of the age of breeding females on the characteristics of the eggs of Japanese quails in Algeria. Livestock Research for Rural Development,30: Article ID: 145. Available at: http://www.lrrd.org/lrrd30/8/amina30145.html

Solomon SE (2010). The eggshell: strength, structure and function. British Poultry Science; 51: 52-59. DOI: https://doi.org/10.1080/00071668.2010.497296

Suarez ME, Wilson HR, Mather FB, Wilcox CJ, and Mcpherson BN (1997). Effect of strain and age of the broiler breeder female on incubation time and chick weight Poultry Science, 76: 1029-1036. DOI: https://www.doi.org/10.1093/ps/76.7.1029.

Travel A, Nys Y and Lopes E (2010). Facteurs physiologiques et environnementaux influençant la production et la qualité de l'œuf. INRA Production Animal, 23: 155-166. DOI: https://www.doi.org/10.20870/productions-animales

Ulmer-Franco AM, Fasenko GM and O'Dea Christopher EE (2010). Hatching egg characteristics, chick quality, and broiler performance at 2 breeder flock ages and from 3 egg weights. Poultry Science, 89: 2735-2742. DOI: https://www.doi.org/10.3382/ps.2009-00403

Vieira SL and Moran Jr (1998). Eggs and chicks from broiler breeders of extremely different age. Journal of Applied Poultry Research, 7: 392-402. DOI: https://www.doi.org/10.1093/japr/7.4.372

Wilson HR, Neuman SL, Eldred AR and Mather FB (2003). Embryonic Malpositions in Broiler Chickens and Bobwhite Quail. Journal of Applied Poultry Research, 12: 14-23. DOI: https://www.doi.org/10.1093/japr/12.1.14

Yambayamba KES, and Chileshe PC (2019). Effect of Increased Photoperiod on Feed Intake, Egg Production and Egg Size in Japanese Quail (*Coturnix japonica*) Under Zambian Conditions. EC Veterinary Science, 4: 334-342. Available at: https://www.ecronicon.com/ecve/pdf/ECVE-04-00109.pdf

Zakaria AH, Miyaki T and Imai K (1983). The effect of aging on the ovarian follicular growth in laying hens. Poultry Science, 62: 670-674. DOI: https://www.doi.org/10.3382/ps.0620670

Zita L, Ledvinka Z and Klesalova L (2013). The effect of the age of Japanese quails on certain egg quality traits and their relationships. Veterinarski Arhiv, 83: 223-232. Available at: https://core.ac.uk/download/pdf/14463833.pdf

To cite this paper: Berrama Z, SouamesS, Merati R, Korteby HM, Chirane MS, Negab N, Hettab K, Idris H, Morzouglal N, and Temim S (2023). Effects of Laying Cycle Periods on Egg Quality, Egg Chemical Composition, and Reproductive Performance of Japanese Quail Breeders Reared in Northern Algeria. *World Vet. J.*, 11 (3): 439-447.

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 448-455, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB)

Febri Ramadhan Batista, Juni Triastuti*, and Kustiawan Tri Pursetyo

Department of Marine Science, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, East Java, Indonesia

*Corresponding author's Email: juni.triastuti@fpk.unair.ac.id; ORCID: 0000-0003-4378-1946

ORIGINAL ARTICLE
pii: S232245682100057-11
Received: 10 July 2021
Accepted: 29 August 2021

ABSTRACT

The decrease in the number of lands for aquaculture will cause the freshwater fish aquaculture, especially tilapia is reared. As one of the efforts to survive, the breeders use the coastal area to anticipate, so the freshwater fish can adapt to the sea waters. Heavy metal pollution near the coasts (or in the coastal waters) has great potential impacts on the environment. The present study aimed to find out the effect of Lead (Pb, heavy-metal) exposure on the Jatimbulan Juvenile tilapia with the different salinity towards histopathology description of the gill, intestine, and the skin tissues. The method used was an experimental method with a completely randomized design using six treatments and three times repetition. The observed parameters included the changes in histopathology description of gill, intestine, and skin organs of Jatimbulan juvenile tilapia. Kruskal-Wallis scoring method was used for data analysis and was continued by Mann-Whitney. The result of the current study showed the effect of salinity towards histopathology description of gill, intestine, and skin of Jatimbulan juvenile tilapia. The effect of changes was proven with the histopathology description in the form of the damage of edema, hyperplasia, and necrosis on the gill tissue; the damage of edema, atrophy, and necrosis on the gill tissue as well as the damage of edema, atrophy, hemorrhagic and necrosis on the gill tissue. From the result of the current study which has been conducted, it can be concluded that the heavy-metal exposure by lead was 0.03 ppm on the salinity of 10 ppt and 20 ppt. given the significant effect on the histopathology description of gill, intestine, and skin of Jatimbulan juvenile tilapia.

Keywords: Histopathology, Lead, Metal, Oreochromis niloticus, Salinity

INTRODUCTION

One of the superior commodities in fisheries aquaculture activities was tilapia aquaculture (*Oreochromis niloticus*). Tilapia has some superiority over other fish for some features; for example, it has rapid growth, is easy to breed, and is efficient for additional feeding, thus it can be a favorite commodity in an aquaculture activity. Along with the increase in the number of tilapia breeders, the need for lands as the aquaculture area highly increases. Based on the data, the potential pond area in East Java in 2014 was decreased by 64% from the previous year to 129518 hectares (FAO, 2005). The decrease in the number of lands for aquaculture threatens the freshwater fish aquaculture business, especially tilapia. As an effort to survive, the breeders utilize the coastal areas to anticipate whether freshwater fish can be adapted to sea waters (Tuiyo, 2016).

Shrimp and milkfish ponds can be used for tilapia aquaculture activities because it has a euryhaline characteristic, thus tilapia can be used as a substitution in the shrimp or milkfish pond that is less productive (Hosseini Aghuzbeni et al., 2017). Nevertheless, coastal waters have a high potential for environmental impacts. The environmental impact meant is heavy metal pollution due to human activities. Heavy metal pollution in coastal areas has a high potential for aquatic toxicity due to industrial waste discarded into the river. One of the heavy metals usually found is Lead (Pb). Water sources, the most vulnerable to the Lead threats, are the rivers which are usually close to the residential areas and the agriculture, and residue of industrial waste that is not favorable and sufficient (Ritonga and Yunasfi, 2014).

Lead (Pb) is a dangerous heavy metal because it is indestructible (non-degradable) for living organisms, and it can accumulate into the environment. Lead settles to the bottom of waters to form complex compounds together with organic and inorganic materials. The Lead continues to enter the tissues of living organs through the respiratory tract, digestion, and skin. Lead dissolved in fat will penetrate the cell membrane, thus the Lead ions will pile up in the cells and organs (Jaishankar et al., 2014; Okereafor et al., 2020).

The presence of salinity in waters has also an impact on the level of Lead toxicity. Salinity is the total volume of K +, Na +, Mg2 +, NO3-, Ca2 +, SO_4 2-, Cl-, and HCO3- in the water. If there is a decrease in salinity due to the desalination process, it will cause an increase in the toxic power of heavy metals, and will cause a greater level of bioaccumulation of heavy metals (Okereafor et al., 2020). Therefore, it is required to conduct a study to determine the

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.,* 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

effect of Lead as a heavy-metal exposure on Jatimbulan juvenile tilapia with different salinity for organ damage, especially in the gill, intestine, and skin.

MATERIALS AND METHODS

The present study was conducted at the Faculty of Fisheries and Marine, Universitas Airlangga (Surabaya, Indonesia) according to the ethical rules of Universitas Airlangga for animal studies. The materials used in this study consisted of Jatimbulan Juvenile tilapia obtained from the Technical Implementation Unit for Freshwater Aquaculture Development, Umbulan, Pasuruan, Indonesia. The selected Jatimbulan Juvenile tilapia was a juvenile which had a body length of 3-7 cm and weighs ± 13 grams as many as 200 fishes. The Lead used was the Lead (II) Nitrate, and the salinity of the water was 0 ppt, 10 ppt, and 20 ppt, distilled water, and nitric acid.

The current study was experimental using a complete randomized design method. Three treatments and six times repetition were used in this study, thus there were 18 test units. Data collection technique was carried out by direct observation, namely by observing directly the behavior of tilapia juvenile after being exposed to Lead (heavy-metal), and histological observation was conducted to determine the description of gill, intestine, and skin tissue due to the effect of salinity on heavy metal exposure. In this study, there were three treatments with six times repetition, namely A (Salinity 0 ppt and using 0.03 mg/l of Pb), B (Salinity 10 ppt and using 0.03 mg/l of Pb), and C (Salinity 20 ppt and using 0.03 mg/l of Pb).

The dose of lead used in the present study was 0.03 mg/l by as suggested by Tarigan and Rozak (2003). The Lead nitrate was weighed, then mixed using distilled water and 60% nitric acid (HNO_3), and after that stored in a bottle; thus the Lead dissolved easily in the water. The water was mixed with the Lead, then aerated, and waited for 2 hours to be mixed properly because the Lead could cause poisoning effects on the fish after the fish has been exposed to the Lead for two hours (Musthapia and Sunarno, 2006). Furthermore, water salinity was made using seawater which was diluted with fresh water from *Perusahaan Daerah Air Minum* (PDAM, Regional Water Company) to obtain the required salinity concentration. The experiment fish that would be used in this study was previously acclimatized so that the fish would not experience stress with the new environment (Rosdianasari and Perbedaan, 2015).

The next stages carried out in this study were measuring the water quality, calculating the survival rates, also making and observing histopathology preparations. The main observed parameter was the histopathology description of the gill, intestine, and the skin of the Jatimbulan tilapia which was exposed to the Lead (heavy-metal) at a different salinity. Supporting the observed parameter was water quality and survival rate. The water quality for observed parameters included pH, temperature, dissolved oxygen, and salinity. The present study used salinity as the independent variable, dose of Lead as a control variable, and the histopathology description of the gill, intestine, and the skin as a dependent variable.

The result in the form of scoring data from the histopathology was then analyzed using analysis of Kruskal-Wallis one-way ranking variant to determine the differences between samples (Dalgaard, 2008), marking real population differences or the differences (p value less than 0.05) due to accidental variation between random samples from the same population. Furthermore, the Mann-Whitney test was carried out to examine whether two independent groups have been drawn from the same population.

RESULTS

The result of the survival rate of Jatimbulan juvenile tilapia *(Oreochromis niloticus)* after exposure to the Lead heavy metal showed that there was no significant difference in each treatment (Table 1). The lowest survival rate was found in treatment B (10 ppt + 0.03 mg/l Pb), while the highest survival rate was found in the control treatment (0 ppt + 0 mg/l Pb). The observation result of gill tissue damage on the Jatimbulan Juvenile tilapia exposed to the Lead at different salinity for 30 days can be seen in Table 2.

Based on the data above, the most severe value of edema was found in treatment A (0 ppt + 0.03 mg/l Pb) with the high level of damage which was 2.6, while the small value of damage of edema was found in the control with the low level of damage (that was 1.4). Considering hyperplasia, the highest damage value was found in treatment A (0 ppt + 0.03 mg/l Pb) with the medium level of damage which was 1.5, while the smallest average of hyperplasia was found in the control treatment with the normal level of damage which was 0.5. While the highest damage value of necrosis was found in treatment A (0 ppt + 0.03 mg/l Pb) with the medium level of damage which was 1.7, the smallest damage value of necrosis was found in the treatment C (20 ppt + 0.03 mg/l Pb) with the normal level of damage which was 0.1.

Histopathological description of the tilapia's gill organ exposed to the Lead (heavy-metal) showed the change in gill tissue structure in Figure 1. In the gill of the tilapia fish, there was a report of edema, hyperplasia, and necrosis. Edema in histopathology was characterized by the rounded shape, and there was liquid in the circle. Hyperplasia was characterized by the finding of thickening or enlargement in an organ. In the gill, it could be recognized by the

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.*, 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

enlargement of the secondary lamella. Meanwhile, the damage of necrosis occurred due to excessive hyperplasia, thus the gill tissue cells were no longer intact. The observation results of the intestinal tissue damage on the Jatimbulan Juvenile tilapia exposed to the Lead at different salinity for 30 days can be seen in Table 3.

Table 1. Survival rate of Jatimbulan Juvenile tilapia exposed to the lead at different salinity

Treatments	Repetition					means (%) ± SD
	1	2	3	4	5	
Control (0 ppt + 0 mg/1 Pb)	90	100	90	100	100	96 ± 4.89
A (10 ppt + 0.03 mg/1 Pb)	100	100	80	80	70	86 ± 12
B (20 ppt + 0.03 mg/1 Pb)	30	100	100	100	90	84 ± 27.27
C (30 ppt + 0.03 mg/1 Pb)	90	100	70	100	100	92 ± 11.66

*SD: Standard error of the means

Table 2. The histopathology damage of gill in each treatment

Treatment	Edema	*Hyperplasia*	Necrosis
Control (0 ppt + 0 mg/1 Pb)	1.4 ± 0.73	0.5 ± 0.70	0.4 ± 0.51
A (10 ppt + 0.03 mg/1 Pb)	2.6 ± 0.51	1.5 ± 0.70	1.7 ± 0.67
B (20 ppt + 0.03 mg/1 Pb)	2.1 ± 0.87	1.3 ± 0.48	0.6 ± 0.69
C (30 ppt + 0.03 mg/1 Pb)	1.6 ± 0.96	1.4 ± 0.84	0.1 ± 0.31

*p < 0.05, Normal: 0-0.75, Low: 0.75-1.5, Medium: 1.5-2.25, High: 2.25-3

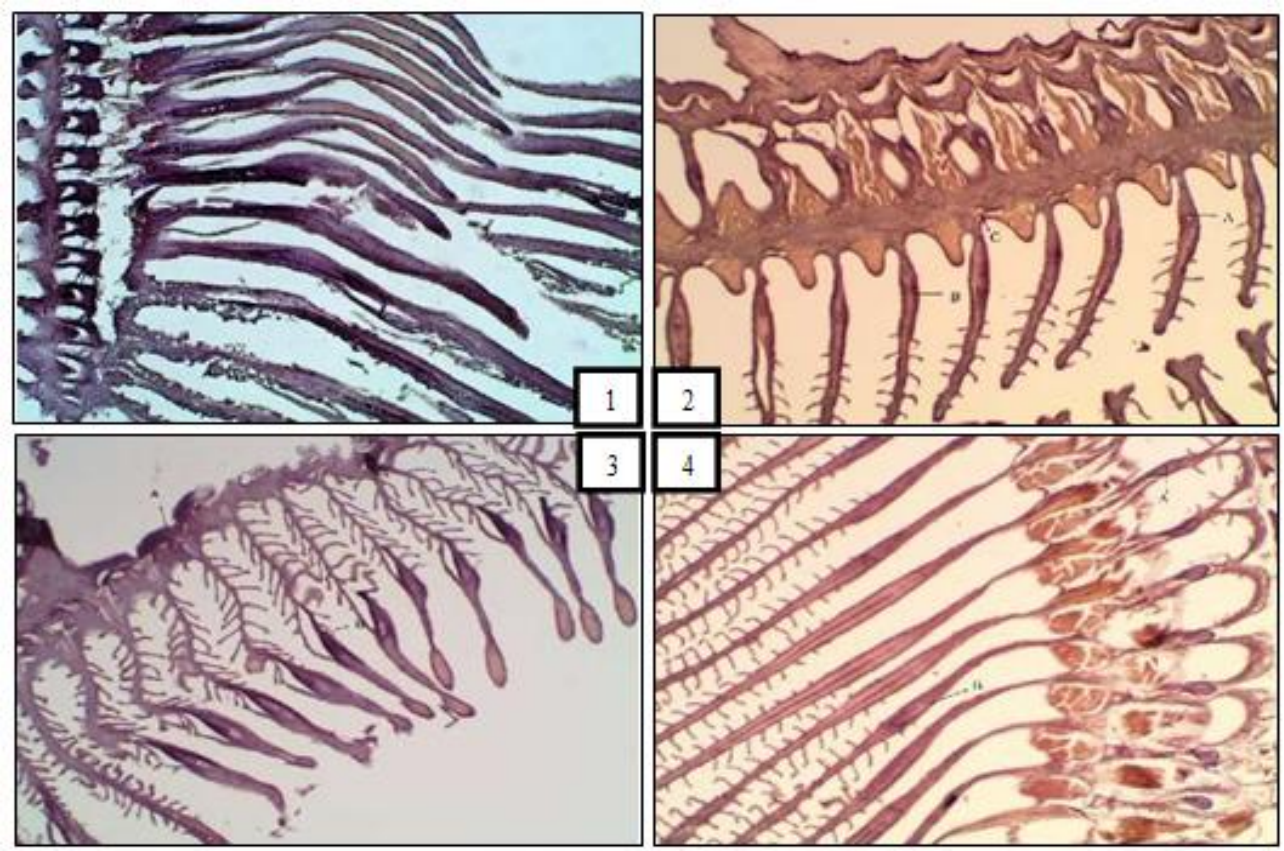


Figure 1. The Histopathologic Description of the gill of Jatimbulan Juvenile Tilapia in the magnification of 400×. **1:** Normal gill, **2:** Treatment A (0 ppt + 0.03 mg/l Pb), **3:** Treatment B (10 ppt + 0.03 mg/l Pb), **4:** Treatment C (20 ppt + 0.03 mg/l Pb); A: Edema, B: Hyperplasia, C: Necrosis, Pb: lead

Table 3. The histopathological damage of the intestine in each treatment

Treatment	Edema	Atrophy	Necrosis
Control (0 ppt + 0 mg/1 Pb)	1.1 ± 0.73	0.4 ± 0.51	1 ± 0.81
A (10 ppt + 0.03 mg/1 Pb)	2.1 ± 0.99	0.7 ± 0.67	0.9 ± 0.99
B (20 ppt + 0.03 mg/1 Pb)	1.6 ± 0.96	0.4 ± 0.51	1.3 ± 0.94
C (30 ppt + 0.03 mg/1 Pb)	1.5 ± 0.70	0.4 ± 0.69	0.9 ± 0.99

p < 0.05, Normal: 0-0.75, Low: 0.75-1.5, Medium: 1.5-2.25, High: 2.25-3

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.*, 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

Based on the data above, the most damage value of edema was found in treatment A (0 ppt + 0.03 mg/l Pb) with the medium level of damage which was 2.1, while the smallest damage value of edema was found in the control treatment with the low level of damage which was 1.1. The most damage value of atrophy was found in treatment A (0 ppt + 0.03 mg/l Pb) with the low damage rate of 0.7, while the average atrophy damage in the control treatment B (10 ppt + 0.03 mg/l Pb) and C (20 ppt + 0.03 mg/l Pb) had the same value of 0.4, and fallen into a normal category. Furthermore, the most damage value of necrosis was found in the treatment B (10 ppt + 0.03 mg/l Pb) with the low level of damage which was 1.3, while the smallest damage value of necrosis was found in treatment A (0 ppt + 0.03 mg/l Pb) with the low level of damage which was 0.9.

The histopathologic description of the intestinal organs of tilapia exposed to the Lead (heavy-metal) revealed the presence of edema, atrophy, and necrosis (Figure 2). Edema was characterized by its rounded shape in an organ due to the liquid entering the cell tissue. Atrophy was characterized by shrinking cells. Meanwhile, the necrotic damage could be found with the characteristic of cells which was blurred or lost because of the inability of these cells to absorb the color when histology staining was done.

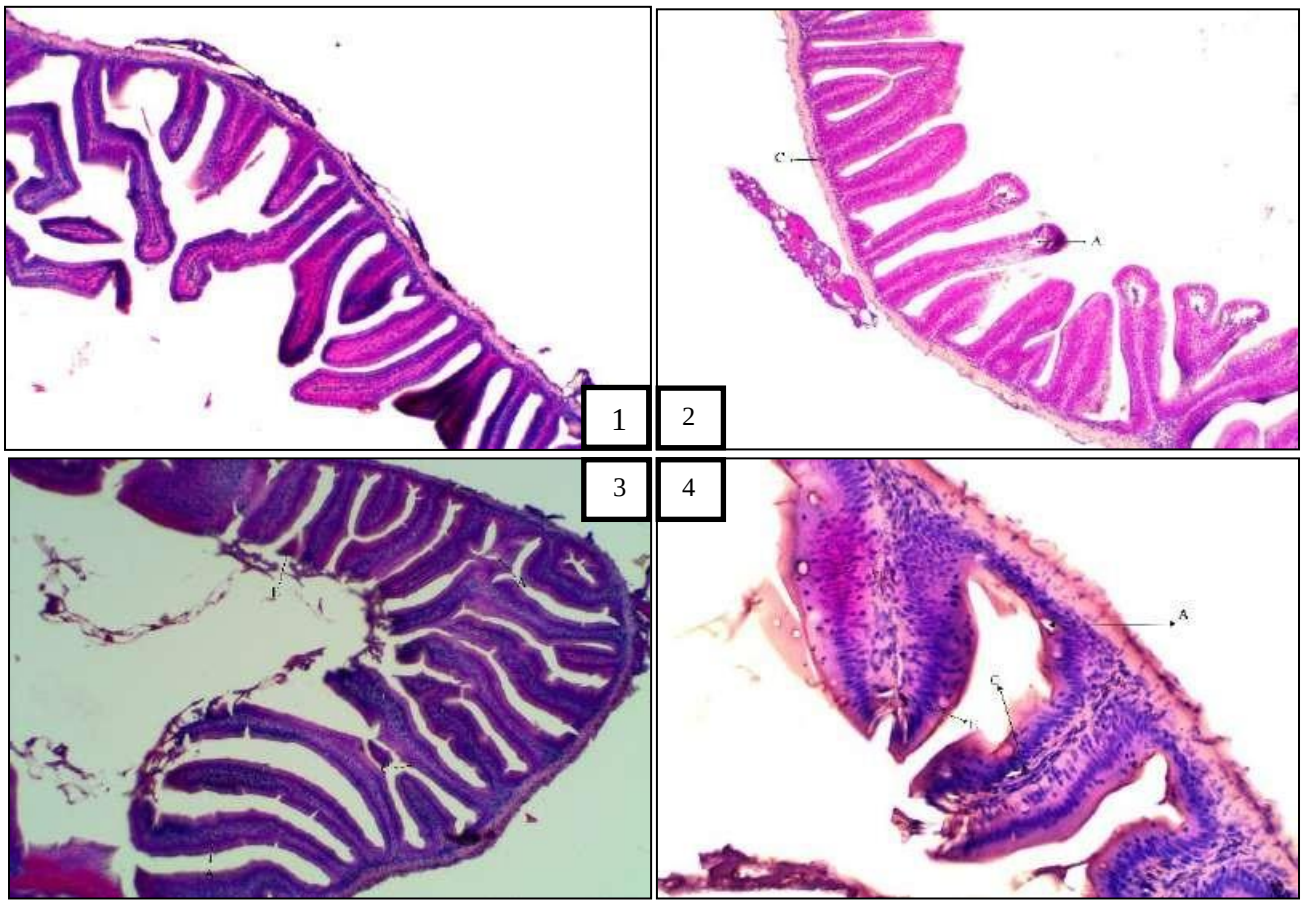


Figure 2. The Histopathologic Description of the intestinal Organs of Jatim Bulan Juvenile Tilapia in the magnification of 100×. **1:** Normal Intestine, **2:** Treatment A (0 ppt + 0.03 mg/l Pb), **3:** Treatment B (10 ppt + 0.03 mg/l Pb), **4:** Treatment C (20 ppt + 0.03 mg/l Pb). A: Edema, B: Hyperplasia, C: Necrosis, D: Hemorrhage, E: Atrophy, Pb: lead

The result of observation of the skin tissue damage on the Jatimbulan Juvenile tilapia exposed to the Lead at different salinity for 30 days can be seen in Table 4 below.

Based on the data above, the highest damage value of edema was found in treatment A (0 ppt + 0.03 mg/l Pb) with the low level of 1.4, while the smallest edema damage was found in the treatment B (10 ppt + 0.03 mg/l Pb) with the normal level of damage which was 0.2. The most hemorrhagic and atrophic damage values were found in the control treatment with the normal level of damage which was 0.6. The most damage value of necrosis was found in treatment A (20 ppt + 0.03 mg/l Pb) with the medium damage level of 1.5, while the smallest damage value of necrosis was found in treatment C (20 ppt + 0.03 mg/l Pb) with the low level of damage which was 0.9.

Histopathologic description of the tilapia's skin organ exposed to the heavy-metal Lead found the presence of edema, atrophy, hemorrhage, and necrosis (Figure 3). Edema was characterized by swelling of cells or excessive accumulation of fluid in the skin tissue. Atrophic damage was characterized by the reduced size of the skin tissue cells. Hemorrhagic damage was characterized by the release of cells from the blood vessels in the skin tissue. Meanwhile, the damage of necrosis was found by the death of skin tissue cells because of the inability to absorb the color when the histology staining was being done.

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.*, 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

Table 4. The histopathological damage of the skin in each treatment

Treatment	Edema	Atrophy	Hemorrhage	Necrosis
Control (0 ppt + 0 mg/1 Pb)	0.9 ± 0,87	0.6 ± 0.82	0.6 ± 0.69	1.1 ± 0.73
A (10 ppt + 0.03 mg/1 Pb)	1.4 ± 0,69	0.5 ± 0.91	0.5 ± 0.70	1.5 ± 0.52
B (20 ppt + 0.03 mg/1 Pb)	0.2 ± 0,94	0.4 ± 0.69	0.4 ± 0.51	1.3 ± 0.82
C (30 ppt + 0.03 mg/1 Pb)	1.1 ± 0.99	0.4 ± 0.51	0.4 ± 0.51	0.9 ± 0.96

$p < 0.05$, Normal: 0-0.75, Low: 0.75-1.5, Medium: 1.5-2.25, High: 2.25-3

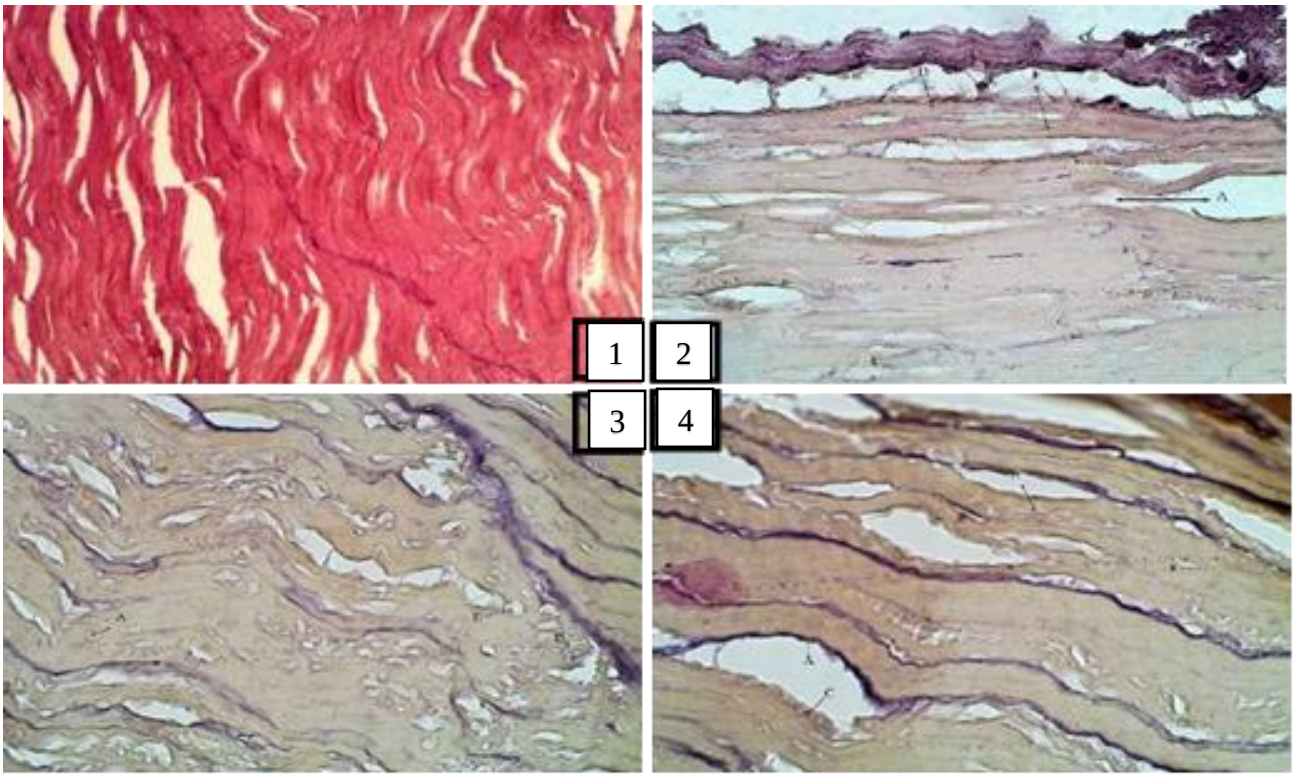


Figure 3. The Histopathologic Description of the skin organ of Jatim Bulan Juvenile Tilapia in the magnification of 400×. **1:** Normal Skin, **2:** Treatment A (0 ppt + 0.03 mg/l Pb), **3:** Treatment B (10 ppt + 0.03 mg/l Pb), **4:** Treatment C (20 ppt + 0.03 mg/l Pb), A: Edema, B: Hyperplasia, C: Necrosis, D: Hemorrhage, E: Atrophy, Pb: lead

The water quality observed in the study included temperature (^{o}C), pH, ammonia (mg/l), and salinity (ppt). The result of the observation of water quality parameters can be seen in Table 5. Based on the data above, it could be seen that the temperature in each treatment had the same range of values among treatments which ranged from 27.5-33℃. In the observation of pH during the maintenance, it was known that the pH among treatments was not significant. In the control treatment and treatment A (0 ppt + 0.03 mg/l Pb), the obtained pH value was the same ranging from 7.7 to 9.1 in the treatment B (10 ppt + 0.03 mg/l Pb), and the lower limit value of the pH was slightly higher than the control treatment and the treatment A (0 ppt + 0.03 mg/l Pb), however the highest value of pH in the treatment B (10 ppt + 0.03 mg/l Pb) was the same as the control treatment (0 ppt) and the treatment A (0 ppt + 0.03 mg/l Pb) which was 7.8-9.1. In the treatment C (20 ppt + 0.03 mg/l Pb), the pH value was higher than the control treatment (0 ppt), A (0 ppt + 0.03 mg/l Pb) and B (10 ppt + 0.03 mg/l Pb), however the pH value from the treatment C (20 ppt + 0.03 mg / l Pb) was not much different from other treatments which was 7.9-9.3. Considering the result of observation of salinity during the maintenance, it was known that the salinity value has increased. It was due to the evaporation during the study. The increase occurred in the treatment B (10 ppt + 0.03 mg/l Pb) and C (20 ppt + 0.03 mg / l Pb). The increase in salinity in both treatments; B (10 ppt + 0.03 mg/l Pb) was 10-12 ppt, treatment C (20 ppt + 0.03 mg / l Pb) was 20-22. From the result of observation of ammonia during the maintenance, it was known that the ammonia value in each treatment was the same as 0-0.5 mg/l, there was no difference at all amongst the treatments.

Table 5. The observation result of the water quality

Treatment / Water Quality Parameter	Control	A	B	C
Temperature (°C)	27.5-33	27.5-33	27.5-33	27.5-33
pH	7.7-9.1	7.7-9.1	7.8-9.1	7.9-9.3
Salinity (ppt)	0	0	10-12	20-22
Ammonia (mg/l)	0-0.5	0-0.05	0-0.05	0-0.05

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.*, 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

DISCUSSION

The results of histopathological examination of gills, intestines, and skin organs in the present study showed the damage caused by exposure to heavy-metal lead with different salinity. The damage in the gill organ included edema, hyperplasia, and necrosis. The initial damage that occurred in the gill was found in the presence of edema, then developed into hyperplasia and necrosis. This could be seen from the histopathologic changes in Jatimbulan Juvenile tilapia during the exposure to the heavy-metal lead at a different salinity. In the intestinal tissue, the changes were found in histopathology in the form of edema and atrophy which then were developed into necrosis. Meanwhile, in the skin tissue, the presence of edema, atrophy, hemorrhage, and necrosis was also found. These conditions indicated that absorption and bioaccumulation of the heavy metal could be stored well in the tissues of skin, gill, stomach, muscles, intestine, liver, brain, kidney, and reproductive organs (Chen and Chen, 2001; Bawuro et al., 2018).

Exposure to the heavy metal lead in treatment A (0 ppt + 0.03 mg/l Pb) had the highest damage effect on the gill organs when being compared with the skin and intestine, especially for edema and necrosis damage. This was because the reaction of the Lead in low salinity resulted in the lead toxicity level became higher (Sullivan, 1977). Besides, the gill was also the first organ to filter water that would enter the body of the fish. Gills were also the organs which were very sensitive to the effect of metal toxicity which would disrupt the normal function of metalloenzyme and metabolism of cells (Darmono , 2006). Therefore, if there was heavy metal in the water sources, the gill would react to the heavy metal.

Edema damage was also found in the intestinal tissue and skin. The highest edema damage to the intestinal tissue and skin occurred when treatment A (0 ppt + 0.03 mg/l Pb) was given. However, from the three tissues, the edema damage was the highest which occurred in the gill with treatment A (0 ppt + 0.03 mg/l Pb) which was 2.6 ± 0.51. Edema was a swelling of cells caused by the entering of the lead into the tissue, thus it resulted in an excessive accumulation of fluid, and was considered as the initial stage of histological damage. The presence of the lead diffused into the gill cells and bounded to the plasma membrane of the lamella epithelial gill cells in synergy, and it caused the lack of Adenosine triphosphate (ATP). The low levels of oxygen in the blood could cause the cells to have insufficient ATP. The low amount of ATP in the cells caused the accumulation of Na + in the cells. This was due to the lack of energy to secrete Na + ions outside the cell. A large amount of Na + could cause osmotic pressure changes, thus water, Ca2 + and Na + could enter and cause swelling or edema (Catron et al., 2015). The high damage value of edema in the gill tissue, intestine, and skin tissue in treatment A (0ppt + 0.03mg/l Pb) with the low salinity value proven that the lower salinity would increase the level of toxicity in the heavy-metal lead toxicity.

The presence of edema in the gill, intestine, and skin was the initial stage of tissue damage and caused other histological damage. As in the gill, hyperplasia and necrosis were found, atrophy and necrosis were found in the intestine, and atrophy, finally hemorrhage, and necrosis were also found in the skin tissue. Excessive edema could cause hyperplasia due to red blood cells coming out of the capillaries, and the cells would be separated from the supporting tissue. Hyperplasia occurred at the lower level of irritation and was usually accompanied by an increase in the number of mucous cells at the base of the lamella, thus resulting in a lamella fusion. The presence of hyperplasia indicated a mechanism of adaptation of gill tissue to protect the tissue from irritants such as heavy metals. The mechanism was as protection and inhibitor of the gill function as a respiratory system because of the process of epithelial cells' cleavage, and excessive chloride cells that disrupted the respiratory system. In this study, the incidence of hyperplasia was found in almost every treatment on the gill tissue. An excessive lead in the gill tissue of Jatimbulan Juvenile tilapia also caused necrosis damage in the gill tissue. The highest necrosis damage was found in treatment A (0 ppt + 0.03 mg/l Pb). This could occur because of the concentration of heavy-metal lead in the water causing continuous absorption into the gill tissue, and causing necrosis (Mulyani et al., 2014).

The lower value of the damage was found in the intestinal and skin tissues. Lead exposure to intestinal tissue caused the damage of edema, atrophy, and necrosis. The intestine has experienced the damage because lead ion was bounded to the food. The results of intestinal histopathology after exposure to the heavy-metal lead did not show a significant difference in each treatment. This occurred because lead exposure entered the intestine through the water and food absorption, where food containing lead was more easily excreted from the body of the fish. Metal absorption through the respiration was usually quite large, while in the digestive tract of fish which most of the metal penetration came from food such as small fishes, aquatic plants, and plankton that have been bound to lead metal ion may not be absorbed by fish and excreted through feces (Darmono , 2006; Kasumyan, 2018).

In the skin tissue which was the outer part of the fish, and at the same time served as a protection against physical, chemical, and biological changes, when it was exposed to the heavy metal lead, it caused damages to edema, atrophy, hemorrhage, and necrosis. The skin experienced damages due to lead exposure that entered through the diffusion process. Histopathological results of the skin observation after the exposure to the heavy-metal lead did not show a significant difference in each treatment. The presence of lead in the skin tissue was able to damage to the necrosis stage or cell death, although Lead was able to cause necrosis, but only with the low value of the damage. This was because the skin had a mechanism for detoxification and excretion so that the toxic effect could still be tolerated (Yulaipi et al.,

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.*, 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

2013). Lead entered the skin organ through the circulatory system, but it took a long time in the distribution process, so only a little Lead and the damage of the skin was found.

The highest mortality rate in Jatimbulan Juvenile tilapia due to exposure to the heavy-metal Lead occurred after getting the treatment B (10 ppt + 0.03 mg/l Pb) with a mortality percentage of 84%, while the highest survival rate occurred after obtaining the control treatment (0 ppt) with a percentage of 96%. This happened because of the efficiency of fish in the utilization of feed, so it did not disrupt the body's metabolic system of fish. The survival rate of fish was also influenced by the condition of water quality which included temperature, pH, salinity, and ammonia. In the result, it could be seen that the parameter of temperature during the study showed an average value of 27.5^{o}C to 33^{o}C. The temperature was in accordance with the condition of the Jatimbulan tilapia fish habitat which was able to grow optimally at the temperature of 14^{o}C to 38^{o}C (FAO, 2018). The result of pH parameter during the study showed an average value ranging from 7.7 to 9.3, which was higher than the optimal PH value which was good for tilapia growth (PH 6 - 8.5; FAO, 2018). Nevertheless, the PH condition did not affect the condition of fish, because Jatimbulan tilapia had a high tolerance to PH. Meanwhile, the results of the ammonia parameter showed the value of 0-0.5, which meant that the waters had a normal ammonia level.

Salinity was also one of the factors highly considered during the study. Salinity is the weight of inorganic halogen salts in gram dissolved in 1kg of water. In this study, the salinity influenced the description of the damage in the gill, intestine, and skin of Jatimbulan Juvenile tilapia. The decrease in salinity caused an increase in the toxicity of heavy metals. The salinity influenced the toxic power of Lead in the body of tilapia which was when the tilapia was placed in the salinity water, then the osmoregulation process of fish tended to maintain the fluid in the body against the changes in salinity. If it was found that Lead has exceeded the limit and could not be tolerated by the body of the fish itself, it would be excreted the xenobiotic substances. The xenobiotic substances in the body of the fish would stimulate the fish to take the physiological resistance to minimize the impact of the poison caused (Yulaipi et al., 2013; Kasumyan, 2018).

CONCLUSION

From the results of the study which has been conducted, it can be concluded that the exposure of heavy-metal Lead was 0.03 ppm at the salinity of 10 ppt and 20 ppt, giving a significant effect on the histopathologic description of the gill, intestine, and the skin of Jatimbulan Juvenile tilapia. The effect of these changes was known with the presence of histopathologic description in the damage of edema, hyperplasia, and necrosis in the gill tissue, the damage of edema, atrophy, and necrosis in the intestinal tissue, and the damage of edema, atrophy, hemorrhage, and necrosis in the skin tissue.

REFERENCES

Bawuro AA, Voegborlo RB, and Adimado AA (2018). Bioaccumulation of Heavy Metals in Some Tissues of Fish in Lake Geriyo, Adamawa State, Nigeria. Journal of Environmental and Public Health, p. 1854892. DOI: https://www.doi.org/10.1155/2018/1854892

Chen YC, and Chen MH (2001). Heavy metal concentrations in nine species of fishes caught in coastal waters off Ann-Ping, SW Taiwan. Journal of Food and Drug Analysis, 9: 107-114. Available at: http://www2.nsysu.edu.tw/mbr/Web/download/P2001-1AnnpingFish.pdf

Cotran RS, Kumar V, and Robbins SL (2015). Robbins y Cotran. Patología estructural y functional 9th Edition. Elsevier, Netherlands. Available at: https://www.elsevier.com/books/robbins-y-cotran-patologia-estructural-yfuncional/kumar/978-84-9022-878-4

Dalgaard P (2008). Analysis of variance and the Kruskal–Wallis test. In: Introductory Statistics with R. Statistics and Computing. Springer, New York, NY. DOI: https://www.doi.org/10.1007/978-0-387-79054-1_7

Darmono LH (2006). Pencemaran Hubungannya dengan Toksikologi Senyawa Logam [Pollution Relation to Toxicology of Metal Compounds]. UI Press, Jakarta.

FAO (2005). Aquaculture production, 2004. Year book of Fishery Statistics - Vol.96/2. Food and Agriculture organization of the United Nations, Rome, Italy. Available at: http://www.fao.org/3/a-y8725t.pdf

FAO (2018). The biology and culture status worldwide of tilapia. Food and Agriculture organization of the United Nations, Rome, Italy. Available at: http://www.fao.org/fi/static-media/MeetingDocuments/TiLV/p2.pdf

Hosseini Aghuzbeni SH, Hajirezaee S, Matinfar A, Khara H, and Ghobadi M (2017). A preliminary study on polyculture of western white shrimp (Litopenaeus vannamei) with mullet (Mugil cephalus): an assessment of water quality, growth parameters, feed intake efficiency and survival. Journal of Applied Animal Research, 45:247-251. DOI: https://www.doi.org/10.1080/09712119.2016.1150845

Jaishankar M, Tseten T, Anbalagan N, Mathew BB, and Beeregowda KN (2014). Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary Toxicology, 7(2): 60-72. DOI: https://www.doi.org/10.2478/intox-2014-0009

Kasumyan AO (2018). The taste system in fishes and the effects of environmental variables. Journal of Fish Biology, 95: 155-178. DOI: https://www.doi.org/10.1111/jfb.13940

Musthapia I, and Sunarno MTD (2006). Dampak polutan timbal pada ikan dan manusia [Impact of lead pollutants on fish and humans] . In: Seminar Nasional Limnologi Widya Graha LIPI Jakarta. 2006. p. 1-12.

Okereafor U, Makhatha M, Mekuto L, Uche-Okereafor N, Sebola T, and Mavumengwana V (2020). Toxic metal implications on agricultural soils, plants, animals, aquatic life and human health. International Journal of Environmental Research and Public Health, 17(7): 2204. DOI: https://www.doi.org/10.3390/ijerph17072204

Ritonga R, and Yunasfi MD (2014). tingkat kandungan logam berat timbal (Pb) dan tembaga (Cu) di sungai Belawan kecamatan Medan Sunggal kota Medan. Journal of Aquacoastmarine, 5: 1-10. Available at: https://jurnal.usu.ac.id/index.php/aquacoastmarine/article/view/8893

Rosdianasari S, and Perbedaan Salinitas (2015). Media terhadap Efisiensi Pemanfaatan Pakan Benih Ikan Nila Gift (Oreochromis sp) [Media on the Efficiency of Feed Utilization of Gift Tilapia Seeds (Oreochromis sp)]. Jurnal PENA Pena J Ilmu Pengetah Dan Teknologi, 25: 1-6. DOI: http://dx.doi.org/10.31941/jurnalpena.v25i2.96

Sullivan JK (1977). Effects of salinty and temperature on the acute toxicity of cadmium to the estuarine crab Paragrapsus gaimardii (Milne Edwards). Australian Journal of Marine and Freshwater Research, 28(6): 739-743. DOI: https://www.doi.org/10.1071/MF9770739

Tarigan Z, and Rozak A (2003). Kandungan logam berat Pb, Cd, Cu, Zn Dan Ni dalam air laut dan sedimen di muara Sungai Membramo, Papua dalam kaitannya dengan kepentingan budidaya perikanan) [The content of heavy metals Pb, Cd, Cu, Zn and Ni in seawater and sediment at the mouth of the Membramo River, Papua in relation to the interests of aquaculture]. Makara Journal of Science, 7: 119-127. DOI: https://www.doi.org/10.7454/mss.v7i3.368

Rahim T, and Tuiyo R (2016). Pengaruh Salinitas Berbeda terhadap Pertumbuhan dan Tingkat Kelangsungan Hidup Benih Ikan Nila Merah (Oreochromis Niloticus) di Balai Benih Ikan Kota Gorontalo [Effect of Different Salinity on Growth and Survival Rate of Red Tilapia (Oreochromis Niloticus) Seeds at the Fish Seed Center of Gorontalo City.]. NIKE Jurnal Ilmiah Perikanan dan Kelautan, 3(1): 39-43. Available at: https://repository.ung.ac.id/get/karyailmiah/590/PENGARUH-SALINITAS-BERBEDA-TERHADAP-PERTUMBUHAN-DAN-TINGKAT-KELANGSUNGAN-HIDUP-BENIH-IKAN-NILA-MERAH-OREOCHROMIS-NILOTICUS-DI-BALAI-BENIH-IKAN-KOTA-GORONTALO.pdf

Yulaipi S, and Aunurohim A (2013). Bioakumulasi Logam Berat Timbal (Pb) dan Hubungannya dengan Laju Pertumbuhan Ikan Mujair (Oreochromis mossambicus) [Bioaccumulation of Heavy Metal Lead (Pb) and Its Relationship with Growth Rate of Tilapia Fish (Oreochromis mossambicus)]. Jurnal Sains dan Seni ITS, 2(2): E166-170. DOI: http://dx.doi.org/10.12962/j23373520.v2i2.3965

To cite this paper: Batista FR, Triastuti J, and Pursetyo KT (2021). The Role of Salinity in Histopathology Description of Jatim Bulan Tilapia Juvenile (*Oreochromis niloticus*) Exposed by Lead (PB). *World Vet. J.*, 11 (3): 448-455. DOI: https://dx.doi.org/10.54203/scil.2021.wvj57

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 456-461, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

In Vitro Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*

Tanawadee Mekvimol[1], Chayanit Chaipunna[1], Gannika Poonthong[1], and Natapol Pumipuntu[1,2*]

[1]*One Health Research Unit, Faculty of Veterinary Sciences, Mahasarakham University, Maha Sarakham, Thailand*
[2]*Faculty of Veterinary Sciences, Mahasarakham University, Maha Sarakham, Thailand*

*Corresponding author's Email: film.natapol@gmail.com; ORCID: 0000-0002-0612-9847

ORIGINAL ARTICLE
pii: S232245682100058-11
Received: 13 July 2021
Accepted: 01 September 2021

ABSTRACT

Bacterial infection is a major global health concern. One of the critical problems is the widespread of antimicrobial-resistant bacteria from inappropriate and prolonged use of antimicrobial agents in both humans and animals. Plant extracts might afford the chance to replace antibiotic drugs and reduce the emerging of antimicrobial-resistant bacteria. This study aimed to examine the antibiotic activity of ethanolic crude extracts of some Thai medicinal plants grouping in their parts as whole onions of red shallot (*Allium ascalonicum*), petals of marigold (*Tagetes erecta*), mulberry leaves, and root barks (*Morus indica*) to inhibit the growth of *Streptococcus pyogenes*. The antibiotic activities of the crude extract of three Thai medicinal plants using absolute ethanol were trialed against *Streptococcus pyogenes* using the disk diffusion method. Erythromycin and Ceftriaxone discs were chosen to be positive control standards as the representative of antibiotic drugs. Each dried plant extracts was prepared to test the inhibition with a concentration at 25, 50, and 75 mg/mL stock solution. The results showed that three groups from all testing groups of Thai medicinal plant extracts had the potential of antibiotic activity against *S. pyogenes*. The highest antibiotic activity against *S. pyogenes* was detected from whole onion extract red shallot followed by the extract of the mulberry leaves and root barks of mulberry strain Nakhon Ratchasima 60 (Nak 60) while the extract of marigold petal did not present antibiotic activity. The results revealed that crude extract of those two Thai medicinal plants, including red shallot and mulberry, had antibiotic activity against bacterial growth of *S. pyogenes* in the experiment and these medical Thai plants had potential benefits for developing as alternative treatment agents for *S. pyogenes* infections in both humans and animals in the future.

Keywords: Antibiotic activity, Ethanolic crude extract, Thai medicinal plants, *Streptococcus pyogenes*

INTRODUCTION

Streptococcus pyogenes (*S. pyogenes*) is known as an important Gram-positive bacteria which display oxidase negative, catalase-negative, and β-hemolytic on blood agar. It is a facultative anaerobe bacteria that usually grows in 5-10% carbon dioxide and forms colonies on blood agar. It is grouped as Group A Streptococci (GAS) by Lancefield serological grouping system (Spellerberg, 2016). In humans, *S. pyogenes* normally colonizes in the throat, skin epithelial surfaces, anus, genital mucosa, and pharynx (Cunningham, 2000), while it was reported to be found in the eye discharge of a dog with conjunctivitis, feces of a dog with colitis, free-living European hedgehog, free-ranging non-human primates and bovine mastitis (Vela et al., 2017). However, the spread of the bacteria to typically sterile body sites can induce invasive conditions that lead to high morbidity and mortality. Therefore, *S. pyogenes* is pathogenic to humans and animals with its contagious infections. *S. pyogenes* can transmit through various routes, including direct contact, droplets, nasal discharge, fomites or surfaces contaminated with this bacteria, skin contact with a contaminated lesion, and by oral route from contaminated foods (Vela et al., 2017).

Currently, the increasing antimicrobial resistance (AMR) is becoming the most important problem for both animal and human health as well as the potential zoonotic infection. The problem can challenge the public health system, cause economic loss, and increase the morbidity rate of infected cases and also the mortality rate of the patients who might not be cured by any antibiotic medications (Shuster et al., 2013). The increase of AMR in *S. pyogenes* has been observed worldwide (Lai et al., 2021). The highest rate of resistance is usually found in erythromycin which was reported in Europe, including Finland, Spain, Italy, Netherlands, Germany, and also the USA (Arvand et al., 2000).

At present, there are many attempts to reduce the usage and for the rational use of antibiotic drugs to treat bacterial infections. Then, the development of alternative agents, including effective natural agents is necessarily required to replace antibiotic drugs. Many studies have addressed the antibiotic activity of plant extracts against the growth of bacteria that could be developed into antibacterial agents or therapeutic drugs (Abiala et al., 2016; Elisha et al., 2017). Marigold petal)*Tagetes erecta*(has revealed a large amount of flavonoids biologically content, especially for patulitrin that is the dominant active substance in the flavonoids containing anti-inflammatory effect and also effective antibiotic

To cite this paper: Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*. *World Vet. J.,* 11 (3): 456-461. DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

activity to destroy bacterial cells (Rhama and Madhavan, 2011; Dasgupta et al., 2012). An earlier study has reviewed the bulbs of red shallot (*Allium ascalonicum* Hort.) entailing a high level of quercetin can inhibit bacterial growth (Fattorusso et al., 2002). The mulberry (*Morus alba* L.) extract was found to have bioactive components, such as flavonol quercetin 3-(6-Malonylglucoside) substance which could inhibit both Gram-negative and Gram-positive bacterial pathogens, including *Escherichia Coli*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Salem et al., 2013; Grajek et al., 2015; Gunjal et al., 2015) and quercetin which could destroy the membranes of bacteria leading to growth inhibitation (Wang et al., 2018). Therefore, the current research aimed to evaluate the antibiotic activity of ethanolic crude extracts from potential Thai medicinal plants, such as red shallot (*Allium ascalonicum*), mulberry leaves and root (*Morus indica*) strain Nakhon Ratchasima 60 (Nak 60), and marigold (*Tagetes erecta*) against *Streptococcus pyogenes* for the potential of usage as an antibiotic agent to reduce the problem of antibiotic resistance in the future.

MATERIALS AND METHODS

Ethical approval

Ethical approval was not required for this research. All experiments and procedures in the current study did not certainly interrupt both humans and animals. All of the experiments and procedures were prepared and processed One Health research unit and Veterinary Public Health (VPH) laboratory room, Faculty of Veterinary Sciences, Mahasarakham University, Maha Sarakham province, Thailand.

Medicinal plant materials

All Thai medicinal plants in this study were identified, housed, harvested, and collected at Mahasarakham University, Maha Sarakham, Northeastern Thailand. Ethanolic crude extracts were prepared from three Thai medicinal plants as were shown their external characteristic in Table 1 and their antibiotic activities were investigated. The selected medicinal plants which used in this research included root barks and leaves of mulberry (*Morus indica*) strain Nakhon Ratchasima 60 (Nak 60), marigold petal (*Tagetes erecta*), and whole onions or bulbs of red shallot (*Allium ascalonicum*) as shown in Figure 1. All medicinal plants were weighed, washed with purified and distilled water, then, they were dried at room temperature for 5 days. After that, they were chopped into 1x1 cm pieces and desiccated in a drying oven for 24 hours at 65°C. The dried medicinal plants were weighed after drying and extracted by soaking with absolute ethanol in a plant-ethanol ratio of 1:5 for 24 hours at room temperature within the cabinet. The medicinal plant extracts were then sieved using a filter paper and concentrated to yield ethanolic crude extracts via evaporation by vacuum rotary evaporator (Heidolph, Germany) at 289 mbar, 60 rpm, and 60°C in a water bath as shown in Figure 2. Each Thai medicinal plant crude extract was then dissolved in dimethyl sulfoxide (DMSO) as solvent before measuring the antibiotic activity test. Each dried plant extracts in this study were used three different concentration tests (low, medium, and high) at 25 mg/ml, 50 mg/ml, and 75 mg/ml, respectively.

Table 1. Thai medicinal plants and their part used for antibiotic activity test

Thai medicinal plants		Part used	Weight before drying (g)	Weight after drying (g)
Common name	Scientific name			
Red shallot	*Allium ascalonicum*	Whole onions	3226	557.5
Mulberry	*Morus indica*	Root barks	345.65	286
		Leaves	340.45	101.78
Marigold	*Tagetes erecta*	Petals	557.5	201.31

Target bacterial strain

This study used a reference strain of *Streptococcus pyogenes* (ATCC19615) as a target microorganism. The bacterial strain was maintained in the Veterinary Public Health Laboratory at the Faculty of Veterinary Sciences, Mahasarakham University, Thiland. *S. pyogenes* was subcultured from the original bacterial culture, stored at -20°C, and streaked on nutrient agar plates (Oxoid, UK) to maintain at 4°C. It was used to test for antibiotic susceptibility by disc diffusion protocol. *S. pyogenes* was cultured on tryptone soy agar (Oxoid, UK) in aerobic incubation at 37°C for 24 hours. Antibiotic activity testing protocol was processed following the methods for antimicrobial dilution and disk susceptibility testing CLSI guidelines M45 (CLSI, 2017). The *S. pyogenes* colony had cultured overnight, then, it was suspended in sterile saline water and the suspension has adjusted the turbidity to equal that of a standard value of 1.5x 10^8 CFU/ml by 0.5 McFarland.

Evaluation of antibiotic activity

Disk diffusion method was processed in triplicate for root barks of mulberry, leaves of mulberry, marigold petal, and whole onions of red shallot ethanolic crude extracts in each concentration. The bacterial suspension was spread onto the surface of the Muller Hinton agar; MHA (Oxiod, UK) that was used to determine the antibiotic activity by sterile cotton swabs. Each dried plant extracts was prepared a concentration at 25, 50, and 75 mg/mL stock solution in DMSO. Wells which were used to place the medicinal plant extracts testing were cut into MHA. The volume of stock solutions

To cite this paper: Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*. *World Vet. J.*, 11 (3): 456-461. DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

about 5 µl in each concentration was loaded to the wells in MHA plates which the target bacterial strain was spread along with representative antibiotic drugs as selected antibiotic assay discs including erythromycin and ceftriaxone (Oxoid, UK) which used as the positive control, then incubated the plates in aerobic incubation for 24 hours at 35°C together with DMSO which was utilized as the solvent control or negative control in this trial. The zone of the inhibition diameters in each plate was interpreted following the recommendation of CLSI guidelines M100 (CLSI, 2020).

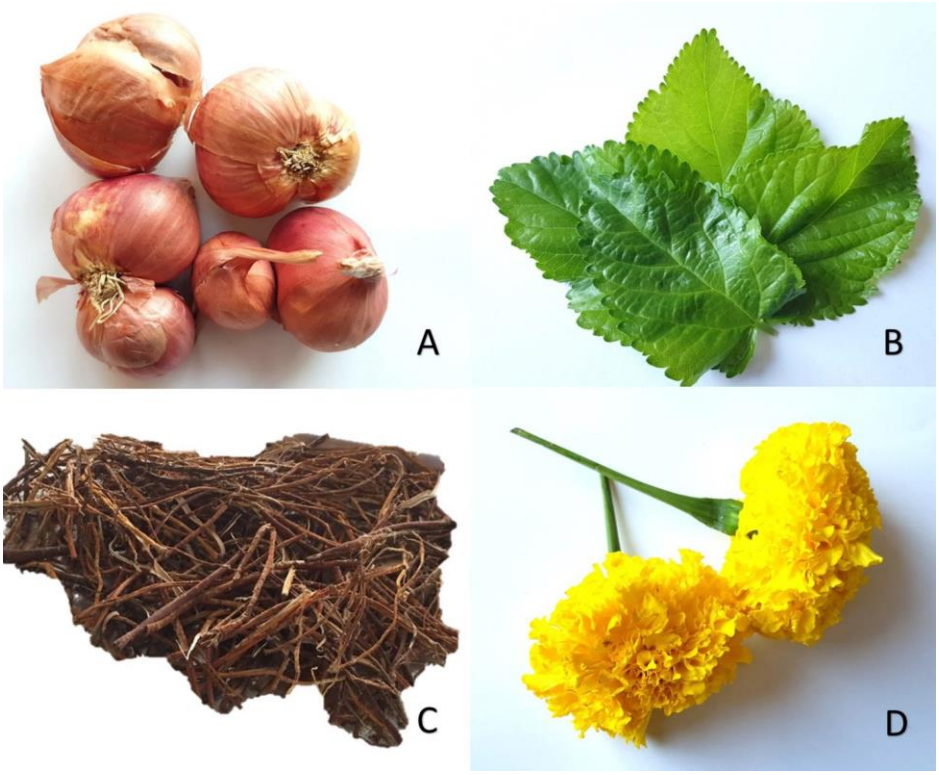


Figure 1. Thai medicinal plants used in the experiment, Whole onions of red shallot **(A)**, Mulberry leaves **(B)**, Root barks of mulberry **(C)**, Petals of marigold **(D)**

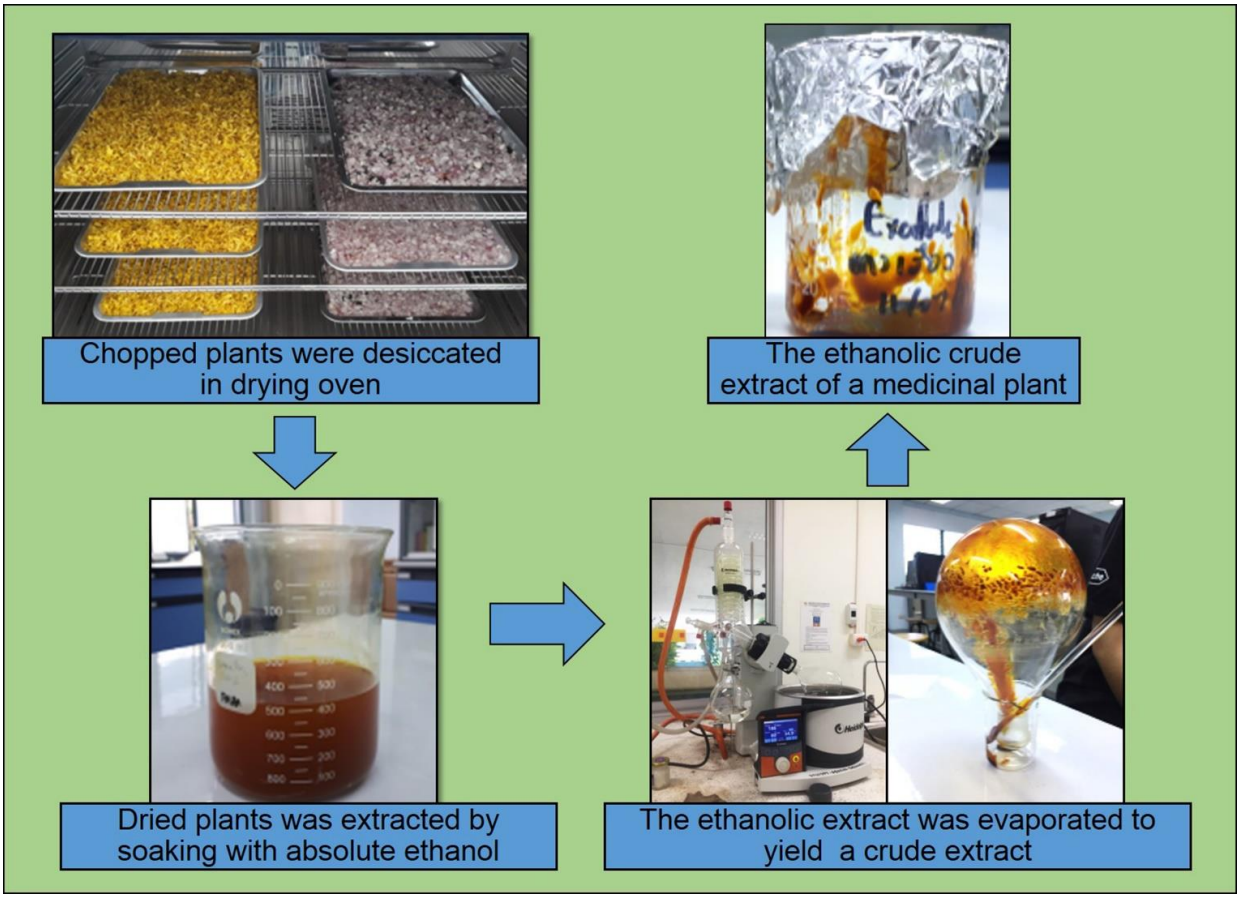


Figure 2. The extraction methods of Thai medicinal plants

To cite this paper: Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*. *World Vet. J.*, 11 (3): 456-461. DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

Statistical analysis

The experimental data were statistically analyzed using differentiation of disk diffusion results by descriptive statistics analysis and one-way analysis of variance (ANOVA) and the least significant difference (LSD) post hoc tests at 95% confidence level. The significance statistical difference was set at $p < 0.05$. The IBM SPSS statistic version 21.0 was used for analysis .

RESULTS

The experimental result from the antibiotic activity trial for inhibiting the growth of *S. pyogenes* by the ethanolic crude extracts of Thai medicinal plants is demonstrated in Table 2 .Ethanolic crude extracts of red shallot and mulberry showed the antibiotic activities on the reference strain of *S. pyogenes*. From the result of inhibition zone performed by the lowest concentration at 25 mg/ml, the average diameters of inhibition zone of whole onions of red shallot extract, root barks of mulberry extract, leaves of mulberry extract, and marigold petal extract were 7.1, 5.17, 5.33, and 2 mm, respectively. At 50 mg/ml concentration, the average diameters of the inhibition zone were 7.83, 6.17, 6.67, and 2.5 mm, respectively. At least, the average diameters of the inhibition zone of the highest concentration at 75 mg/ml were 8.67, 7, 7.83, and 3 mm, respectively.

Thai medicinal plant extracts comprising red shallot, root bark, and leaves of mulberry showed good antibiotic activity against *S. pyogenes*, while marigold extract rarely had antibiotic activity. Red shallot ethanolic extracts appeared the most antibiotic activity following by the crude extract of mulberry leaves and root bark of mulberry extract, respectively. The inhibition zones of erythromycin and ceftriaxone which were used as the positive control in this trial were 15.67 mm and 24.42 mm in diameter, respectively. There were statistically significant differences among the four categories of Thai medicinal plant extracts in all concentration tests when analyzed through one-way ANOVA and the least significant difference (LSD) post hoc tests at 95% confidence level. The obtained results from LSD post hoc test indicated that the ethanolic extracts of red shallot bulbs were the most effective against *S. pyogenes*, while the ethanolic extracts of marigold showed the lowermost antimicrobial activity at all concentration tests.

Table 2. Antibiotic activity of Thai medicinal plant ethanolic crude extracts demonstrated as the diameter of inhibition zone

Thai medicinal plants extract	Zone of inhibition diameter (mm)		
	Plant extracts concentration)mg/ml)		
	25	**50**	**75**
Red shallot	7	7.5	8.5
	7.5	8	8.5
	7	8	9
Average	7.16	7.83	8.67
Mulberry (root bark)	5	6	7.5
	5	6	6.5
	5.5	6.5	7
Average	5.17	6.17	7
Mulberry (leaves)	6	7	8
	5	6	7.5
	5	7	8
Average	5.33	6.67	7.83
Marigold	2	2.5	3
	2.5	3	3.5
	1.5	2	2.5
Average	2	2.5	3

DISCUSSION

The outcomes of the study revealed the potential antibiotic activity derived from ethanolic crude extracts of Thai medicinal plants comprising red shallot, mulberry leaves, and mulberry roots strain Nakhon Ratchasima 60 (Nak 60) inhibiting the growth of *S. pyogenes*. Ethanolic crude extracts of bulbs or whole onions part of red shallot expressed the highest potential inhibitory activity against *S. pyogenes* which is consistent with a previous study from Mekvimol et al. (2020) that mentioned the high antibiotic activity of red shallot and mulberry leaves extracts against the growth of Gram-positive bacteria, such as *Streptococcus agalactiae*. The findings of the current research indicated that the bulbs or whole

To cite this paper: Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*. *World Vet. J.*, 11 (3): 456-461. DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

onions part of red shallot and the leaves and rook barks part of mulberry ethanolic crude extracts have the possibility to inhibit the growth of *S. pyogenes* in all concentrations. Some previous studies reported that the shallot bulbs and mulberry leaves contain a high level of active phenolic compound which demonstrated to have antimicrobial activities, including flavonoid substance which can inhibit the growth of bacteria (Butkhup et al., 2013; Mozin et al., 2015). The major active ingredient of flavonoid substances from these herb extracts is quercetin with a bactericidal effect by demolishing bacterial cell membranes (Mozin et al., 2015). Therefore, this result is consistent with many previous studies indicating that quercetin was a crucial bioactive compound and plays a role in inhibiting Gram-positive and Gram-negative bacteria by prohibiting the activity of extracellular proteins (Koo and Jeon, 2009; Mozin et al., 2015; Fredotović et al., 2021). Nevertheless, some reports revealed that quercetin could not inhibit the growth of some Gram-negative organisms include *E. coli* and *Salmonella typhi* (Souza et al., 2010; Mozin et al., 2015). The bioactivity compounds of red shallot and mulberry which were revealed the potential antibiotic activity on *S. pyogenes* were likely more effective to inhibit bacterial growth, while ethanolic extract of marigold seemed to have no effect on the bacterial growth. The *in vitro* study of antibiotic activity from Thai medicinal plant extracts is a crucial phase for the alternative treatment strategy as surrogate antibacterial agents replacing antibiotic drugs for several bacterial pathogens, including *S. pyogenes*. The findings of the current *in vitro* study can be well used for alternative medicine. These medicinal plants in urban communities can be developed into therapeutic drugs as plant-based antimicrobial agents. This evidence is in accordance with many previous studies that revealed the success of herbal medicine in curing bacterial infectious diseases (Palombo, 2011; Abiala et al., 2016; Elisha et al., 2017). More than that, the current research seems to be the first investigation that discovered ethanolic crude extracts from various Thai medicinal plants, including whole onions of red shallot, root bark, and leaves of mulberry had antibiotic activity against the growth of *S. pyogenes*.

CONCLUSION

The current *in vitro* study revealed that red shallot (*Allium ascalonicum*), leaves part and root barks part of mulberry (*Morus indica*) strain Nakhon Ratchasima 60 (Nak 60) ethanolic extracts expressed the antibiotic activity against *S. pyogenes*. Thus, the future development of red shallot and mulberry leaves ethanolic extracts as an alternative treatment for *S. pyogenes* infections in both humans and animals are recommended for resolving the antimicrobial resistance in the prospect. Ethanolic crude extract of red shallot exhibited strong activity against *S. pyogenes* reference strain ATCC19615. Further investigation should be aimed to analyze the quantity of active ingredients and action mode of these plant extracts against bacterial clinical strains. It would be interesting to evaluate *in vivo* trial for developing and applying these medicinal plants as natural therapeutic products for both humans and animals for medical and veterinary use.

DECLARATIONS

Competing interest

The authors declare that they have no competing interests.

Authors' contribution

Natapol Pumipuntu designed and supervised the research project, supported in the experimental study and data analysis. Tanawadee Mekvimol, Chayanit Chaipunna, and Gannika Poonthong designed the research experiment and collected the data. All authors interpreted the data, wrote the manuscript, and approved the final version.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

Acknowledgments

This research project was financially supported by Mahasarakham University (Fast Track 2021). The authors are grateful to the Faculty of Veterinary Sciences, Mahasarakham University for the use of the laboratory to carry out this experiment. The authors would like to thank Asst. Prof. Dr. Prasoborn Rinthong, Faculty of Pharmacy, Mahasarakham University for her advice. All authors are also grateful to Miss Thamonphan Wimonsrinarachai, a veterinary student at the Faculty of Veterinary Sciences, Mahasarakham University for the graphical abstract design and illustration.

REFERENCES

Abiala M, Olayiwola J, Babatunde O, Aiyelaagbe O, and Akinyemi S (2016). Evaluation of therapeutic potentials of plant extracts against poultry bacteria threatening public health. BMC Complementary and Alternative Medicine, 16(1): 417. DOI: https://www.doi.org/10.1186/s12906-016-1399-z

To cite this paper: Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*. *World Vet. J.*, 11 (3): 456-461. DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

Arvand M, Hoeck M, Hahn H, and Wagner J (2000). Antimicrobial resistance in *Streptococcus pyogenes* isolates in Berlin. Journal of Antimicrobial Chemotherapy, 46(4): 621-624. DOI: https://www.doi.org/10.1093/jac/46.4.621

Butkhup L, Samappito W, and Samappito S (2013). Phenolic composition and antioxidant activity of white mulberry (*Morus alba* L.) fruits. International Journal of Food Science and Technology, 48: 934-940. DOI: https://www.doi.org/10.1111/ijfs.12044

Clinical and Laboratory Standards Institute (CLSI) (2017). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. 3rd ed. CLSI guideline M45. Wayne, PA: Clinical and Laboratory Standards Institute, 5(7): 7-8. Available at: https://goums.ac.ir/files/deputy_treat/md_labs_ef39a/files/CLSI-M45ed3e-2018(1).pdf

Clinical and Laboratory Standards Institute (CLSI) (2020). Performance standards for antibiotic susceptibility testing. 31st ed. Informational supplement CLSI document M100. Wayne, PA: clinical and laboratory standards institute, 40(1): 88-94. Available at: https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf

Cunningham MW (2000). Pathogenesis of group a streptococcal infections. Clinical Microbiology Reviews, 13(3): 470-511. DOI: https://www.doi.org/10.1128/cmr.13.3.470-511.2000

Dasgupta N, Ranjan S, Saha P, Jain R, Malhotra S, and Saleh MAAM (2012). Antibiotic activity of leaf extract of Mexican marigold (*Tagetes erecta*) against different gram positive and gram negative bacterial strains. Journal of Pharmacy Research, 5(8): 4201-4203. Available at: http://jprsolutions.info/files/final-file-57944be92c4dd0.83730986.pdf

Elisha IL, Botha FS, McGaw LJ, and Eloff JN (2017). The antibacterial activity of extracts of nine plant species with good activity against *Escherichia coli* against five other bacteria and cytotoxicity of extracts. BMC Complementary and Alternative Medicine, 17(1): 133. DOI: https://www.doi.org/10.1186/s12906-017-1645-z

Fattorusso E, Iorizzi M, Lanzotti V, and Taglialatela-Scafati O (2002). Chemical composition of shallot (*Allium ascalonicum* Hort.). Journal of Agricultureal and Food Chemistry, 50(20): 5686-5690. DOI: https://www.doi.org/10.1021/jf020396t

Fredotović Ž, Puizina J, Nazlić M, Maravić A, Ljubenkov I, Soldo B, Vuko E, and Bajić D (2021). Phytochemical characterization and screening of antioxidant, antimicrobial and antiproliferative properties of *Allium* × *cornutum* Clementi and two varieties of *Allium cepa* L. peel extracts. Plants, 10(5): 832. DOI: https://www.doi.org/10.3390/plants10050832

Gunjal S, Ankola AV, and Bhat K (2015). *In vitro* antibiotic activity of ethanolic extract of *Morus alba* leaf against periodontal pathogens. Indian Journal of Dental Research, 26(5): 533-536. Available at: https://www.ijdr.in/article.asp?issn=0970-9290;year=2015;volume=26;issue=5;spage=533;epage=536;aulast=Gunjal

Grajek K, Wawro A, and Kokocha D (2015). Bioactivity of *Morus alba* L. extracts – an overview. International Journal of Pharmaceutical Sciences and Research, 6: 3110-3122. Available at: https://ijpsr.com/bft-article/bioactivity-of-morus-alba-l-extracts-an-overview/?view=fulltext

Koo H, and Jeon J G (2009). Naturally occurring molecules as alternative therapeutic agents against cariogenic biofilms. Advances in Dental Research, 21(1): 63–68. DOI: https://www.doi.org/10.1177/0895937409335629

Lai CC, Chen SY, Ko WC, and Hsueh PR (2021). Increased antimicrobial resistance during the COVID-19 pandemic. International Journal of Antimicrobial Agents, 57(4): 106324. DOI: https://www.doi.org/10.1016/j.ijantimicag.2021.106324

Mekvimol T, Poonthong G, Chaipunna C, and Pumipuntu N (2020). Antimicrobial activity of marigold (*Tagetes erecta*), mulberry (*Morus indica*), and red shallot (*Allium ascalonicum*) extracts against *Streptococcus agalactiae*. International Journal of One Health, 6(1): 56-60. DOI: https://www.doi.org/10.14202/IJOH.2020.56-60

Mozin S, Rosyidi D, Sjofjan O, and Widodo E (2015). The effect of shallot (*Allium ascalonicum* L.) by-product as an antibiotic and alternative phytobiotic on characteristics of small intestine of broiler. Livestock Research for Rural Development, 27(4): Article #78. Available at: http://www.lrrd.org/lrrd27/4/mozi27078.htm

Palombo E (2011). Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. Evidence-based Complementary and Alternative Medicine, Article ID 680354. DOI: https://www.doi.org/10.1093/ecam/nep067

Rhama S, and Madhavan S (2011). Antibiotic activity of the flavonoid, patulitrin isolated from flowers of *Tagetes erecta* L. International Journal of Pharm Tech Research, 3(3): 1407-1409. Available at: https://www.sphinxsai.com/Vol.3No.3/pharm/pdf/PT=30(1407-1409)JS11.pdf

Salem M, Aly H, Gohar Y, and El-Sayed A (2013). Biological activity of extracts from *Morus alba* L., *Albizzia lebbeck* (L.) Benth and *Casuarina glauca* Sieber against the growth of some pathogenic bacteria**.** International Journal of Agriculture Food Science and Technology, 2(1): 9-22. DOI: https://www.doi.org/10.24102/ijafr.v2i1.122

Shuster KA, Hish GA, Selles LA, Chowdhury MA, Wiggins RC, Dysko RC, and Bergin IL (2013). Naturally occurring disseminated group B streptococcus infections in postnatal rats. Comparative Medicine, 63(1): 55-61. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3567377/

Souza LD, Wahidulla S, and Devi P (2010). Antibacterial phenolics from the Mangrove *Lumnitzera racemosa*. Indian Journal of Marine Sciences, 39(2): 294-298. Available at: http://nopr.niscair.res.in/handle/123456789/10027

Spellerberg BC (2016). Laboratory Diagnosis of *Streptococcus pyogenes* (group A streptococci). *Streptococcus pyogenes* : Basic biology to clinical manifestations. Oklahoma City, pp. 10-11. Available at: https://www.ncbi.nlm.nih.gov/books/NBK343617/

Vela AI, Villalon P, Saez-Nieto JA, Chacon G, Dominguez L, and Fernandez-Garayzabal JF (2017). Characterization of *Streptococcus pyogenes* from Animal Clinical Specimens, Spain. Emerging Infectious Diseases, 23(12): 2013-2016. DOI: https://www.doi.org/10.3201/eid2312.151146

Wang S, Yao J, Zhou B, Yang J, Chaudry MT, Wang M, Xiao F, Li Y, and Yin W (2018). Bacteriostatic effect of quercetin as an antibiotic alternative *in vivo* and its antibiotic mechanism *in vitro*. Journal of Food Protection, 81(1): 68-78. DOI: https://www.doi.org/10.4315/0362-028X.JFP-17-214

To cite this paper: Mekvimol T, Chaipunna Ch, Poonthong G, and Pumipuntu N (2021). *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*. *World Vet. J.*, 11 (3): 456-461. DOI: https://dx.doi.org/10.54203/scil.2021.wvj58

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 462-468, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*

Rasha Elsabagh[1*], Shaimaa M. Nada[2], and Elsayed M. Abd-Elaaty[2]

[1]*Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Benha University, 13736 Qaluobia, Egypt*
[2]*Food Control, Animal Health Research Institute, Agriculture Research Center of Egypt,13829 Shebin El koom Branch, Egypt*

*Corresponding author's Email: Rasha.alsbagh@fvtm.bu.edu.eg; ORCID: 0000-0002-1759-7443

ORIGINAL ARTICLE
pii: S232245682100059-11
Received: 13 July 2021
Accepted: 04 September 2021

ABSTRACT

Lactobacillus plantarum (*L. plantarum*) bacteria is generally recognized as safe and widely used in the food industry. The current study aimed to study the antimicrobial effects of *L. plantarum* against some food poisoning microorganisms, such as *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), and *Escherichia coli* (*E. coli*) in oriental fermented chicken sausage for 18 days of storage at 4°C. The *L. plantarum* has broad-spectrum antimicrobial effects that enhance the quality and safety of food products. *L. plantarum* reduced the count of *S. aureus*, *B. cerus*, and *E. coli* to 1.54, 4.26, and 3.03 Log_{10}, respectively, after 18 days of refrigerated storage. Moreover, there were significant effects of *L. plantarum* on pH, thiobarbituric acid, total volatile basic nitrogen, and sensory attributes of fermented sausage samples during storage time. It was revealed that *L. plantarum* enhanced the physic-chemical, sensory attributes, and shelf life of fermented chicken sausage. Moreover, *L. plantarum* inhibited the inoculated food poisoning bacteria in fermented chicken sausage. In conclusion, it is recommended to use *L. plantarum* in fermented meat products as a starter and a bio-preservative to enhance the quality of the fermented chicken sausage.

Keywords: Chicken sausage, Food safety, *Lactobacillus plantarum*, Probiotic

INTRODUCTION

Food safety is a key concern in the food industry since it has serious and long-term consequences for public health, particularly when people consume food contaminated with harmful bacteria (FDA, 2020). Moreover, meat products with functional ingredients are considered a demand in the meat industry to reduce the risk of food-borne diseases and enhance health conditions (Sirini et al., 2020). In this regard, fermented sausages fortified with probiotic bacteria are considered a functional food with several health-promoting benefits (Lafarga and Hayes, 2017).

Probiotics, such as lactic acid bacteria are generally recognized as safe and are widely used in the food industry (Oleksy and Klewicka, 2018). It has been applied in human medicine for treatment or alternative in the treatment of chronic inflammation, cancer, cardiovascular diseases, and Alzheimer's disease (Woo et al., 2014; Kurhan and Çakir, 2016), as it can enhance individuals' health, physiology, and immunity (Arasu et al., 2016). Moreover, it is used for food fermentation, improving the texture and flavor of sausages, suppressing the spoilage bacteria of food, and prolonging the shelf-life (Lin and Pan, 2017).

Lactobacillus plantarum is one of the Lactic acid bacteria, that has promising characteristics to be applied in the commercial fermented meat industry as well as it has a great antioxidant effect in the fermentation of camel sausages (Ayyash et al., 2019).

Although the effect of *L. plantarum* inoculation on the quality of fermented meat products was evaluated before (Sun et al., 2016), the current study aimed to focus on the evaluation of not only the antimicrobial effect of *L. plantarum* against some food poisoning microorganisms, such as *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cerus*), and *Escherchia coli* (*E. coli*) but also changes in physicochemical and sensory attributes of inoculated oriental fermented chicken sausage stored at refrigerated temperature for 18 days.

MATERIALS AND METHODS

Ethical approval

The present study did not involve either humans or animals as an experimental setup. The experiment was conducted at Animal Health Research Institute, Egypt.

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

Bacterial strains

Referenced pathogenic bacterial strains, including *E. coli* (Lot No: 020090, Des: NCTC: 12241 and ATCC: 25922), *S. aureus* (Lot No: 460074, Des: NCTC: 10788 and ATCC: 6538), *B. cereus* (Lot No: 02900402, Des: NCTC: 10400 and ATCC: 6633), and *L. plantarum* local strain used in the current study were obtained from Media Unit, Food Hygiene Department, Animal Health Research Institute, Dokki, Giza, Egypt.

Pathogenic strains were adjusted to obtain a count of 6 $\log_{10}$ CFU mL^{-1} and *L. plantarum* was adjusted at 8 $\log_{10}$ CFU mL^{-1}.

Assessment of *in vitro* antimicrobial activity of *L. plantarum* against different food poisoning bacteria

Disc diffusion method was used following Toure et al. (2003) to assess the inhibitory range of *L. plantarum* (10^6, 10^7, and 10^8 CFU ml^{-1}) against *E. coli*, *S. aureus*, and *B. cereus* at 10^6 CFU ml^{-1} concentration.

Preparation of chicken sausage

Minced chicken breast meat was purchased from markets in Elminofia governorate, Egypt. The sausage formulations were prepared according to the formulae detailed by Mejri et al. (2017) with minor modifications using chicken meat instead of camel meat. Briefly, sausage batters were prepared for each experiment based on chicken meat content, mincing it with a meat mincer (MK-G20NR-W, Panasonic, Osaka, Japan). The added ingredients to the minced meats included 25 g kg^{-1} NaCl, 10 g kg^{-1} garlic, 4 g kg^{-1} sucrose, and mixed spices 30 g kg^{-1}.

Before the experiment, the meat was surface treated with ultraviolet light (UV) (wavelength 385 nm) for 15 minutes to minimize background micro-flora according to Morsy et al. (2018).

Challenge study

The prepared mixture was divided into seven groups, then inoculated with cultured bacteria adjusted at 10^6 for pathogenic bacteria and 10^8 for *L. Plantarum.* The first group named control entailed UV treated but not inoculated, the second group included *E. coli* inoculated group, the third group had *E. coli* + *L. plantarum*, the fourth group composed of *S. aureus* included group, the fifth group was *S. aureus* + *L. plantarum*, the sixth group contained *B. cereus* infected group, and the seventh group included *B. cereus* + *L. plantarum* inoculated group. After inoculation, samples were kept at room temperature (22°C) for 15 minutes for cell attachment and then stuffed into a sterile polyethylene sausage casing using a handheld sausage filling machine. Samples were kept at 4 ± 1°C for 18 days, and they were analyzed at the beginning of the study as well as days 3, 6, 9, 12, 15, and 18 for remaining microbial populations. The current experiment was repeated three times for each group to obtain mean values for statistical analysis (n = 3).

Microbiological assay

At each sampling day, samples were opened and then 10 g from each one of them was aseptically transferred into 90 mL of 0.1% buffered peptone water (BPW, Biolife) and stomached (model G-560E, Bohemia) for 1 minute. Ten-fold serial dilutions were made in BPW (Biolife) and 1 ml was poured on Eosin methylene blue (EMB, Biolife) for *E. coli* (ISO 21150, 2006), Baird parker (LO, Biolife) for *S. aureus* (ISO 6888-1, 2003) and *B. Cereus*, agar base-MYP (BC-MYP, Biolife) with polymyxin B sulphate supplement (Code 4240001) and egg yolk emulsion (Code 42111601) for *B. cereus* (ISO 7932, 2004). Colonies were counted after 24 hours of incubation at 37°C and expressed as log10 CFU gm^{-1}.

Physico-chemical evaluation

The measured parameters were included pH value using a digital pH-meter (model P107, Consort, Belgium), total volatile base nitrogen (TVB-N, N/100 g of sample), and Thiobarbituric acid reactive substances (TBARS, MDA kg-1) using spectrophotometric (CE 599Universal, USA, AOAC, 2005).

Sensory evaluation

Sensory evaluation of fermented sausage (control and inoculated groups) was performed under the controlled conditions of temperature 22 °C and humidity 55% by seven well-trained panelists who were working in Food Hygiene and Control Department, Animal health research institute, Egypt. The criteria used as the basis of the descriptive organoleptic assessment (color, odor, and texture) with triangle test and the hedonic rating system to score on numerical and continuous scales from 0 (the lowest score for each attribute, very bad) to 9 (the highest score for each attribute, very good). The scale points were used according to ISO 13299 (2003).

Statistical analysis

Results of physicochemical properties and sensory attributes were tested for normality and homogeneity. Then, a one-way analysis of variance was applied to evaluate the statistical significance of differences between groups followed by an LSD test as post hoc for making multiple comparisons by the Statistical Package for Social science Software

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

(Version 25, SPSS Inc.; Chicago, IL, USA). The values were expressed as the mean ± standard error. A significant difference was used at the $p \leq 0.05$ probability level. Statistical analysis of concerning results of the effects of *L. plantarum* on food poisoning bacteria was carried out using student's T-test according to Steel and Torrie (1980). Significant differences were calculated at degree of freedom at p values 0.05.

RESULTS AND DISCUSSION

Natural bio-control demand has raised with varying efficacy and impacts on food quality and consumer health (Al-Juhaimi et al., 2018). Minimum inhibitory concentration of *L. plantarum* was evaluated *in vitro*, results in Table 1 showed that zones of inhibition differed according to the use of *L. plantarum* concentration. It was found 10^8 CFU ml^{-1} concentration had the widest inhibitory zone against *S. aureus*, *B. cereus*, and *E. coli.*

Challenge study

Based on the results in Table 2 *L. plantarum* showed antimicrobial effect against *S. aureus*, *B. cerus*, and *E. coli* selected for the challenge study, there was a significant difference between groups inoculated with pathogenic bacteria only and those treated with *L. plantarum* ($p \leq 0.05$). It was found the pathogenic bacteria of inoculated groups reached a count of 8.61, 7.94, and 8.61 $\log_{10}$ for *S. aureus*, *B. cereus*, and *E. coli* over the course of the experiment, respectively. *L. plantarum* decreased count of *S. aureus*, *B. cereus*, and *E. coli* in treated groups to reach 1.54, 4.26, and 3.03 $\log_{10}$ respectively, after day 18 of refrigerated storage. The *L. plantarum* reduced the microbial load of inoculated pathogens mainly against *S. aureus*, followed by *E. coli*, and *B. cereus*. The *L. plantarum* showed antimicrobial properties in fermented chicken sausage (Yadav and Pipaliya, 2017). This might be due to the low pH level of the product that affects directly inoculated bacteria or the metabolites secreted as organic acids, fatty acids, exopolysaccharides, and bacteriocins (Oleksy and Klewicka, 2018).

Table 1. Antimicrobial activity assessment of *Lactobacillus plantarum* against *Staphylococcus aureus*, *Bacillus cerus*, and *Escherichia coli* using disc diffusion method

Pathogenic bacteria	*Lactobacillus plantarum*			
	10^6	10^7	10^8	
Staphylococcus aureus (10^6)	8 ± 0.22	12 ± 0.10	18 ± 0.15	mm
Bacillus cereus (10^6)	ND*	9 ± 0.23	13 ± 0.11	mm
Escherichia coli (10^6)	ND	10 ± 0.10	15 ± 0.14	mm

ND*: Not detected

Table 2. Effect of *Lactobacillus plantarum* on different food poisoning bacteria (log CFU/gm) inoculated in chicken sausage stored at 4°C

Groups	Storage period (day)						
	1	3	6	9	12	15	18
Staphylococcus aureus (10^6)	6.38 ± 0.09	6.89 ± 0.02	7.12 ± 0.05	7.56 ± 0.06	7.88 ± 0.07	8.20 ± 0.04	8.61 ± 0.06
Staphylococcus aureus (10^6) + *Lactobacillus plantarum*	6.35 ± 0.05	5.60 ± 0.09 ***	5.12 ± 0.03 ***	4.81 ± 0.03 ***	4.00 ± 0.08 ***	3.73 ± 0.09 ***	1.54 ± 0.13 ***
Bacillus cereus (10^6)	6.50 ± 0.08	6.79 ± 0.02	7.07 ± 0.03	7.36 ± 0.04	7.71 ± 0.04	7.81 ± 0.02	7.94 ± 0.03
Bacillus cereus (10^6) + *Lactobacillus plantarum*	6.40 ± 0.05	6.20 ± 0.00***	5.99 ± 0.02***	5.55 ± 0.03***	5.13 ± 0.03***	4.99 ± 0.07***	4.26 ± 0.02***
Escherichia coli (10^6)	6.38 ± 0.09	6.89 ± 0.02	7.12 ± 0.05	7.56 ± 0.06	7.88 ± 0.07	8.20 ± 0.04	8.61 ± 0.06
Escherichia coli (10^6) + *Lactobacillus plantarum*	6.28 ± 0.15	5.83 ± 0.08***	5.37 ± 0.04***	4.86 ± 0.03***	4.92 ± 0.03***	3.54 ± 0.08***	3.03 ± 0.12***

Data are presented as (Mean ± S.E). S. E: Standard error. *** Represents statistical significance at $p \leq 0.05$

Physico-chemical evaluation

In this phase of the study, the effect of *L. plantarum* on freshness, shelf life time, and chemical quality of chicken sausage was evaluated. Results in Table 3 revealed that pH values in groups that inoculated with *L. plantarum* were significantly different ($p \leq 0.05$) than the control one. Moreover, there was a significant difference ($p \leq 0.05$) in the pH value over the storage period (18 days) in the same group. The *L. plantarum* decreases pH values in sausage due to the growth of lactic acid bacteria (Slima et al., 2017). This acidification is a straightforward metric for assessing a starter's effectiveness. Organic acids formation during fermentation reduces pH and prevents pathogens' growth (Mataragas et al., 2015). Moreover, it has positive effects on the flavor, as it strengthens the perception of aroma (Bonomo et al., 2009).

Concerning TVB-N results during challenge study in Table 4 revealed that TVB-N increased gradually during chilling storage at 4°C with significant difference ($p \leq 0.05$) between groups inoculated with pathogenic bacteria and

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

those treated with *L. plantarum* due to bacterial or enzymatic actions on protein degradation. There was also a significant difference ($p \leq 0.05$) in TVB-N values over the storage period (18 days), with prolonged storage time the TVB-N values increased in groups inoculated with pathogenic bacteria, whereas in those treated with *L. plantarum* decreased. *L. plantarum* maintains lipid oxidation in fermented sausage (Slima et al., 2017).

Regarding Thiobarbituric acid (TBA), a typical indicator of lipid rancidity in meat products provides useful information on lipid oxidation (Tornuk et al., 2015). Results in Table 5 revealed that TBA increased gradually during extended chilling storage at 4 °C with significant difference ($p \leq 0.05$) between groups inoculated with pathogenic bacteria and those treated with *L. plantarum* due to oxidative action effect on fatty acids. There was a significant difference ($p \leq 0.05$) in TBA values over the refrigerated storage period, with prolonged storage time TBA values increased in groups inoculated with pathogenic bacteria, compared to *L. plantarum* treated groups. The *L. plantarum* in camel sausages (Ayyash et al., 2019) and chicken sausages (Yadav and Pipaliya, 2017) has a significant antioxidant effect.

Physico-chemical properties, including pH, TBA, and TVN, in ~~all~~ pathogenic bacteria inoculated groups were evaluated until nine days of storage While, those groups treated with *L. plantarum* were evaluated till 18 days of refrigerated storage (4°C) when samples undergo spoilage.

Table 3. Effect of *Lactobacillus plantarum* on pH of chicken sausage stored at refrigerator temperature 4°C

Groups	Storage period (day)						
	1	3	6	9	12	15	18
Control	5.90 ± 0.04	6.34 ± 0.04	6.97 ± 0.07	S	S	S	S
Staphylococcus aureus (10^6)	5.86 ± 0.02	6.51 ± 0.05[a]	7.01 ± 0.16	S	S	S	S
Staphylococcus aureus (10^6) + *Lactobacillus plantarum*	5.74 ± 0.01[ab]	4.87 ± 0.03[ab]	4.50 ± 0.04[ab]	4.47 ± 0.04	4.23 ± 0.06	5.00 ± 0.06	5.35 ± 0.07
Bacillus cereus (10^6)	5.90 ± 0.03	6.51 ± 0.05[a]	7.01 ± 0.16	S	S	S	S
Bacillus cereus (10^6) + *Lactobacillus plantarum*	5.79 ± 0.02[ab]	4.67 ± 0.03[ab]	4.55 ± 0.04[ab]	4.48 ± 0.04	4.26 ± 0.06	5.00 ± 0.06	5.32 ± 0.16
Escherichia coli (10^6)	5.97 ± 0.03	6.60 ± 0.05[a]	7.22 ± 0.08	S	S	S	S
Escherichiacoli (10^6) + *Lactobacillus plantarum*	5.84 ± 0.03[b]	4.80 ± 0.03[ab]	4.76 ± 0.03[ab]	4.46 ± 0.04	4.20 ± 0.05	5.51 ± 0.07	5.80 ± 0.08

Data are presented as (Mean ± S.E). S.E: Standard error, S: Spoilage depending on sensory evaluation. Significance at $p \leq 0.05$. [a] Significant in the control group, [b] Significant within corresponding bacterial groups

Table 4. Effect of *Lactobacillus plantarum* on TVB-N of chicken sausage stored at 4°C

Groups	Storage period (day)						
	1	3	6	9	12	15	18
Control	1.65 ± 0.03	18.33 ± 0.33	27.72 ± 0.67	S	S	S	S
Staphylococcus aureus (10^6)	1.71 ± 0.05	19.60 ± 0.32[a]	30.26 ± 0.65[a]	S	S	S	S
Staphylococcus aureus (10^6) + *Lactobacillus plantarum*	1.57 ± 0.03	4.80 ± 0.20[ab]	9.47 ± 0.25[ab]	13.66 ± 0.26	16.01 ± 0.51	19.19 ± 0.34	27.72 ± 0.67
Bacillus cereus (10^6)	1.79 ± 0.05[a]	21.00 ± 0.55[a]	32.06 ± 0.63[a]	S	S	S	S
Bacillus cereus (10^6) + *Lactobacillus plantarum*	1.63 ± 0.03[b]	5.54 ± 0.28[ab]	10.25 ± 0.36[ab]	14.39 ± 0.33	17.26 ± 0.36	19.93 ± 0.47	29.67 ± 1.41
Escherichia. coli (10^6)	2.09 ± 0.07[a]	21.66 ± 0.66[a]	32.95 ± 0.53[a]	S	S	S	S
Escherichiacoli (10^6) + *Lactobacillus plantarum*	1.78 ± 0.04[b]	6.10 ± 0.10[ab]	10.93 ± 0.33[ab]	15.06 ± 0.59	18.20 ± 0.39	20.88 ± 0.61	32.95 ± 0.53

Data are presented as (Mean ± S.E). S.E: Standard error, S: Spoilage depending on sensory evaluation. Significance at $p \leq 0.05$. [a] Significant in the control group, [b] Significant within corresponding bacterial groups

Table 5. Effect of *Lactobacillus plantarum* on Thiobarbituric acid of chicken sausage stored at refrigerated temperature 4°C.

Groups	Storage period (day)						
	1	3	6	9	12	15	18
Control	0.05 ± 0.00	0.81 ± 0.03	1.07 ± 0.06	S	S	S	S
Staphylococcus aureus (10^6)	0.05 ± 0.00	0.90 ± 0.02	1.23 ± 0.07	S	S	S	S
Staphylococcus aureus (10^6) + *Lactobacillus plantarum*	0.04 ± 0.01	0.14 ± 0.02[ab]	0.32 ± 0.04[ab]	0.49 ± 0.04	0.66 ± 0.04	0.81 ± 0.03	1.07 ± 0.06
Bacillus cereus (10^6)	0.06 ± 0.01	0.97 ± 0.04	1.37 ± 0.06[a]	S	S	S	S
Bacillus cereus (10^6) + *Lactobacillus plantarum*	0.05 ± 0.01	0.19 ± 0.03[ab]	0.40 ± 0.06[ab]	0.57 ± 0.06	0.74 ± 0.04	0.88 ± 0.04	1.37 ± 0.06
Escherichia coli (10^6)	0.06 ± 0.01	1.05 ± 0.07[a]	1.34 ± 0.09[a]	S	S	S	S
Escherichia coli (10^6) + *Lactobacillus plantarum*	0.05 ± 0.00	0.35 ± 0.14[ab]	0.53 ± 0.05[ab]	0.64 ± 0.07	0.79 ± 0.05	1.00 ± 0.07	1.49 ± 0.07

Data are presented as (Mean ± S.E). S.E: Standard error, S: Spoilage depending on sensory evaluation. Significance at $p \leq 0.05$. [a] Significant in the control group, [b] Significant within corresponding bacterial groups

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

Sensory evaluation

Sensory evaluation performed to assess the overall acceptability (odor, texture, and color) of chicken sausage during storage at 4°C is shown in Table 6. Results showed that there was a significant difference ($p \leq 0.05$) between samples inoculated with pathogenic bacteria only and treated groups with *L. plantarum* on overall acceptability (odor, texture, and color). Inoculated pathogenic bacteria groups in the current study spoiled at day nine.

Over the refrigerated storage period, there was a significant difference ($p \leq 0.05$) in overall acceptability values between the first group (control one) and groups inoculated with pathogenic bacteria and that inoculated with pathogenic bacteria and *L. Plantarum*. During storage, fermented chicken sausage became unaccepted or rejected in groups without *L. plantarum* at day 9 of storage and still acceptable in those treated with *L. plantarum* until day 18. Generally, all organoleptic data were in agreement with microbiological, physical, and chemical quality indices present in Tables 1, 2, 3, and 4. Changes in color, odor, and texture during refrigerated storage occurred due to lipid oxidation and protein degradation (Sirocchi et al., 2017). Changes can also be attributed to the ability of lactic acid bacteria to produce small organic substances prevent oxidation mechanism, so enhance the aroma and give specific organoleptic attributes of the products (Tagg et al., 1976). Consumers' demand for foods depends mainly on sensory attributes (Fernández-López et al., 2005). The current study results were in agreement with Slima et al. (2017) who used *L. plantarum* to evaluate the enhanced quality and safety of beef sausages stored at 4℃ for 10 days.

Table 6. Effect of *Lactobacillus plantarum* on sensory attributes of chicken sausage stored at 4°C

Groups	Storage period (day)						
	1	3	6	9	12	15	18
Control	9.11 ± 0.14	6.44 ± 0.13	3.80 ± 0.21	R	R	R	R
Staphylococcus aureus (10^6)	8.44 ± 0.21 [a]	6.44 ± 0.13	3.80 ± 0.21	R	R	R	R
Staphylococcus aureus (10^6) + *Lactobacillus plantarum*	9.22 ± 0.18 [b]	8.70 ± 0.10 [ab]	7.23 ± 0.07 [ab]	6.91 ± 0.02 [ab]	6.14 ± 0.08 [ab]	5.30 ± 0.14 [ab]	4.93 ± 0.18 [ab]
Bacillus cereus (10^6)	9.01 ± 0.09	6.44 ± 0.13	3.47 ± 0.13	R	R	R	R
Bacillus cereus (10^6) + *Lactobacillus plantarum*	9.06 ± 0.06	8.81 ± 0.04 [ab]	7.74 ± 0.10 [ab]	7.31 ± 0.12 [ab]	6.84 ± 0.06 [ab]	6.06 ± 0.05 [ab]	5.07 ± 0.09 [ab]
Escherichia coli (10^6)	9.47 ± 0.20	6.10 ± 0.46	2.80 ± 0.39 [a]	R	R	R	R
Escherichia coli (10^6) + *Lactobacillus plantarum*	9.22 ± 0.18	8.70 ± 0.10 [ab]	7.73 ± 0.13 [ab]	6.91 ± 0.02 [ab]	6.14 ± 0.08 [ab]	5.30 ± 0.14 [ab]	4.62 ± 0.14 [ab]

Data are presented as (Mean ± S.E). S.E: Standard error, R: Rejected depending on sensory evaluation. Significance at $p \leq 0.05$. [a] Significant in the control group, [b] Significant within corresponding bacterial groups

CONCLUSION

Results of the present study revealed that *L. plantarum* has broad *in vitro* and *in vivo* antimicrobial effects against *S. aureus, B. cereus*, and *E. coli*. The use of *L. plantarum* in chicken sausage enhances shelf lifetime, physicochemical properties, sensory attributes, and safety until day 18, whereas infected groups inadmissible at day 9 of storage 4°C. It is recommended to use *L. plantarum* in fermented meat products as a starter and a bio-preservative to enhance the quality.

DECLARATION

Authors' contributions

Rasha Elsabagh designed the plan of study, revised the research article. Shaimaa M. Nada and Elsayed M. Abd-Elaaty analyzed the data, performed laboratory experiments, and drafted the manuscript. Rasha Elsabagh provided the experimental tools. All authors checked the statistical results and approved the final version of the article.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

Acknowledgments

The authors express their sincere gratitude to DR\ Abd El-Aziz M.A, *Food Control, Animal Health Research Institute, Agriculture research center,* for all the support provided.

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

REFERENCES

Al-Juhaimi FY, Ghafoor K, Özcan MM, Jahurul MHA, Babiker EE, Jinap S, Sahena F, Sharifudin MS, and Zaidul ISM (2018). Effect of various food processing and handling methods on preservation of natural antioxidants in fruits and vegetables. Journal of Food Science and Technology, 55: 3872-3880. DOI: https://www.doi.org/10.1007/s13197-018-3370-0

Arasu M, Al-Dhabi N, Ilavenil S, Choi K, and Srigopalram S (2016). *In vitro* importance of probiotic *L. plantarum* related to medical field. Saudi Journal of Biological Sciences, 23: 6-10. DOI: https://www.doi.org/10.1016/j.sjbs.2015.09.022

Association of Official Agricultural Chemists (AOAC) (2005). Official methods of analysis (18thedn). Association of Official Analytical, Chemists, Washington DC. Available at: https://www.scirp.org/(S(351jmbntvnsjt1aadkposzje))/reference/ReferencesPapers.aspx?ReferenceID=2033299

Ayyash M, Shao-Quan L, Aysha A, Mouza A, Bakhita R, Anas A, Tareq O, and Amin O (2019). *In vitro* investigation of health-promoting benefits of fermented camel sausage by novel probiotic *Lactobacillus. plantarum*: A comparative study with beef sausages. LWT-Food Science and Technology, 99: 346-354. DOI: https://www.doi.org/10.1016/j.lwt.2018.09.084

Bonomo MG, Ricciardi A, Zotta T, Sico MA, and Salzano G (2009). Technological and safety characterization of coagulase-negative *staphylococci* from traditionally fermented sausages of Basilicata Region (Southern Italy). Meat Science, 83: 15-23. DOI: https://www.doi.org/10.1016/j.meatsci.2009.03.006

Fernández-López J, Zhi N, Aleson-Carbonell L, Pérez-Alvarez JA, and Kuri V (2005). Antioxidant and antibacterial activities of natural extracts: Application in beef meatballs. Meat Science, 69(3): 371-380. DOI: https://www.doi.org/10.1016/j.meatsci.2004.08.004

Food and Drug Administration (FDA) (2020). Outbreaks of foodborne illness. Available at: https://www.fda.gov/food/recalls-outbreaks-emergencies/outbreaks-foodborne-illness

International Organization for Standardization (ISO) 13299 (2003). Sensory analysis – Methodology - General guidance for establishing a sensory profile. Available at: https://www.iso.org/standard/37227.html

International Organization for Standardization (ISO) 21150 (2006). Cosmetics. Microbiology - Detection of *Escherichia coli.* Available at: https://www.iso.org/obp/ui/es/#iso:std:iso:21150:ed-1:en

International Organization for Standardization (ISO) 6888-1 (2003). Horizontal methods for the enumeration of coagulase-positive Staphylococci (*Staphylococcus aureus* and other species), Technique using Baird-Parker medium. Available at: http://www.oxoid.com/pdf/iso-food-safety-brochure.pdf

International Organization for Standardization (ISO) 7932 (2004). Horizontal method for the enumeration of presumptive *Bacillus cereus*–colony-count technique at 30°C. Available at: http://www.oxoid.com/pdf/iso-food-safety-brochure.pdf

Kurhan Ş, and Çakir I (2016). NA-bioprotective effects of lactic acid bacteria against aflatoxin B1. Current Research in Nutrition and Food Science, 4(2): 87-91. DOI: https://www.doi.org/10.1016/j.meatsci.2004.08.004

Lafarga T, and Hayes M (2017). Bioactive protein hydrolysates in the functional food ingredient industry: Overcoming current challenges. Food Reviews International, 33(3): 217-246. DOI: https://www.doi.org/10.1080/87559129.2016.1175013

Lin TH, and Pan TM (2017). Characterization of an antimicrobial substance produced by *L. plantarum* NTU 102. Journal of Microbiology, Immunology and Infection, 52(3): 409-417. DOI: https://www.doi.org/10.1016/j.jmii.2017.08.003

Mataragas M, Bellio A, Rovetto F, Astegiano S, Decastelli L, and Cocolin L (2015). Risk-based control of food-borne pathogens *L. monocytogenes* and *Salmonella* enterica in the Italian fermented sausages Cacciatore and Felino. Meat Science, 103: 39-45. DOI: https://www.doi.org/10.1016/j.meatsci.2015.01.002

Mejri L, Ziadi A, El Adab S, Boulares M, Essid I, and Hassouna M (2017). Effect of commercial starter cultures on physicochemical, microbiological and textural characteristics of a traditional dry fermented sausage reformulated with camel meat and hump fat. Journal of Food Measurement and Characterization, 11(2): 758-767. https://www.doi.org/10.1007/s11694-016-9445-6

Morsy MK, Elsabagh R, and Trinetta V (2018). Evaluation of novel synergistic antimicrobial activity of nisin, lysozyme, EDTA nanoparticles, and/or Zn Oxide nanoparticles to control foodborne pathogens on minced beef. Food Control, 92: 249-254. DOI: https://www.doi.org/10.1016/j.foodcont.2018.04.061

Oleksy M, and Klewicka E (2018). Exopolysaccharides produced by *Lactobacillus* sp.: Biosynthesis and applications. Crit. Rev. Food Science and Nutrition, 58: 450-462. DOI: https://www.doi.org/10.1080/10408398.2016.1187112

Sirini N, Frizzo LS, Aleu G, Soto LP, and Rosmini MR (2020). Use of probiotic microorganisms in the formulation of healthy meat products. Current Opinion in Food Science, 38: 141-146. DOI: https://www.doi.org/10.1016/j.cofs.2020.11.007

Sirocchi V, Devlieghere F, Peelman N, Sagratini G, Maggi F, Vittori S, and Ragaert P (2017). Effect of rosmarinus officinalis L. essential oil combined with different packaging conditions to extend the shelf life of refrigerated beef meat. Food Chemistry, 221: 1069-1076. DOI: https://www.doi.org/10.1016/j.foodchem.2016.11.054

Slima SB, Naourez K, Imen T, Mehdi T, Feki-Tounsi M, Hafedh M, Iskandar M, Ana H, Francisco J, Claudia P et al. (2017). Effect of partial replacement of nitrite with a novel probiotic *Lactobacillus plantarum* TN8 on color, physico-chemical, texture and microbiological properties of beef sausages. LWT-Food Science and Technology, 86: 219-226. DOI: https://www.doi.org/10.1016/j.lwt.2017.07.058

Steel R, and Torrie J (1980). Principles and procedures of statistics: A biometrical approach. New York: McGraw and Hill, p. 593. Available at: http://www.sidalc.net/cgi-bin/wxis.exe/?IsisScript=ACERVO.xis&method=post&formato=2&cantidad=1&expresion=mfn=048115

Sun Q, Chen Q, Li F, Zheng D, and Kong B (2016). Biogenic amine inhibition and quality protection of Harbin dry sausages by inoculation with Staphylococcus xylosus and *Lactobacillus plantarum*. Food Control, 68: 358-66. DOI: https://www.doi.org/10.1016/j.foodcont.2016.04.021

Tagg JR, Dajani AS, and Wannamaker LW (1976). Bacteriocins of gram-positive bacteria. Bacteriological Reviewers, 40: 722-756. DOI: https://www.doi.org/10.1128/mr.59.2.171-200.1995

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

Tornuk F, Hancer M, Sagdic O, and Yetim H (2015). LLDPE based food packaging incorporated with nanoclays grafted with bioactive compounds to extend shelf life of some meat products. LWT-Food Science and Technology, 64(2): 540-546. DOI: https://www.doi.org/10.1016/j.lwt.2015.06.030

Toure R, Kheadr E, Lacroix C, Moroni O, and Fliss I (2003). Production of anti-bacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogen*. Journal of Applied Microbiology, 95(5): 1058-1069. DOI: https://www.doi.org/10.1046/j.1365-2672.2003.02085.x

Woo JY, Gu W, Kim KA, Jang SE, Han MJ, and Kim DH (2014). *Lactobacillus.pentosus var Lactobacillus. plantarum* C29 ameliorates memory impairment and inflammaging in a D-galactose-induced accelerated aging mouse model. Anaerobe, 27: 22-26. DOI: https://www.doi.org/10.1016/j.anaerobe.2014.03.003

Yadav AS, and Pipaliya G (2017). Antioxidant and antimicrobial profile of chicken sausages prepared after fermentation of minced chicken meat with *Lactobacillus. plantarum* and with additional dextrose and starch. LWT-Food Science and Technology, 77: 249-258. DOI: https://www.doi.org/10.1016/j.lwt.2016.11.050

To cite this paper: Elsabagh R, Nada ShM, and Abd-Elaaty EM (2021). Controlling Food Poisoning Bacteria in Fermented Chicken Sausage Using *Lactobacillus plantarum*. *World Vet. J.*, 11 (3): 462-468. DOI: https://dx.doi.org/10.54203/scil.2021.wvj59

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 469-473, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj60

The Effect of the Different Artificial Insemination Time Periods on the Pregnancy Rate of Sapudi Ewes

Cristy Dwi Putri[1], Ismudiono[1*], and Emmanuel Djoko Poetranto[2]

[1]*Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya-60115, Indonesia*
[2]*Department of Veterinary Clinic, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya-60115, Indonesia*

*Corresponding author's Email: ismudiono@yahoo.co.id ; ORCID: 0000-0002-9705-5176

ABSTRACT

Artificial insemination is required to increase the reproduction rate in ruminant breeding. The artificial insemination success rate in sheep only reaches 47.6%, whereas the proposed ideal rate is 70%. One of the factors influencing the artificial insemination success rates in sheep is improper estrus detection, resulting in no fertilization. The present study aimed to determine the effect of different artificial insemination time periods on the pregnancy rates of Sapudi ewes**.** The research design was based on a completely randomized design. A total of 20 female Sapudi sheep were divided into four treatment groups with five repetitions (for each group). In addition, the observed variables were artificial insemination time in Sapudi ewes. Estrus synchronization in ewes was conducted by injecting $PGF_{2\alpha}$. The results of the research indicated that ewes subjected to artificial insemination 6, 12, 18, and 24 hours after estrus had a pregnancy rate of 20%, 100%, 60%, and, 60%, respectively. It can be concluded that the time differences in artificial insemination significantly influence the pregnancy rate in Sapudi ewes'.

Keywords: Artificial insemination, Estrus, Pregnancy rate, Sapudi ewes

SHORT COMMUNICATION
pii: S232245682100060-11
Received: 16 July 2021
Accepted: 31 August 2021

INTRODUCTION

Sapudi sheep is a family of native Indonesian sheep with the characteristics of a larger tail size than other sheep strains. This different characteristic provides benefits to farmers during the dry season, since Sapudi sheep can reserve energy in their tails (Tanziila, 2018). However, the big size of Sapudi sheep's tails is also one of the problematic factors to conduct natural mating. Therefore, human assistance is required to conduct artificial insemination in order to improve the reproduction rate (Hafez, 2000).

Artificial insemination is a reproductive bio-technique that relies on improving livestock genetic quality, controlling reproductive infectious diseases, and optimizing the reproductive appearance (Ramadhani, 2016). However, the insemination success rate of sheep only reaches 47.6%, whereas the ideal rate is believed to be 70% (Rizal, 2006). This situation can be affected by many factors, including the quality of the frozen semen which experiences a quality decrease after thawing, incorrect implementation of artificial insemination, and incorrect estrus detection which results in a fertilization failure (Abebe and Alemayehu, 2021). The estrus period detection is highly complicated to perform, which results in difficulties in determining the ovulation period (Miguel-Cruz et al., 2019). The length of ewes' estrus period is around 24-36 hours, and the sheep's ovulation period begins approximately 24-30 hours from the beginning of the estrus time (Miguel-Cruz et al., 2019). Determining the ovulation period is considered important for an exact time for artificial insemination (Ramadhani, 2016).

One of the alternative methods to overcome the problems above is conducting an estrus synchronization. Estrus synchronization is a method to hormonized insemination (insemination at the same time), within a short period of time, so can be estimated in animal models (Mirshamsollahi, 2016). As a result, livestock can be inseminated at the same time. The estrus synchronization commonly is achieved using prostaglandin F2α or $PGF_{2\alpha}$ hormone (Mirshamsollahi, 2016; Miguel-Cruz et al., 2019). The hormone acts by lysing the *corpus luteum*, which leads to a decreased level of the progesterone hormone (Ferrag et al., 2017). The low progesterone is followed by an FSH increase, which stimulates the development of matured follicles causing the heat in sheep (Stenbak et al., 2001). The $PGF_{2\alpha}$ hormones can only regress the active corpus luteum rather than starting or growing corpus luteum (Sudarmaji et al., 2004). The implementation of massive estrus synchronization will improve the reproductive efficiency of the livestock, optimize the artificial insemination implementation, and increase the group fertility (Sardi, 2011).

Pregnancy failure often occurs due to an inappropriate estrus detection, which results in incorrect artificial insemination time (Sigit et al., 2014). Based on the background above, the present study was done for determining the effects of insemination time on the pregnancy rate of Sapudi ewes.

To cite this paper: Putri CD, Ismudiono, and Poetranto ED (2021). The Effect of the Different Artificial Insemination Time Periods on the Pregnancy Rate of Sapudi Ewes. *World Vet. J.,* 11 (3): 469-473. DOI: https://dx.doi.org/10.54203/scil.2021.wvj60

MATERIAL AND METHODS

The research design in this study was a completely randomized design. The current study was conducted at Dinas Peternakan Provinsi Jawa Timur, Garahan, Jember from April to June 2017.

Ethical approval

All processes and experiments of the present study (*in vivo* and *in vitro*) were approved by the ethical committee of Universitas Airlangga.

Estrus detection and synchronization

This research comprised of 20 unfertilized Sapudi ewes, which were examined with Ultrasound guided (USG) device. Estrus detection was done using USG according to the method of Macías et al. (2017). The estrus synchronization is achieved by intramuscular administration of prostaglandin F2α or PGF2α at the dose of 7.5 mg/sheep. In addition, the PGF2α injection in the current study was carried out twice to obtain simultaneous sheep estrus with an interval of 11 days. The sheep would be sexually excited two days after $PGF_{2\alpha}$ injection (Srianto et al., 2010).

Artificial insemination

The artificial insemination was carried out by employing the frozen semen of Sapudi sheep obtained from Farm animal Institute, Universitas Airlangga of Indonesia. In addition, this research utilized 0.25 ml of mini straws with the concentration of each straw ± 50x106 spermatozoa (Hardijanto et al., 2010). The number of straws was adjusted to the required spermatozoa concentration for artificial insemination on the ewes. Afterward, the straw was thawed on the water at room temperature for 30 seconds, and then it was inserted into the artificial insemination gun. Next, the straw tip was cut using a pair of scissors. After that, the plastic sheath was installed on an artificial insemination gun, which already contained the straw (Hardijanto et al., 2010).

The 20 ewe samples were divided into four groups. The artificial insemination in the first group was carried out 6 hours after the estrus occurred (P1). Meanwhile, artificial insemination in the second group was conducted 12 hours after the estrus was detected (P2). The artificial insemination of the third group was performed 18 hours after the sheep were in estrus (P3). Finally, the artificial insemination in the fourth group was carried out 24 hours after the estrus signs appeared (P4). Furthermore, the semen was sprayed in the area before the uterine cornua branch with a sperm concentration of approximately $\pm 50x10^6$ on each spraying (Satiti et al., 2014). The ewes' pregnancy diagnosis was conducted when the pregnancy reached 60 days by employing Carelife portable USG device.

Data analysis

The research data were the pregnancy rate analyzed by using F-test or One-way Factorial ANOVA, and followed by Duncan's Multiple Range Test if there were differences in the ANOVA Factorial test ($p < 0.05$).

RESULTS

The pregnancy diagnosis of the examined Sapudi ewes was performed using a USG device with different artificial insemination times generated the pregnancy rate (Table 1). The results identified there was a significant difference between P1, where the artificial Insemination was conducted 6 hours after the initial estrus, and P2, where the artificial Insemination was administered 12 hours after the initial estrus, on the pregnancy success rate. On the other hand, the results obtained from P2, and from P3 where the artificial insemination was performed 18 hours after the initial estrus, were not significantly different in affecting the pregnancy success rate. Similarly, the results achieved from the P2 group and the P4 group where the artificial insemination was carried out 24 hours after the initial estrus, were not significantly different regarding the pregnancy success rate. As can be seen in Table 1, Sapudi ewes in P1 exhibited a pregnancy rate of 20%. Meanwhile, the ewes in P2 were fertilized 100% after being inseminated artificially 12 hours after the estrus. The artificial insemination conducted in Sapudi sheep 18 hours after the estrus (P3) resulted in the pregnancy rate of 60%. In addition, the pregnancy rates of the ewes artificially inseminated 24 hours after estrus (P4) amounted to 60%.

Table 1. Pregnancy Rates of Sapudi Ewes' with different artificial insemination times

Group	Repetition	Pregnancy Rate (%)
P1	5	(20%) [a]
P2	5	(100%) [b]
P3	5	(60%) [ab]
P4	5	(60%) [ab]

P1: Artificial Insemination was conducted 6 hours after estrus, P2: Artificial Insemination was conducted 12 hours after estrus, P3: Artificial Insemination was conducted 18 hours after estrus, P4: Artificial Insemination was conducted 24 hours after estrus

To cite this paper: Putri CD, Ismudiono, and Poetranto ED (2021). The Effect of the Different Artificial Insemination Time Periods on the Pregnancy Rate of Sapudi Ewes. *World Vet. J.*, 11 (3): 469-473. DOI: https://dx.doi.org/10.54203/scil.2021.wvj60

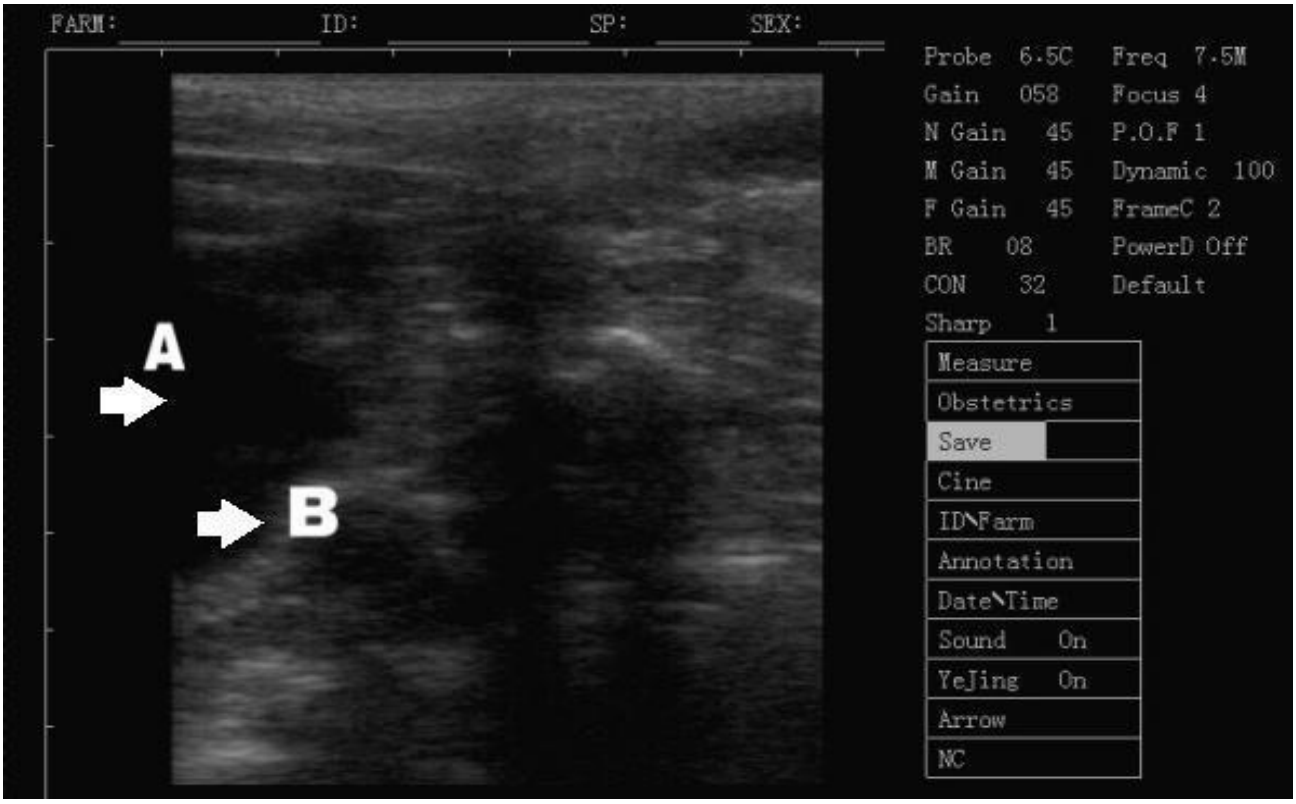


Figure 1. The result of Ultrasound-guided examination on Sapudi ewes 60 days after artificial insemination. **A**: Amniotic fluid is black, **B**: The fetus appears white in amniotic fluid

DISCUSSION

Findings demonstrated that the highest pregnancy rate appeared if the artificial insemination was carried out 12 hours (P2) after the estrus was detected. The results of pregnancy diagnosis employing USG device in Sapudi sheep is illustrated in Figure 1.

The $PGF_{2\alpha}$ Injection was carried out on day 1 and day 11 with the same dose, according to Toelihere (1981), stating that PGF2α injection can be performed with one injection or two injections with an interval of 11-12 days. The PGF2α injection can lyse the corpus luteum, causing a follicular period process. It causes estrus and ovulation symptoms on the ewes. The ewes will be in estrus one to three days after the hormone treatment was administered (Martinez-Ros et al., 2018). Ewes indicate the signs of estrus if they keep bleating frequently, and it can be identified through the physical condition where transparent mucus comes from their genital organs, the vulva looks swollen, and the vulvar mucosa appears purplish-red and warm when touched. Moreover, if they are grouped with other ewes, they indicate standing heat (Martinez-Ros et al., 2018).

The high pregnancy success rate can be influenced by controlled estrus and the insemination time. The number of ovulatory follicles increases estrogen levels in serum, and this condition can prolong the estrus (Setiatin, 2015; Martinez-Ros et al., 2018). Estrus synchronization in ewes is carried out by injecting PGF2α, which can lyse the corpus luteum, and cause a follicular period. These factors cause estrus and ovulation symptoms of the ewes. One to three days after being administered with hormonal treatment, the ewes will be in estrus (Martinez-Ros et al., 2018).

The artificial insemination conducted 12 hours after the initial estrus signs resulted in a higher pregnancy rate, compared to the other treatments (Table 1). It implies that the proper artificial insemination timing is 12 hours after the initial estrus signs are detected (Srianto et al., 2010). The ewes' estrus period is around 24-36 hours, and the ovulation period begins approximately 24-30 hours after the estrus (Srianto et al., 2010). The artificial insemination in the other treatments identified imperfect pregnancy results because, in the first treatment, where artificial insemination was conducted 6 hours after estrus, the fusion between spermatozoa and egg cells was too early. The spermatozoa cells can only survive in the female genital tract for 12-24 hours, while ovulation occurs 24- 30 hours after the initial estrus signs appear (Table 1). Hence, fertilization cannot occur. At the third treatment where the artificial insemination was conducted 18 hours after estrus, and the fourth treatment where the artificial insemination was conducted 24 hours after estrus, the pregnancy rate was lower. It occurred because the spermatozoa cells stayed in the female genital tract for 30 hours with a capacitation period of 1.5 hours in the oviduct (Bedford, 1970). Moreover, ovulation happens 24 hours after the initial estrus sign; therefore, the possibility of fertilization is not strong.

The samples of the experiment were limited to 20 ewes of productive age so that the expected value obtained was less than or equal to five (≤ 5). This fact might be caused by the feed given, the estrus time, the estrus period, and the

To cite this paper: Putri CD, Ismudiono, and Poetranto ED (2021). The Effect of the Different Artificial Insemination Time Periods on the Pregnancy Rate of Sapudi Ewes. *World Vet. J.*, 11 (3): 469-473. DOI: https://dx.doi.org/10.54203/scil.2021.wvj60

individual response of the Sapudi ewes to each different treatment. Each animal may respond differently to the treatment provided due to the livestock conditions and feed given (Toelihere, 1981; Thornton, 2010).

CONCLUSION

The difference in artificial insemination time influenced Sapudi sheep pregnancy rate. The highest pregnancy rate was obtained in the second treatment, namely Sapudi sheep, received artificial insemination 12 hours after estrus signs appeared. Therefore, the best time to carry out artificial insemination seems to be12 hours after the estrus signs were detected.

DECLARATION

Author's contribution

All authors had equal roles in conducting, writing, and editing the manuscript.

Competing interests

The author did not report any conflicts of interest in the current research.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Abebe B, and Alemayehu M (2021). Challenges and Opportunities on Estrus Synchronization and Mass Artificial Insemination in Dairy Cows for Smallholders in Ethiopia. International Journal of Zoology, Article ID: 9914095. DOI: https://doi.org/10.1155/2021/9914095

Bedford JM (1970). The saga of mammalian sperm from ejaculation to syngamy. In: Mammalian reproduction. Springer, Berlin, Heidelberg, pp. 124–82. DOI: https://www.doi.org/10.1007/978-3-642-64993-6_10

Farrag B, El-Hawy AS, and El-Bassiony MF (2017). Improving Reproductive and Productive Efficiency of Barki Sheep by using GnRH and Selenium. World Veterinary Journal, 7(4): 128-136. Available at: http://wvj.science-line.com/attachments/article/48/WVJ%207(4)%20128-136,%20Dec%2025,%202017.pdf

Hafez ESE (2000). Reproduction in Farm Animals. 7th Ed. Lippincott Williams and Wilkins, A Wolters Kluwer Company, Philadelphia. pp. 451-470. Available at: https://www.wiley.com/en-us/Reproduction+in+Farm+Animals%2C+7th+Edition-p-9781118710289

Hardijanto SS, Sardjito T, Hernawati T, and Suprayogi TW (2010). Inseminasi Buatan [Artificial insemination]. Fakultas Kedokteran Hewan. Airlangga University Press. Surabaya. Availble at: https://sinta.ristekbrin.go.id/authors/detail?id=6055753&view=documentsgs

Martinez-Ros P, Rios-Abellan A, and Gonzalez-Bulnes A (2018). Influence of Progesterone-Treatment Length and eCG Administration on Appearance of Estrus Behavior, Ovulatory Success and Fertility in Sheep. Animals (Basel), 9(1): 9. DOI: https://dx.doi.org/10.3390%2Fani9010009

Macías A, Ferrer LM, Ramos JJ, Lidón I, Rebollar R, Lacasta D, and Tejedor MT (2017). Technical Note: A new device for cervical insemination of sheep - design and field test. Journal of Animal Science, 95(12): 5263–5269. DOI: https://www.doi.org/10.2527/jas2017.1951

Miguel-Cruz E, Mejía-Villanueva O, and Zarco L (2019). Induction of fertile estrus without the use of steroid hormones in seasonally anestrous Suffolk ewes. Animal Bioscience, 32(11): 1673-1685. DOI: https://www.doi.org/10.5713/ajas.18.0769

Mirshamsollahi A (2016). Comparison of different methods of oestrus synchronization on reproductive performance of farahani sheep in Iran. Iranian Journal of Applied Animal Science, 6(4): 849-853. Available at: http://ijas.iaurasht.ac.ir/article_526630.html

Ramadhani AZS (2016). Status reproduksi dan potensi populasi berbagai bangsa sapi di desa karang endah kecamatan terbanggi besar kabupaten lampung tengah [Reproductive status and population potential of various breeds of cattle in Karang Endah Village, Sub-District of Teranggi Besar, Central Lampung Regency]. Jurnal Ilmiah Peternakan Terpadu, 4(1): 55-62. DOI: http://dx.doi.org/10.23960/jipt.v4i1.p%25p

Rizal M (2006). Fertility of Frozen-Thawed Semen From Ejaculation and Frozen-Thawed Spermatozoa From Cauda Epididymis of Garut Rams. Jurnal Sain Veteriner, 24: 49-57. Available at: https://jurnal.ugm.ac.id/jsv/article/view/347/236

Sardi M (2011). Respon estrus kerbau lumpur terhadap injeksi pgf2α di kecamatan bangkinang seberang kabupaten kampar. Universitas Islam Negeri Sultan Sarif Kasim Riau, Indonesia. Available at: http://repository.uin-suska.ac.id/2227/

Satiti D, Triana IN, and Rahardjo AP (2014). Pengaruh Penggunaan Kombinasi Progesteron (Medroxy Progesterone Acetate) dan Prostaglandin (PGF2α) Injeksi Terhadap Persentase Birahi dan Kebuntingan pada Domba Ekor Gemuk [Effect of Using a Combination of Progesterone (Medroxy Progesterone Acetate) and Prostaglandin (PGF2α) Injection on the Percentage of Erection and Pregnancy in Fat Tailed Sheep]. Veterinaria, 7(2): 23-33. Available at: http://repository.unair.ac.id/21487/

Setiatin ET (2015). Pemanfaatan Ekstrak Hipotalamus Kambing Sebagai Upaya Optimalisasi Kesuburan Kambing Kejobong Betina (the use of goat hypothalamic extract to optimalize fertility of female kejobong goats). Jurnal Veteriner, 16(3): 343–350.

Available at: https://ojs.unud.ac.id/index.php/jvet/article/view/16248

Sigit S, Lamid M, and Hermadi HA (2014). Production of progesterone implants to mass estrus synchronization and optimization levels pregnancy on fat-tailed sheep of strains sapudi Madura Island. International Journal of Academic Research, 6(6): 235–238. Available at: http://repository.unair.ac.id/63532/13/Abstrak%20016.pdf

Srianto P, Anwar H, Pantja S, Samik A, and Safitri E (2010). Buku Ajar Fisiologi Reproduksi pada Ternak. Airlangga University Press, Airlangga. Available at: https://ditjenpkh.pertanian.go.id/perpustakaan/pusvetma/index.php?p=show_detail&id=2356

Stenbak TK, Redmer DA, Berginsk HR, Erickson AS, Navanukraw C, Toutges MJ, and Grazul-Bilska AT (2001). Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology, 56(1): 51-64. DOI: https://doi.org/10.1016/S0093-691X(01)00542-8

Sudarmaji S, Malik ABD, and Gunawan AAM (2004). Pengaruh Penyuntikan Prostaglandin terhadap Persentase Birahi dan Angka Kebuntingan Sapi Bali dan PO di Kalimantan Selatan [The Effect of Prostaglandin Injection on Percentage of Erection and Pregnancy Rates for Bali and PO Cattle in South Kalimantan]. Majalah Ilmiah Peternakan, 10: 1-10. Available at: https://ojs.unud.ac.id/index.php/mip/article/view/1751

Tanziila M (2018). Karakteristik Sifat Kualitatif Dan Kuantitatif Domba Sapudi H. Universitas Brawijaya, Indonesia. Available at: http://repository.ub.ac.id/13599/

Thornton PK (2010). Livestock production: recent trends, future prospects. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 365(1554): 2853–2867. DOI: https://www.doi.org/10.1098/rstb.2010.0134

Toelihere MR (1981). Fisiologi Reproduksi pada Ternak [Reproductive Physiology in Livestock]. Angkasa, Bandung, Indonesia. Available at: http://library.um.ac.id/free-contents/index.php/buku/detail/fisiologi-reproduksi-pada-ternak-mozes-r-toelihere-1857.html

To cite this paper: Putri CD, Ismudiono, and Poetranto ED (2021). The Effect of the Different Artificial Insemination Time Periods on the Pregnancy Rate of Sapudi Ewes. *World Vet. J.*, 11 (3): 469-473. DOI: https://dx.doi.org/10.54203/scil.2021.wvj60

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 474-483, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt

Nadia Hamdi Fahim[1]*, Mohamed Abdel-Aziz Mohamed Ibrahim[1], Ahmed Hassan Amin[2], and Rabie Ragab Sadek[1]

[1]*Animal Production Department, Faculty of Agriculture, Cairo University, Giza, Egypt*
[2]*Company of 4Genetics for Dairy Solutions, Cairo, Egypt*

*Corresponding author's Email: Nadiaamn@agr.cu.edu.eg; ORCID:0000-0002-0380-2338

ORIGINAL ARTICLE
pii: S232245682100061-11
Received: 02 July 2021
Accepted: 21 August 2021

ABSTRACT

The study aimed to identify the culling reasons of Holstein cows raised in a large commercial herd in Egypt with emphasis on the performance of retained and culled cows. A total of 31534 complete lactation records for 10994 cows calved from 2008 to 2019 were used. The overall rate of culling per lactation was 61.1%. Involuntary culling represented 92% of all culling cases. The reasons for culling included mastitis and udder problems (24.2%), reproductive disorders (18.7%), metabolic and digestive disorders (13.6%), lameness (13%), endemic diseases (10.8%), low milk yield (8.1%), respiratory diseases (4.3%) and unknown causes (7.3%). Means of 305-day milk yield and daily milk yield were significantly lower in culled cows than the retained ones. On the other hand, no significant differences were observed between culled and retained cows for days open and the number of services/conception. The high involuntary culling rate of Holstein under the Egyptian conditions revealed that management practices regarding mastitis prevention and reproductive efficiency should be improved.

Keywords: Culling reasons, Egypt, Holstein, Milk production, Reproductive performance

INTRODUCTION

Culling refers to the process of removing animals from the herd. Culling could be voluntary due to low milk production or sale of excess animals or could be involuntary, as a result of illness, injury, infertility, or death. Both types of culling are applied to keep herd economics high. However, a high rate of involuntary culling reduces the herd profit, particularly for high-yielding cows. Replacement of heifers necessitates replacing culled cows. which adds a financial load on the dairy unit where rearing replacement of heifers represents about 20% of total variable costs (Karszes, 2014).

Culling strategies vary from one farm to another. These strategies can be also modified within the same farm over time. Culling decision is a complicated process since one should consider the price of culled cows, availability of replacement heifers within the farm, and the cost of rearing replacement heifers. Pinedo et al. (2010) studied cows calved between 2001 and 2006 in the Eastern United States and found that the main culling causes were death (20.6%), reproductive problems (17.7%), injury/other (14.3%), low production, and mastitis (12.1% for each). However, in the Polish population of Holstein, Adamczyk et al. (2017) found that the primary reasons for culling were reproductive disorders (39.6%) and udder problems (15.5%). In Spanish dairy cattle farms (2006-2016), Armengol and Fraile (2018) found that reproductive disorders were the most frequent reason for cow removal (30.2%), followed by low milk yield (23.4%), accidents (7.7%), diseases (7.2%), locomotor disorders (2.4%), and obstetrics (2.4%). Doornewaard et al. (2018) also stated that the main reasons for culling dairy cows in Wageningen, Netherland were reproduction failure, mastitis udder health, low production, and leg diseases. The rate of culling varies due to parity, stage of lactation, breed, and management strategies (Pinedo et al., 2014). De Vries (2017) reported that the rate of culling was within the range of 28-64%.

In Egypt, Holstein cattle are the main dairy cattle in commercial farms which supply the local market with milk stably and steadily. Egypt lies in the semi-arid subtropical zone where the temperature and humidity are high mainly in the summer season, besides, there are limited feed and water resources. Thus, dairy farms in Egypt have to pay for imported feeds and vaccinations and also for installing and maintaining cooling systems. These harsh conditions markedly increase culling rates, shorten Holstein's longevity, and rise the cost of raising replacement heifers. Breeding programs should take into consideration the way to diminish the causes of involuntary culling. Therefore, it is necessary to identify the reasons for culling Holstein cows raised in Egypt.

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

Although the performance of Holstein in Egypt has been intensively studied, few available reports are found concerning culling reasons, therefore, the objective of this study was to identify the culling reasons, as well as, investigating the performance of retained and culled cows in large Holstein herds in Egypt.

MATERIALS AND METHODS

Source of data

Data used in the present study were collected from Dina farm, one of the largest commercial dairy farms in Egypt. This farm is placed in the Menoufia governorate in northern Egypt. The geographical coordinates of the farm are 30.14°N, 30.33°E. The location is characterized by a subtropical climate with humid summers and relatively mild winters. Data of 38060 lactation records for 12259 Holstein cows were obtained from the herd management software of Dairy Comp 305 (Valley Ag Software, Tulare, CA). A total number of 31534 records of complete lactations for 10994 cows, calved from 2008 to 2019 were extracted from the whole data and used in the current study.

Herd management

Cows were housed in shaded free stall barns with a concrete floor. Heifers were served for the first time at about 370 kg body weight and frozen semen was used in insemination. Machine-milking three times/day was performed at eight-hour intervals and cows were dried off two months before the expected calving dates. The farm was provided with a cooling system. All cows were housed, fed, and milked under the same conditions and received the same management.

Studied traits

The productive traits: included the standard lactation length (305-dMY, kg) and daily milk yield (DMY, kg).

The reproductive traits: included days open; the days from calving until conception (DO, days), and the number of services per conception for each cow (NSPC).

Data analysis

The statistical analysis was performed using the XLSTAT version 2020.3.1. The following model was applied to analyze productive and reproductive traits:

$$Y_{ijklm} = \mu + L_i + P_j + Y_k + S_l + (L_i \times P_j) + b\,(x_{ijklm} - \bar{X}) + e_{ijklm},$$

Where, Y_{ijklm} is an observation of productive or reproductive trait, μ denotes the overall mean, L_i refers to the fixed effect of the i^{th} longevity class, (i=1,2), 1 explains cows have subsequent record(s) (retained), and 2 means cows have no subsequent records (culled), P_j is the fixed effect of the j^{th} parity order, (j = 1, 2,…, and 6), 1=1^{st}, 2=2^{nd}, 3=3^{rd}, 4=4^{th}, 5=5^{th} and 6=6^{th}, Y_k serves as the fixed effect of the k^{th} period of calving, (k = 1, 2, 3), 1 = 2008-2011, 2 = 2012-2015, and 3 = 2016-2019, S_l stands for the fixed effect of the season of calving (l = 1, 2, 3, 4), l refers to Winter (December-February), 2 denotes Spring (March-May), 3 explains Summer (June-August) and 4 indicates Autumn (September-November), L_iXP_j is the interaction between longevity class and parity order, b accounts for the linear regression coefficient of the studied trait on age at first calving, x_{ijklm} shows the age at first calving, $\bar{X}$ signifies the overall average of age at first calving (AFC, month), and e_{ijklm} is random residual effect.

All possible interactions were tested, but only the interaction between longevity class and parity order was significant, therefore, the used model included only this interaction.

RESULTS AND DISCUSSION

Culling rate per lactation

The numbers and percentages of retained and culled cows across parities are shown in Table 1. The overall average culling rate was high (61.1%). The semi-arid subtropical climate prevailing in the farm location, particularly in summer times, may cause heat stress as well as adverse fertility and milk production process, and encourage causes of various diseases. Haine et al. (2017) and Rilanto et al. (2020) reported lower culling rates (35% and 26.2%, respectively) than that obtained in the present study. Bahrampour et al. (2016) stated that culling risk was clear in warm, dry, and semi-arid regions, compared to temperate or cold and wet regions. Moreover, cows in large herd sizes are more exposed to be culled as a result of stressful mechanization, less individual care, and greater levels of physiological pressure. Jankowska et al. (2014) stated that culling rates were mainly high (92%) in herds keep > 100 cows.

The culling rate increased with the parity order until the fourth one (58.1%, 59.9%, 63.4%, and 68% in the first four parities). Culling rates in the fourth to the sixth parities were higher than those in the first to third parities. The high culling rate in older cows found in the present study was also declared by Mostafa (2009), Ansari-Lari et al. (2012), and Bahrampour et al. (2016). Old cows are more susceptible to culling since they gradually consume the energy reserve in

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

their bodies during milk synthesis, and pregnancy as well as overcoming diseases until they become unable to continue in the herd.

Culling reasons across parities

Table 2 presents the culling reasons across parities. The involuntary reasons formed about 92% of total culling cases. Mastitis and udder problems, as well as reproductive disorders, were the primary reasons forcing to cull cows, representing about 43% of culling cases. The same results were reported by Ansari-Lari et al. (2012) although the overall value of culling was lower (17.5%). Metabolic and digestive disorders and lameness had almost the same contribution in culling cases (about 13%). Endemic diseases were responsible for one-tenth of culling cases, whereas culling due to respiratory diseases was the lowest (4.3%). Reproductive disorders were the primary reasons for culling in the first and second lactations indicating 27.2% and 21.5% of culling cases, respectively.

Regardless of the parity order, reproductive disorders represented the second main reason for culling in this study (18.7%). The same result was obtained by Boujenane (2017) and Karrar et al. (2017). They stated that reproductive disorders were responsible for 12.5-47% of culling cases. The high risk of culling due to reproduction failure in dairy cattle has increased throughout the world as a result of intensive selection for milk yield for decades. An increase in milk yield is accompanied by a reproduction decrease due to the negative correlation between production and reproduction (Knob et al., 2016). In the present study, the proportion of culling due to reproduction disorders decreased with the increase in parity number. It is similar to the finding of Armengol and Fraile (2018) who declared that the role of reproductive failure as a culling reason greatly decreased after the third lactation. This is because cows with reproductive problems are gradually excluded from the herd.

Mastitis and udder problems were the first reasons for culling in the third till the sixth parity. The culling due to mastitis and udder problems increased as the cows got older, representing one-third of total culling in the sixth parity. The increase of culling due to mastitis and udder problems with parities was also stated by Esslemont and Kossaibati (1997). The culling of cows infected with frequent mastitis aims to reduce the risk of new infections within the herd. Mastitis is not only accompanied by a reduction in milk yield and quality, but also by its negative effects on reproduction (Pinedo et al., 2016). As it is associated with long DO, high NSPC, decrease in conception rate, and increase in early abortion (Kumar et al., 2017). The change in percentage of culling due to low milk yield or respiratory diseases across parities was narrow. The percentage of culled cows due to metabolic and digestive disorders was almost constant throughout lactations, ranging from 13% to 15%. Metabolic disorders as a reason for culling were also reported by Chiumia et al. (2013) and Rilanto et al. (2020). High milk-producing cattle are challenged by high metabolism which could put stress on these cattle causing metabolic disorders.

Lameness is the second reason for culling in the fifth and sixth parities. It is highly related to the number of parity. Similarly, Egger-Danner et al. (2015) and Gross et al. (2016) reported lameness as a reason causing culling. In the present study, the overall percentage of culls that occurred due to lameness (13%) was higher than 9.9%, 3.5%, and 2% reported by Olechnowicz and Jaskowski (2011), Ansari-Lari et al. (2012), and Karrar et al. (2017), respectively. The high rate of lameness may be rooted in the high humidity in stall barns and poor hygienic conditions surrounding cows, where cows stand in the slurry for a time on the concrete floor. The increase of culling due to lameness in old cows was also observed by Olechnowicz and Jaskowski (2011). They reported that culling due to lameness increased from 3.5% in the first lactation to 9% in the seventh one. Lameness and locomotive disorders make cows unable to perform their daily activities. So, it negatively affects milk production and reproductive performance (Pinedo et al., 2016).

Endemic diseases are viral infectious diseases. They are represented by lumpy skin disease (LSD, 42%), bovine ephemeral fever (BEF, 39%), and foot and mouth disease (FMD, 19%). They recorded the greatest percentage (16.5%) in the first parity and reached the lowest in the sixth one (6.3%). The endemic diseases are one of the causes that raise the involuntary culling rates in dairy herds in endemic countries, compared with other countries as the case in the current study. Outbreaks of these diseases occur in summer when the weather is hot and humid. The LSD and BEF are transmitted by biting insects (Sprygin et al., 2019) and FMD is spread by contact, polluted farming equipment, vehicles, clothing, or feed, and by domestic and wild predators (Salam et al., 2015). They greatly cause economic losses due to decrease in milk yield and fertility, increase in abortions, and embryo losses as well as difficulty in animal movement, vaccination, and treatment cost, all in all, may sometimes cause mortality induced by secondary bacterial infections (Ahmed and Dessouki, 2013; Knight-Jones and Rushton, 2013). Although vaccination is obligatory in Egypt, outbreaks of LSD and FMD still occur. The vaccine used against LSD depends on using strains derived from sheep. It was confirmed that these strains are not effective enough to beat the virus (Allam et al., 2020). Moreover, with the import of animals from endemic African countries and the high mutation rate, there is a possibility to develop new strains of FMD that could resist the currently used vaccine. Until developing proper vaccines for endemic diseases, management procedures should prevent the spread of infections. Applying programs that include biosafety and insect control could help in reducing the infection rate.

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

Table 1. Numbers and percentages of retained and culled Holstein cows raised in a large herd in Egypt during 2008-2019

Longevity class	Overall rate (%)	Parity												Total
		First		Second		Third		Fourth		Fifth		Sixth		
		N	%	N	%	N	%	N	%	N	%	N	%	
Retained	38.9	1583	41.9	1345	40.1	775	36.6	338	32	166	34.7	66	31.9	4273
Culled	61.1	2199	58.1	2009	59.9	1342	63.4	718	68	312	65.3	141	68.1	6721
Total	100	3782	100	3354	100	2117	100	1056	100	478	100	207	100	10994

Table 2. Culling reasons across parities for Holstein cows raised in a large herd in Egypt during 2008-2019

Culling reason	Overall average (%)	Parity												Total
		First		Second		Third		Fourth		Fifth		Sixth		
		N	%	N	%	N	%	N	%	N	%	N	%	
-Mastitis and udder problems	24.2	320	14.5	424	21.2	336	25.1	201	28.1	79	25.3	44	31.2	1404
-Reproductive disorders*	18.7	598	27.2	433	21.5	230	17.2	120	16.7	50	16.1	19	13.5	1450
-Metabolic & digestive disorders	13.6	289	13.1	285	14.3	176	13.1	95	13.2	41	13.2	21	14.8	907
-Lameness	13.0	178	8.1	188	9.3	162	12.1	83	11.5	55	17.6	27	19.2	693
-Endemic diseases**	10.8	362	16.5	246	12.2	129	9.6	82	11.4	27	8.6	9	6.3	855
-Respiratory diseases	4.3	110	5.1	83	4.2	62	4.6	32	4.4	16	5.2	3	2.3	306
-Unknown	7.3	179	8.1	174	8.6	129	9.6	44	6.2	18	5.7	8	5.6	552
Subtotal	91.9	2036	92.6	1833	91.3	1224	91.3	657	91.5	286	91.7	131	92.9	6167
-Low milk yield	8.1	163	7.4	176	8.7	118	8.7	61	8.5	26	8.3	10	7.1	554
Total	100	2199	100	2009	100	1342	100	718	100	312	100	141	100	6721

*****Reproductive diseases included dystocia, metritis, pregnancy toxemia, uterus prolapse, uterus adhesion, and repeat breeder. ******Endemic diseases included lumpy skin disease (LSD), Bovine ephemeral fever (BEF), and Foot and mouth disease (FMD)

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

Table 3. Least squares means of productive and reproductive traits for retained and culled Holstein cows raised in a large herd in Egypt during 2008-2019

Classification	N	305-dMY		DMY		DO		NSPC	
		LSM	SE	LSM	SE	LSM	SE	LSM	SE
Overall mean	10994	9321	28	26.2	0.05	161	1.0	3.4	0.03
Longevity class		***		***		NS		NS	
Retained	4273	9465 b	49	26.4 b	0.12	162 a	2.7	3.5 a	0.07
Culled	6721	9177 a	29	26.1 a	0.09	160 a	2.0	3.4 a	0.05
Parity order		***		***		***		***	
1	3782	9063 a	28	27.9 b	0.09	216 d	1.9	4.6 d	0.05
2	3354	9080 a	26	25.9 a	0.08	183 c	1.8	4.0 c	0.05
3	2117	9308 b	33	25.8 a	0.10	163 b	2.2	3.6 b	0.06
4	1056	9364 bc	109	26.2 a	0.15	141 a	3.4	3.0 a	0.09
5	478	9509 c	49	26.2 a	0.22	140 a	4.9	3.0 a	0.13
6	207	9605 c	71	25.7 a	0.34	124 a	7.5	2.5 a	0.20
Period of calving		***		***		***		***	
2008-2011	1833	9163 a	40	25.7 a	0.12	168 b	2.8	3.2 a	0.07
2012-2015	4620	9270 b	29	25.8 a	0.09	177 a	2.0	4.0 b	0.05
2016-2019	4541	9530 c	36	27.3 b	0.11	138 c	2.5	3.1 a	0.06
Season of calving		***		***		***		***	
Winter	2873	9192 a	35	25.9 a	0.11	163 b	2.4	3.4 b	0.06
Spring	2350	9266 a	36	25.9 a	0.11	174 c	2.5	3.8 c	0.06
Summer	2535	9393 b	35	26.5 b	0.11	160 b	2.5	3.5 b	0.06
Autumn	3236	9435 b	36	26.7 b	0.11	147 a	2.3	3.0 a	0.06
Longevity class X Parity		***		***		***		***	
Reg. on AFC		***		**		***		**	

LSM: Least squares means, SE: Standard error, 305-dMY: 305-day milk yield, DMY: Daily milk yield, DO: Days open, NSPC: Number of services per conception, AFC: age at first calving. Within each classification in the same column means followed by different letters differ significantly, **: ($p < 0.001$) and ***: ($p < 0.0001$).

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

Milk production and reproductive performance of retained and culled cows

According to Table 3, culled cows recorded significantly lower means of 305-dMY and DMY than retained ones ($p < 0.0001$). All productive and reproductive traits were significantly affected by parity order ($p < 0.0001$). Primiparous cows recorded the lowest values of 305-dMY (9063 kg), then the value increased gradually till reached 9605 kg at the sixth lactation. The DMY decreased by an increase in parity, the primiparous had the highest value (27.9 kg/day), compared to those in the other parities. The effect of year and season of calving and the regression on age at first calving were significant on all investigated productive and reproductive traits.

Although the productive performance of culled cows was significantly lower than retained ones (Table 3), there were no significant differences in either DO or NSPC. Kalantari and Cabrera (2015) proposed that the relationship between infertility level and farm economics may not be linear and many aspects are included when estimating the losses. Moreover, the increase in NSPC and DO which result in longer calving intervals may lead to prolonged lactations as it is a herd management approach followed in the intensive dairy system in hot regions (Flores et al., 2019). The reason is that the hot climate adversely affects the conception rate and more services are needed for conception (Mellado et al., 2016). In this case, extending the calving interval may not negatively influence the profits of the farm (Inchaisri et al., 2011). Conversely, the extension of calving intervals may offer economic advantages and permits the herd managers an option for decisions regarding high-yielder cows having reproductive disorders. This could reduce the involuntary cow culling (Rodríguez-Godina et al., 2021). With the progress of parity, lower NSPC and shorter DO were recorded which was similar to the results reported by Wondossen et al. (2018). This may explain the decrease in the proportion of cows culled due to reproductive disorders with the increase of parity number. Wondossen et al. (2018) attributed the longer DO in younger cows to the requirements needed for maintenance, growth, and milk production at an early age.

The interaction effect of longevity × parity order

Effects of longevity class × parity on productive and reproductive traits are presented in Figures 1-4. In the first parity, significant differences appear clearly between retained and culled cows for all the studied traits. Figure 1 shows that values of 305-dMY of retained cows were higher than those of culled ones in all parities except for the sixth one. The same trend is also displayed in Figure 2 for DMY. Figures 3 and 4 show the traits of DO and NSPC, respectively. Their values were both significantly higher in the first parity in culled cows, compared to the retained ones ($p < 0.0001$). Their values became almost similar in the subsequent lactations compared to retained ones. Significantly greater values of DO and NSPC in culled cows were also reported by Mostafa (2009). . Moreover, it is observed that curves for both productive (Figures 1 and 2) and reproductive traits (Figures 3 and 4) belonging to retained cows show more fluctuations than curves of culled cows. This may express the effort done by retained cows to overcome challenges using different internal mechanisms to keep their performance high. So, Adriaens et al. (2021) reported that perturbation in production curves is a good approach to measure how cows respond to challenges.

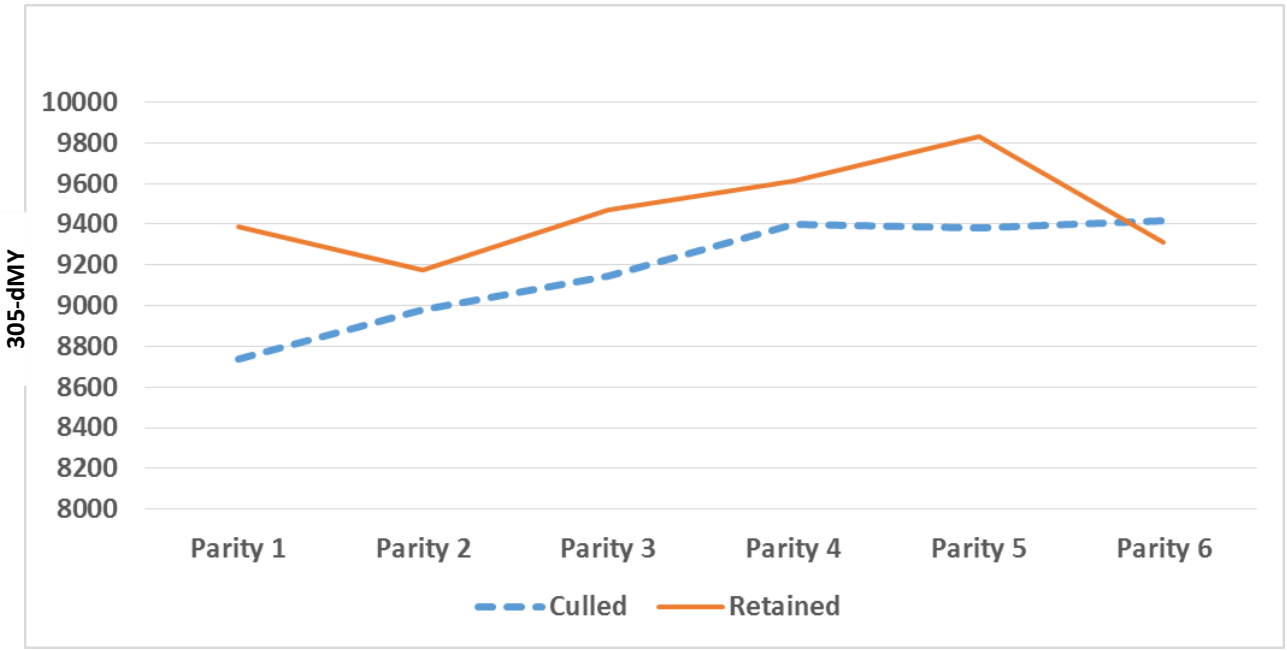


Figure 1. The interaction effect of longevity class x parity order on 305-day milk yield of Holstein cows raised in a large herd in Egypt during 2008-2019

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

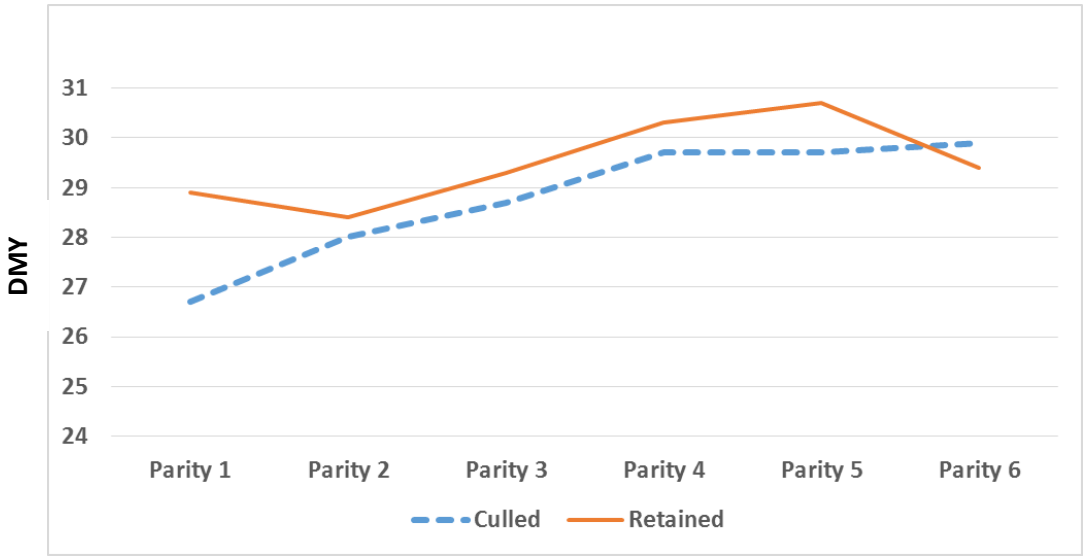


Figure 2. The interaction effect of longevity class × parity order on daily milk yield of Holstein cows raised in a large herd in Egypt during 2008-2019

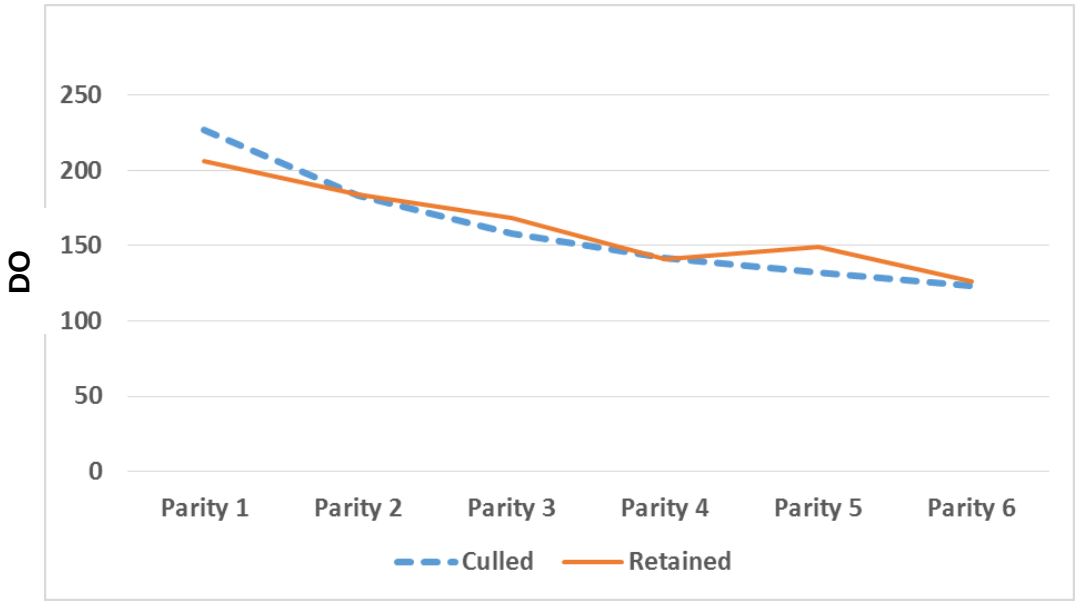


Figure 3. The interaction effect of longevity class × parity order on days open of Holstein cows raised in a large herd in Egypt during 2008-2019

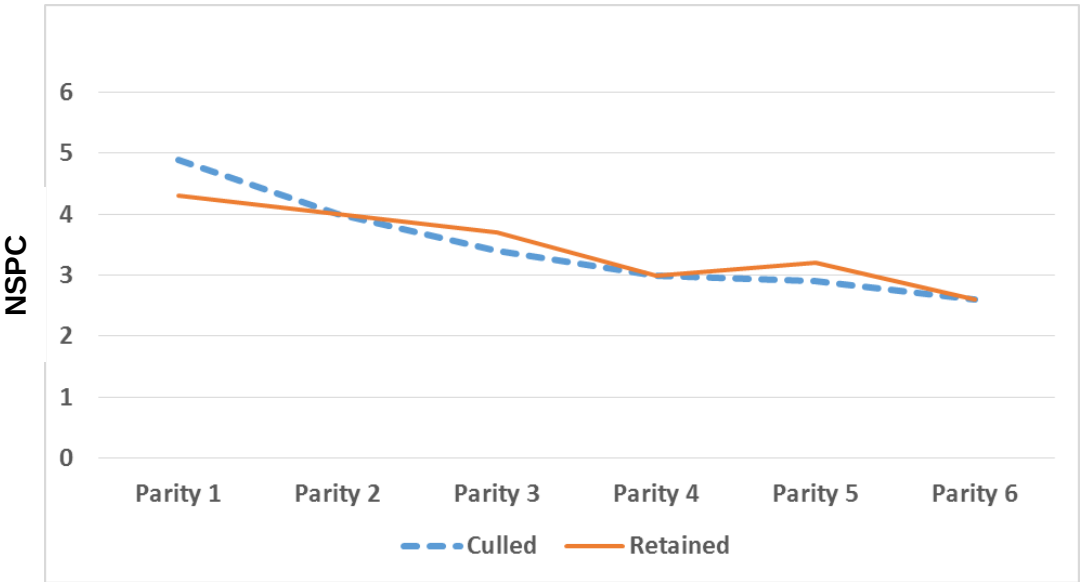


Figure 4. The interaction effect of longevity class × parity order on the number of services per conception of Holstein cow raised in a large herd in Egypt during 2008-2019

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

CONCLUSION

In Egypt, the culling rate for Holstein cows was higher than expected. Mastitis and udder problems and reproductive disorders were the primary causes for involuntary culling. Milk production performance of culled cows was significantly lower than that of retained ones. However, there was no significant difference between retained and culled cows regarding reproductive performance. To reduce the culling rate, it is important to set management programs that include good farming practices for mastitis prevention and improvement of reproductive efficiency.

DECLARATIONS

Authors' contribution

Nadia H. Fahim analyzed the results and wrote the manuscript, M. A. M. Ibrahim performed the statistical analysis, A. H. Amin collected the data and R. R. Sadek suggested the topic and revised the manuscript.

Competing interests

The authors certify that there is no conflict of interest.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

Acknowledgments

The authors express their appreciation to Dr. Ali Nigm, Professor of Animal Breeding, Department of Animal Production, Faculty of Agriculture, Cairo University, for the revision of the manuscript. Also, the authors would like to extend their gratitude to the board of Dina Company for Agricultural Investment (Dina Farms) for providing the data.

REFERENCES

Adamczyk K, Makulska J, Jagusiak W, and Węglarz A (2017). Associations between strain, herd size, age at first calving, culling reason and lifetime performance characteristics in Holstein-Friesian cows. Animal, 11(2): 327-334. DOI: https://www.doi.org/10.1017/S1751731116001348

Adriaens I, Van Den Brulle I, D'Anvers L, Statham JM, Geerinckx K, De Vliegher S, Piepers S, and Aernouts B (2021). Milk losses and dynamics during perturbations in dairy cows differ with parity and lactation stage. Journal of dairy science, 104(1): 405-418. DOI: https://www.doi.org/10.3168/jds.2020-19195

Ahmed AM, and Dessouki AA (2013). Abattoir-based survey and histopathological findings of lumpy skin disease in cattle at Ismailia abattoir. International Journal of Bioscience, Biochemistry and Bioinformatics, 3(4): 372. DOI: https://www.doi.org/10.7763/IJBBB.2013.V3.235

Allam AM, Elbayoumy MK, Abdel-Rahman EH, Hegazi AG, and Farag TK (2020). Molecular characterization of the 2018 outbreak of lumpy skin disease in cattle in Upper Egypt. Veterinary World, 13(7): 1262-1268. DOI: https://www.doi.org/10.14202/vetworld.2020.1262-1268

Ansari-Lari M, Mohebbi-Fani M, and Rowshan-Ghasrodashti A (2012). Causes of culling in dairy cows and its relation to age at culling and interval from calving in Shiraz, Southern Iran. Veterinary Research Forum, 3(4): 233-237. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4313041/

Armengol R, and Fraile L (2018). Descriptive study for culling and mortality in five high-producing Spanish dairy cattle farms (2006–2016). Acta Veterinaria Scandinavica, 60(1): 1-11. DOI: https://www.doi.org/10.1186/s13028-018-0399-z

Bahrampour J, Danesh Mesgaran M, Arabpour AR, Vakili A, and Khezri A (2016). Risk factors affecting the culling of Iranian Holstein dairy cows. Journal of Livestock Science and Technologies, 4(2): 15-23. DOI: https://www.dx.doi.org/10.22103/jlst.2016.1510

Boujenane I (2017). Reasons and risk factors for culling Holstein dairy cows in Morocco. Journal of Livestock Science and Technologies, 5(1): 25-31. DOI: https://www.dx.doi.org/10.22103/jlst.2017.1661

Chiumia D, Chagunda MG, Macrae AI, and Roberts DJ (2013). Predisposing factors for involuntary culling in Holstein–Friesian dairy cows. Journal of Dairy Research, 80(1): 45-50. DOI: https://www.doi.org/10.1017/S002202991200060X

De Vries A (2017). Economic trade-offs between genetic improvement and longevity in dairy cattle. Journal of Dairy Science, 100(5): 4184-4192. DOI: https://www.doi.org/10.3168/jds.2016-11847

Doornewaard JL, Reijs JW, Beldman ACG, Jager JH, and Hoogeveen MW (2018). Sectorrapportage Duurzame Zuivelketen: prestaties 2017 in perspectief. No. 2018-094. Wageningen Economic Research, Wageningen, the Netherlands. Available at: https://edepot.wur.nl/466401

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

Egger-Danner C, Cole JB, Pryce JE, Gengler N, Heringstad B, Bradley A, and Stock KF (2015). Invited review: Overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. Animal, 9(2): 191-207. DOI: https://www.doi.org/10.1017/S1751731114002614

Esslemont RJ, and Kossaibati MA (1997). Culling in 50 dairy herds in England. Veterinary Record, 140(2): 36-39. DOI: https://www.doi.org/10.1136/vr.140.2.36

Flores J, García JE, Mellado J, Gaytán L, De Santiago A, and Bosque MM (2019). Effect of growth hormone on milk yield and reproductive performance of subfertile Holstein cows during extended lactations. Spanish Journal of Agricultural Research, 17(1): 11. DOI: https://www.doi.org/10.5424/sjar/2019171-13842

Gross JJ, Grossen-Rösti L, Schmitz-Hsu F, and Bruckmaier RM (2016). Metabolic adaptation recorded during one lactation does not allow predicting longevity in dairy cows. Schweizer Archiv fur Tierheilkunde, 10: 1-0. DOI: https://www.doi.org/10.17236/sat00078

Haine D, Cue R, Sewalem A, Wade K, Lacroix R, Lefebvre D, Rushton J, Arsenault J, Bouchard É, and Dubuc J (2017). Culling from the actors' perspectives—decision-making criteria for culling in Quebec dairy herds enrolled in a veterinary preventive medicine program. Preventive Veterinary Medicine, 148: 1-9. DOI: https://www.doi.org/10.1016/j.prevetmed.2017.09.015

Inchaisri C, Jorritsma R, Vos PL, Van der Weijden GC, and Hogeveen H (2011). Analysis of the economically optimal voluntary waiting period for first insemination. Journal of Dairy Science, 94(8): 3811-3823. DOI: https://www.doi.org/10.3168/jds.2010-3790

Jankowska M, Sawa A, and Kujawska J (2014). Effect of certain factors on the longevity and culling of cows. Acta Scientiarum Polonorum Zootechnica, 13(2): 19-30. Available at: https://asp.zut.edu.pl/2014/13_2/asp-2014-13-2-233.pdf

Kalantari AS, and Cabrera VE (2015). Stochastic economic evaluation of dairy farm reproductive performance. Canadian Journal of Animal Science, 95(1): 59-70. DOI: https://www.doi.org/10.4141/cjas-2014-072

Karrar MH, KhM O, and Sulieman MS (2017). Culling in dairy cattle farms of Khartoum, Sudan. Online Journal of Animal and Feed Research, 7: 1-8. Available at: www.ojafr.ir

Karszes J (2014). Dairy replacement programs: Costs and analysis 3rd quarter 2012. Extension Bulletin, 2014-02. Available at: https://ecommons.cornell.edu/bitstream/handle/1813/36889/DairyReplaceCost12-3.pdf?sequence=1&isAllowed=y

Knight-Jones TJ, and Rushton J (2013). The economic impacts of foot and mouth disease–What are they, how big are they and where do they occur?. Preventive Veterinary Medicine, 112: 161-173. DOI: https://www.doi.org/10.1016/j.prevetmed.2013.07.013

Knob DA, Alessio DR, Neto AT, and Mozzaquatro FD (2016). Reproductive performance and survival of Holstein and Holstein× Simmental crossbred cows. Tropical Animal Health and Production, 48(7): 1409-1413. DOI: https://www.doi.org/10.1007/s11250-016-1103-9

Kumar N, Manimaran A, Kumaresan A, Jeyakumar S, Sreela L, Mooventhan P, and Sivaram M (2017). Mastitis effects on reproductive performance in dairy cattle: A review. Tropical Animal Health and Production, 49(4): 663-673. DOI: https://www.doi.org/10.1007/s11250-017-1253-4

Mellado M, Flores JM, De Santiago A, Veliz FG, Macías-Cruz U, Avendaño-Reyes L, and García JE (2016). Extended lactation in high-yielding Holstein cows: Characterization of milk yield and risk factors for lactations> 450 days. Livestock Science, 189: 50-55. DOI: https://www.doi.org/10.1016/j.livsci.2016.05.004

Mostafa MA (2009). Genetic parameters and breeding values for productive reproductive and persistency of milk yield traits of culled cows and their contemporaries retained in herd of Friesian cattle at different lactations. Journal of Animal and Poultry Production, 34(9): 9389-9404. DOI: https://www.dx.doi.org/10.21608/jappmu.2009.119188

Olechnowicz J, and Jaskowski JM (2011). Reasons for culling, culling due to lameness, and economic losses in dairy cows. Medycyna Weterynaryjna, 67(9): 618-621. Available at: http://www.medycynawet.edu.pl/archives/189-summary-2011/summary-2011-9/3920-summary-medycyna-wet-67-9-618-621-2011

Pinedo PJ, Daniels A, Shumaker J, and De Vries A (2014). Dynamics of culling for Jersey, Holstein, and Jersey× Holstein crossbred cows in large multibreed dairy herds. Journal of Dairy Science, 97(5): 2886-9285. DOI: https://www.doi.org/10.3168/jds.2013-7685

Pinedo PJ, De Vries A, and Webb DW (2010). Dynamics of culling risk with disposal codes reported by dairy herd improvement dairy herds. Journal of dairy science, 93(5): 2250-2261. DOI: https://www.doi.org/10.3168/jds.2009-2572

Pinedo PJ, Melendez P, Paudyal S, Krauss R, Arias F, Lopez H, Luco A, and Vergara CF (2016). Association between disease occurrence and fertility of dairy cows in three geographic regions of Chile. Theriogenology, 86(3): 817-823. DOI: https://www.doi.org/10.1016/j.theriogenology.2016.03.001

Rilanto T, Reimus K, Orro T, Emanuelson U, Viltrop A, and Mõtus K (2020). Culling reasons and risk factors in Estonian dairy cows. BMC Veterinary Research, 16: 1-6. DOI: https://www.doi.org/10.1186/s12917-020-02384-6

Rodríguez-Godina IJ, García JE, Mellado J, Morales-Cruz JL, Contreras V, Macías-Cruz U, Avendaño-Reyes L, and Mellado M (2021). Permanence time in the herd and milk production of Holstein cows with up to five successive

extended lactations. Tropical Animal Health and Production, 53(1): 1-7. DOI: https://www.doi.org/10.1007/s11250-021-02581-x

Salam HM, Mahmoud AA, Khadr AM, Salem SA, Elshemey TM, Abdelrahman AH, and Mahmoud MA (2015). Isolation, sequencing and phylogenetic analysis of FMD strains during 2011-2012 outbreaks in Egypt. International Journal of Agriculture Innovations and Research, 3(4): 2319-2473. Available at: https://ijair.org/administrator/components/com_jresearch/files/publications/IJAIR-1052_final.pdf

Sprygin A, Pestova Y, Wallace DB, Tuppurainen E, and Kononov AV (2019). Transmission of lumpy skin disease virus: A short review. Virus Research, 269: 197637. DOI: https://www.doi.org/10.1016/j.virusres.2019.05.015

Wondossen A, Mohammed A and Negussie E (2018). Reproductive performance of Holstein Friesian dairy cows in a tropical highland environment. Journal of Advances in Dairy Research, 6(2): 203. DOI: https://www.doi.org/10.4172/2329-888X.1000203

To cite this paper: Fahim NH, Ibrahim MA-AM, Amin AH, and Sadek RR (2021). Milk Production and Reproductive Performance of Retained and Culled Cows in a Large Holstein Herd in Egypt. *World Vet. J.*, 10 (3): 474-483. DOI: https://dx.doi.org/10.54203/scil.2021.wvj61

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 484-488, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*)

Fernanda Elfan[1], Suryo Kuncorojakti[1]*, and Nusdianto Triakoso[2]

[1]*Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia*
[2]*Department of Clinical Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia*

*Corresponding author's Email: suryokuncorojakti@fkh.unair.ac.id; ORCID: 0000-0001-6240-0155

SHORT COMMUNICATION
pii: S232245682100062-11
Received: 09 July 2021
Accepted: 30 August Jan 2021

ABSTRACT

The present study aimed to determine the effects of borax ($Na_2B_4O_7.10H_2O$) addition on the changes of histological cerebrum imaging in the brains of white mice (*Rattus norvegicus*). The current research was an experimental study with randomization of 24 white mice that were divided into four treatment groups with five replications. Borax was dissolved for each treatment with a dose of 19 mg/mouse/day, 26 mg/mouse/day, and 37 mg/mouse/day, and it was administered orally for 14 days. Then, it was analyzed statistically using the Kruskal-Wallis test. The statistical analysis results suggested that there were significantly different results in each treatment group. The control treatment with an administration dose of 26 mg/rat/day had a significantly different result in the worst cloudy swelling degeneration of cerebrum in histopathology imaging on Wistar rats (*Rattus norvegicus*). Using the Mann-Whitney test, it was found that the dose of borax at 37 mg/rat/day led to significant difference, compared to the other treatment groups, which means that 37 mg/rat/day of borax caused the worst pyramidal cell necrosis in histopathology imaging of the cerebrum on white mice. Borax exposure on Wistar rats (*Rattus norvegicus*) can cause cloudy swelling at a dose of 26mg/head/day, and pyramidal cell necrosis at a dose of 37 mg/head/day.

Keywords: Borax, Cerebrum, Cloudy swelling, Necrosis

INTRODUCTION

Borax is a component of many detergents, cosmetics, and enamel glazes. It is used to make buffer solutions in biochemistry, as a fire retardant, as an anti-fungal compound, in the manufacture of fiberglass, as a flux in metallurgy, neutron-capture shields for radioactive sources, a texturing agent in cooking, as a cross-linking agent in slime, as an alkali in photographic developers, as a precursor for other boron compounds, and is useful as an insecticide (similarly to boric acid, Levy and Lisensky, 1978). The use of borax on a large scale as a food preservative can be profitable in terms of production since borax can be purchased at a relatively cheap price. However, the negative impact resulting from excessive use can cause health problems. Borax is used excessively as preservatives in the food-making process of some foods, such as in *bakso*, noodles, and dumplings (Sugiyatmi, 2015). In a small amount, borax can create a chewy effect in the food, making the food stickier, long-lasting, and feel tasty. Compared to other preservatives, borax can maintain the food texture, which results in the longer maintenance of food without any change in chewiness and appearance. Borax can also maintain acidity causing the food not to spoil quickly.

The use of borax on a large scale as a food preservative can certainly be profitable in production since borax can be purchased at a relatively cheap price, compared to other harmless preservatives. In fact, even non-hazardous preservatives must be used in the right dosage. Borax even needs more cautious treatment since people cannot estimate the dosage used as a food preservative (Meacham et al., 2010).

The direct danger caused by the excessive use of borax or the residual left can have a systemic impact on the body (Dourson et al., 1998). Visible effects of borax include skin hardening or dead skin. The more dangerous effects of borax include making damage to the liver, stomach, small intestine, large intestine, testicular, and ovarian organ infertility, and it stimulates cancer cell growth (Nasution, 2009). Borax attacks cells, especially in the mitochondria, and it is accumulated in the cytoplasm and can disturb the cells' metabolism function. The study aimed to determine the effects of borax ($Na_2B_4O_7.10H_2O$) addition on the changes of the histological imaging of cerebrum in Wistar rats' brains (*Rattus norvegicus*).

To cite this paper: Elfan F, Kuncorojakti S, and Triakoso N (2021). The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*). *World Vet. J.*, 11 (3): 484-488. DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

MATERIALS AND METHODS

Ethical approval

All experimental protocols and procedures were approved by the Institutional Animal Care of Indonesia. The present research was conducted in experimental animal cages in the Faculty of Veterinary of Universitas Airlangga in Indonesia. The making of histopathological preparations of mice brains was carried out at the Veterinary Pathology Laboratory of the Faculty of Veterinary of Universitas Airlangga.

Research material

The experimental animals used in the current research were 24 healthy male Wistar rats (*Rattus norvegicus*) aged 1.5 months old and weighed approximately 100 grams. The rats were obtained from the Faculty of Medicine, Universitas Airlangga, Indonesia. The equipment used in the current study included 40 cm × 25 cm × 12 cm plastic cages, wire mesh for cage cover, feeding and drinking places, digital scales, feeding needles, surgical scissors, sterile scalpel, sterile tweezer, object glass, cover glass, tray as a container, small pot, lid as organ storage, Bunsen burner, oven, aluminum foil, microtome, staining jar, refrigerator, camera, and Eclipse C® 140 with NIS application (Nikon imaging system) BR40. The materials of the current study consisted of borax ($Na_2B_4O_7.10H_2O$) obtained from a chemical store on Jalan Tidar (Surabaya), sterile aqua dest as a borax solvent, 511 pellet chicken feed from PT Charoen Pokphand (Surabaya), drinking water, husk as the base for an individual cage, sterile cotton, ketamine, and 10% formalin.

Research design

The current study employed a laboratory experimental method. The research design used was a completely randomized design of various borax doses of 19 mg/rat/day, 26 mg/rat/day, and 37 mg/rat/day (Wagner and Wolff, 1976). The treatment groups were defined as P0 including Wistar rats treated with 0.5 ml/rat/day sterile aqua dest, P1 entailing Wistar rats treated with 0.5 ml/rat/day sterile aquadest + 19 mg/mouse/day borax, P2 having Wistar rats treated with 0.5 ml/rat/day sterile aquadest + 26 mg/mouse/day borax, and P3 including Wistar rats treated with 0.5 ml/rat/day sterile aquadest + 37 mg/mouse/day borax.

On day 15 of the experiment, the rats were intramuscularly anesthetized using ketamine. After that, fixation and dissection were conducted to separate the cerebrum from the brain organ. Furthermore, the pathology of anatomy was observed and histopathology preparation of the cerebrum was performed. The observed changes on the histopathologic imaging included cloudy swelling and pyramidal cell necrosis aspects (Sairazi et al., 2017). The damage level scoring (cloudy swelling and necrosis degenerations) in one visual field can be seen in Table 1 as follows (Purnima et al., 2013):

Table 1. Histopathological scoring of Borax exposure in the cerebrum of rats

Score	Degeneration	Necrosis
0	If the degenerative cloudy swelling of the pyramidal cell was not found at a third of the observed visual field of the cerebrum.	If the pyramidal cell necrosis was not found at a third of the observed the visual field of the cerebrum.
1	If the cloudy swelling degeneration of the pyramidal cell was found at a third of the observed visual field of the cerebrum.	If the pyramidal cell necrosis was found at a third of the observed visual field of cerebrum.
2	If the cloudy swelling degeneration of the pyramidal cell was found at a third until two-third of the observed visual field of the cerebrum.	If the pyramidal cell necrosis was found at a third until two-third of the observed power field of the cerebrum.
3	If the cloudy swelling degeneration of the Pyramidal cell was found at two-third until the whole of the observed visual field of the cerebrum.	If the pyramidal cell necrosis was found at two-third until the whole of the observed power field of the cerebrum.

Data analysis

The present study was analyzed using the Kruskal-Wallis test ($p < 0.05$). If significant differences were found, it would be continued with the Mann-Whitney test (Purnima et al., 2013).

RESULTS

The results of statistical analysis with the Kruskal-Wallis test suggested that there was a significantly different cloudy swelling degeneration in each treatment group ($p < 0.05$). Based on the results of statistical analysis with the Kruskal-

To cite this paper: Elfan F, Kuncorojakti S, and Triakoso N (2021). The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*). *World Vet. J.*, 11 (3): 484-488. DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

Wallis test, it was found that there were significantly different results in each treatment group ($p < 0.05$). In the Mann-Whitney test, it was found that P0, P1, and P3 were not significantly different statistically; values of P0 and P2 were significantly different; values of P1, P2, and P3 were not significantly different ($p > 0.05$); and values of P2 and P3 were not significantly different either ($p > 0.05$). In Table 2, it was identified that most cloudy swelling degeneration occurred in P2, whereas, the pyramidal cell that had cloudy swelling degeneration was hardly found in P3, whom the highest dose was used since more than two-thirds of the whole of the pyramidal cell had necrosis in one visual field of the Wistar rats.

Meanwhile, the results of the statistical analysis of the Kruskal-Wallis test indicated that there were significantly different results of necrosis in the pyramidal cell of Wistar rats' cerebrum in each treatment group. Regarding the Mann-Whitney test, P3 was significantly different ($p<0.05$), compared to the whole treatment groups. An insignificant difference ($p > 0.05$) only occurred in P1 and P2.

Table 2. Histopathological Imaging Analysis of the Cloudy Swelling Level and Necrosis of the pyramidal Cell of Cerebrum in each treatment group of Wistar rat (*Rattus norvegicus*)

Treatment	Cloudy Swelling Degeneration Mean ± SD	Necrosis Mean ± SD
P0: Control of 0.5ml/rat/day sterile aquadest	$0.10^{a} \pm 0$	$1^{a} \pm 0$
P1: 0.5 sterile aquadest + 19 mg/rat/day borax	$0.33^{abc} \pm 0.516$	$1.5^{b} \pm 0.54$
P2: 0.5 sterile aquadest + 26 mg/rat/day borax	$0.83^{c} \pm 0.408$	$1.5^{bc} \pm 0.548$
P3: 0.5 sterile aquadest + 37 mg/rat/day borax	$0.38^{acd} \pm 0.495$	$1.63^{d} \pm 0.71$

Different superscript in the same column indicates that there were significant differences ($p < 0.05$), SD: Standard deviation

DISCUSSION

Brain damage caused by borax exposure can pass through various pathophysiological ways. Borax which is orally got into the stomach would react with hydrochloric acid (HCl) causing faster synthesis to become boric acid (Sugiyatmi, 2015). Furthermore, the boric acid would be absorbed by the intestines, then it gets into the blood circulation until reaching the organs, and causes some damages including brain damage.

Boric acid attacks cells from the body's systemic process, especially mitochondria (Dourson et al., 1998). Normally, mitochondria function to produce energy for cell activities through the oxidative phosphorylation process (Nielsen, 1994; Pizzorno, 2015). Boric acid will inhibit H^+ ion that makes a co-enzyme bound between NAD^+ and H^+ ion. The H+ ion which bounds with boric acid will cause the failure of reduction-oxidation reaction in mitochondria (Litovitz et al., 1988). In addition, boric acid is bounded with proteins and lipids causing malfunction and forming Reactive Oxygen Species (ROS, Tanaka et al., 2016). The failure of reduction-oxidation reaction in mitochondria and the increase of ROS can cause a decrease in neuron cell viability caused by the inhibition of the electron transport chain. That process could disturb Adenosine triphosphate (ATP) formation that causes cell degeneration or even necrosis. The ATP is required for the fluency of sodium (NA^+) and potassium (K^+) pumps.

The mechanism of the sodium-potassium pump's disturbance starts with the shifting of sodium ion. Then, H_2O and calcium (Ca^+) get into the cell, while potassium and magnesium (Mg+) move out from the cell increasing the intracellular fluid influx, changes of the cell form, and acute cell swelling (Price and Wilson, 1986). The process of cloudy swelling degeneration occurs due to the failure of ATP formation as the implication of mitochondria function experiencing energy synthesis disturbance (Jamison, 1974). The failure of energy synthesis is caused by the boric acid and co-enzyme bond of NAD^+ and H^+ ion. Therefore, it inhibits the sodium-potassium pump to maintain intracellular stability. The cells which are supposed to release metabolic energy to pump sodium ion out of the cells cannot function properly. This situation increases sodium ion concentration inside the cells followed by water entrance causing cell inflammation and cloudy cytoplasm. If the influence of toxic substances can be removed, the cell would return to its normal situation (Nielsen, 1994).

Necrosis is the cell destruction caused by pathological damage, such as toxin exposure (Kumar, 2007). Some biochemical mechanisms of necrosis include ATP depletion, oxygen deprivation, or the formation of Reactivated Oxygen Species, the loss of calcium homeostasis, the defect in plasma membrane permeability, and mitochondrial damage (Heindel et al., 1992). The cerebrum pyramidal cell necrosis of the Wistar rats' brains with borax exposure started with boric acid and co-enzyme bound of NAD^+ and H^+ ion. It led to the failure of a reduction-oxidation reaction inside the mitochondria (Nielsen, 1994). The manifestations of failure in mitochondrial function would disturb glycolysis, energy synthesis, and ATP formation. The ATP is required to ease the sodium-potassium pump. If ATP formation is obstructed, water accumulation will occur inside the cytoplasm since sodium absorbs water. Next, cloudy swelling occurs in the cell. Extended and continuous effects of toxic substances make the cell unable to metabolize resulting in cell death or necrosis (Price and Wilson, 1986).

To cite this paper: Elfan F, Kuncorojakti S, and Triakoso N (2021). The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*). *World Vet. J.*, 11 (3): 484-488. DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

Extended cell degeneration disruption and the relatively large effect of toxic substances in cells will surpass the interference compensation limit causing necrosis to take place. Necrosis is marked by the morphological changes of the cell nucleus, including pyknosis, karyorrhexis, and karyolysis. Pyknosis is the depreciation and compaction process of the cell nucleus causing it to be more basophilic with bluer cell color with H & E coloring. Microscopically, it was marked by the cell nucleus looking more compact and darker. Karyorrhexis is marked by the spread of broken nucleus and chromatin fragments formation, while karyolysis is marked by the nucleus dissolution (Kumar, 2007). Necrosis process also increases the bloodstream, and is followed by capillary vasodilation that occurs actively inside the microcirculation. Cell swelling around the capillary will press and cause lumen narrowing, causing the blood unable to stream smoothly, commonly referred to as congestion (Bezabeh et al., 2004).

In imaging pyramidal cells of the cerebrum caused by borax exposure, the cells experienced widespread necrosis that caused systemic damage or cell injury around it, followed by an inflammatory reaction. The capillary was blocked by cell inflammation-causing blood vessel dilatation, followed by increasing blood amount. In this stage, the erythrocytes would accumulate in the capillary as congestion. If the capillary is broken, it will be followed by the spread of erythrocytes around the cell, resulting in hyperemia (Bezabeh et al., 2004). Borax can also cause brain, heart, fat, and kidney disturbance. In addition, borax can cause comma, central nervous system disruption, depression, and even mortality (Murray, 1998). The exposure of 20.8 mg/kg BW of borax in Wistar rats for 14 days increased the dehydrogenase inside the cerebrum (Parks and Edwards, 2005). Histologically, degeneration, congestion edema in the brain, meninges, perivascular hemorrhage, and intravascular thrombosis may occur.

CONCLUSION

Borax addition can cause cerebrum cloudy swelling on Wistar rats (*Rattus norvegicus*) at the dose of 26 mg/rat/day. Meanwhile, borax addition at the dose of 37 mg/rat/day can cause necrosis in the pyramidal cells of Wistar rats' cerebrum.

DECLARATION

Authors' contribution

All authors contributed equally to conduct this study.

Competing interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Bezabeh M, Tesfaye A, Ergicho B, Erke M, Mengistu S, Bedane A and Desta A (2004). General Pathology: Lecture Notes for Health Science Students. Ethiopia: Jimma University, Gondar University, Haramaya University, Dedub University, pp. 68-69. Available at: https://www.cartercenter.org/resources/pdfs/health/ephti/library/lecture_notes/health_extension_trainees/GeneralPathology.pdf

Dourson M, Maier A, Meek B, Renwick A, Ohanian E and Poirier K (1998). Boron tolerable intake: re-evaluation of toxicokinetics for data- derived uncertainty factors. Biological Trace Element Research, 66(1-3): 453–63. DOI: https://www.doi.org/10.1007/BF02783155

Heindel JJ, Price CJ, Field EA, Marr MC, Myers CB, Morrissey RE and Schwetz BA (1992). Developmental toxicity of boric acid in mice and rats. Fundamental and Applied Toxicology, 18(2): 266–277. DOI: https://www.doi.org/10.1016/0272-0590(92)90055-m

Jamison RL (1974). The role of cellular swelling in the pathogenesis of organ ischemia. The Western Journal of Medicine, 120(3): 205-218. DOI: https://www.doi.org/10.1172/JCI106781

Kumar S (2007). Caspase function in programmed cell death. Cell Death and Differentiation, 14(1): 32-43. DOI: https://www.doi.org/10.1038/sj.cdd.4402060

Levy HA and Lisensky GC (1978). Crystal structures of sodium sulfate decahydrate (Glauber's salt) and sodium tetraborate decahydrate (borax). Redetermination by neutron diffraction. Acta Crystallographica Section B. 34 (12): 3502–3510. DOI: https://www.doi.org/10.1107/S0567740878011504

Litovitz TL, Klein-Schwartz W, Oderda GM and Schmitz BF (1988). Clinical manifestations of toxicity in a series of 784 boric acid ingestions. The American Journal of Emergency Medicine, 6(3): 209–213. DOI: https://www.doi.org/10.1016/0735-6757(88)90001-0

To cite this paper: Elfan F, Kuncorojakti S, and Triakoso N (2021). The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*). *World Vet. J.*, 11 (3): 484-488. DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

Meacham S, Karakas S, Wallace A and Altun F (2010). Boron in human health: Evidence for dietary recommendations and public policies. The Open Mineral Processing Journal, 3(1): 36-53. DOI: https://www.doi.org/10.2174/1874841401003010036

Murray FJ (1998). A comparative review of the pharmacokinetics of boric acid in rodents and humans. Biological Trace Element Research, 66(1-3): 331-341. DOI: https://www.doi.org/10.1007/BF02783146

Nasution A (2009). Analisa kandungan boraks pada lontong di kelurahan padang bulan kota medan tahun 2009. Universitas Sumatra Utara. Available at: http://repository.usu.ac.id/handle/123456789/17797

Nielsen FH (1994). Biochemical and physiologic consequences of boron deprivation in humans. Environ Health Perspect, 102: 59-63. DOI: https://www.doi.org/10.1289/ehp.94102s759

Parks JL and Edwards M (2005). Boron in the environment. Critical Reviews in Environmental Science and Technology, 35(2): 81-114. DOI: https://www.doi.org/10.1080/10643380590900200

Pizzorno L (2015). Nothing boring about boron. Integrative Medicine (Encinitas, Calif.), 14(4): 35-48. Available at: https://pubmed.ncbi.nlm.nih.gov/26770156/

Price SA and Wilson LM (1986). Phatophysiologi: clinical concepts of disease processes. The Journal of Pathology: Clinical Research, 150(3): 223-223. DOI: https://www.doi.org/10.1002/path.1711500312/

Purnima MTE, Plumeriastuti H and Widjaja NMR (2013). The effect of borax on duodenal histopathological changes in rats (Rattus norvegicus). Antimicrobial Resistance on Livestock Production Veterinary Reproduction.

Sairazi NSM, Sirajudeen KNS, Asari MA, Mummedy S, Muzaimi M and Sulaiman SA (2017). Effect of tualang honey against KA-induced oxidative stress and neurodegeneration in the cortex of rats. BMC Complementary and Alternative Medicine, 17: 31. DOI: https://www.doi.org/10.1186/s12906-016-1534-x

Sugiyatmi S, Sulistiyani S and Hanani DY (2015). Analisis faktor risiko pencemaran bahan toksik boraks dan pewarna pada makanan jajanan tradisional yang dijual di pasar-pasar kota semarang tahun 2006. Jurnal Kesehatan Lingkungan Indonesia, 5(1): 29-38. DOI: https://www.doi.org/10.14710/jkli.5.1.29-38

Tanaka M, Sotta N, Yamazumi Y, Yamashita Y, Miwa K, Murota K, Chiba Y, Hirai MY, Akiyama T, Onouchi H, Naito S, and Fujiwara T (2016). The minimum open reading frame, AUG-Stop, induces boron-dependent ribosome stalling and mRNA degradation. The Plant Cell, 28(11): 2830-2849. DOI: https://www.doi.org/10.1105/tpc.16.00481

Wagner HK and Wolff PM (1976). New natural products and plants drugs with pharmacologycal biologycal or therapeutical activity. Proceedings of the First International Congress on Medicinal Plant Research, Section A, held at the University of Munich, Germany, 37-38. Available at: https://www.springer.com/gp/book/9783642666841

To cite this paper: Elfan F, Kuncorojakti S, and Triakoso N (2021). The Effects of Borax ($NA_2B_4O_710H_2O$) on Histopathology of Wistar Rats' Cerebrum (*Rattus norvegicus*). *World Vet. J.*, 11 (3): 484-488. DOI: https://dx.doi.org/10.54203/scil.2021.wvj62

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 489-497, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt

Rabie Ragab Sadek[1], Samy Abou-Bakr[1], Ali Attia Nigm[1], Mohamed Abd El-Aziz Mohamed Ibrahim[1], Mohamed Magdy Badr[2], and Mohamed Attia Ahmed Awad[1]*

[1]*Department of Animal Production, Faculty of Agriculture, Cairo University, Giza 12613, Egypt*
[2]*Company of 4Genetics for Dairy Solutions, Cairo, Egypt*

*Corresponding author's Email: mohamed.awad@agr.cu.edu.eg; ORCID: 0000-0001-7443-8724

ORIGINAL ARTICLE
pii: S232245682100063-11
Received: 08 July 2021
Accepted: 29 August 2021

ABSTRACT

The present study was carried out to compare the milk yield and reproductive performance of pure Holstein (HO) cows with those of their first generation (F1) crossbreds with Montbeliarde cows (MO) in four commercial dairy herds under Egyptian conditions. Data used in the current study comprised 2268 records for the first four lactations of 531 HO and 536 MO × HO F1 crossbred cows during the period between 2012 and 2020. Data were analyzed using the least squares method by XLSTAT software. The MO × HO crossbred cows were significantly superior compared with pure HO cows for 305-day milk yield, scoring 9210 ± 96 kg versus 7987 ± 149 kg. Moreover, MO × HO F1 crossbred cows had a significantly higher daily milk yield (30.0 ± 0.45 kg) than pure HO cows (25.9 ± 0.52 kg). However, pure HO cows had significantly greater days in milk (399 ± 6 days) than MO × HO crossbred cows (341 ± 5.2 days). With regard to reproductive performance, MO × HO F1 crossbred cows had significantly less number of services per conception and days open than pure HO cows (2.6 ± 0.16 vs. 3.7 ± 0.18) and (132 ± 5.2 days vs. 190 ± 6 days), respectively. However, the statistical difference between MO × HO F1 crossbred cows and pure HO cows for age at first calving was not significant (22.9 ± 0.11 vs. 23.1 ± 0.15 months, respectively). It can be concluded that under Egyptian subtropical conditions, the first generation of MO × HO crossbred cows exhibit better performance, compared to pure HO cows in milk yield and reproductive traits. These findings could provide an effective strategic option for the genetic improvement of dairy cattle in hot subtropical regions.

Keywords: Crossbreeding, Egypt, Holstein, Milk Yield, Montbeliarde, Reproduction

INTRODUCTION

Over the last decades, worldwide milk production has been dominated by the Holstein (HO) breed due to the intensive continuous selection for milk production (Heins et al., 2012). However, the superiority in milk production had direct negative effects on other functional traits related to survival and reproduction as a result of consistent increases of inbreeding leading to higher rates of culling and reduction of profitability (Heins et al., 2012; Puppel et al., 2018).

To overcome these challenges, dairy cattle holders have tended to use the crossbreeding between pure Holstein and other dairy breeds. Crossbreeding dairy breeds may be a feasible way to achieve significant improvement in milk yield, fertility, and health characteristics more quickly than pure breeding (Dezetter et al., 2017). Crossbreeding seeks to take advantage of positive heterosis effects and complementarity between dairy breeds by introducing the desirable genes and decreasing the rate of inbreeding (Sørensen et al., 2008; Knob et al., 2020; Clasen et al., 2021).

Several previous studies have been conducted to compare the performance of pure Holstein with their first generation (F1) crosses, including HO × Simmental cows (Knob et al., 2020; Knob et al., 2021), HO ×Viking Red cattle (Hazel et al., 2017a), HO × Nordic Red cattle (Clasen et al., 2018), HO × Jersey cows (Prendiville et al., 2010), and HO × Brown Swiss (Blöttner et al., 2011; El-Tarabany et al., 2016). These studies had shown positive outcomes of crossbred cows compared with pure HO cows especially for fertility, health, and survival traits with the possibility to increase the rate of genetic gain for these economically valuable traits (Shonka-Martin et al., 2019; Clasen et al., 2021).

Recently, the Montbeliarde (MO) breed has received considerable interest as the best complement with Holstein in crossbreeding programs (Hazel et al., 2017a). Montbeliarde is a French breed that was subjected to heavy selection for fertility, health traits, body condition, and milk quality traits (Heins and Hansen, 2012; Hazel et al., 2017a). Numerous comparative studies have been conducted between pure HO and MO × HO crossbreds to assess their productive and reproductive performances. The productive traits in terms of milk, fat, and protein yields were very close between the two genetic groups with slightly higher estimates for pure HO cows (Hazel et al., 2013; Buckley et al., 2014; Hazel et al., 2014).

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

However, MO × HO crossbred cows revealed superiority compared to pure HO cows for fertility traits in terms of first-service conception rates, days open, days to first breeding, and a number of services per conception (Hazel et al., 2014; Hazel et al., 2017a). Likewise, MO × HO crossbred cows had advantages over pure HO cows for survival traits, including survival of subsequent calving and mortality rates (Heins et al., 2012; Hazel et al., 2014; Hazel et al., 2017a). Consequently, MO × HO crossbred cows had greater longevity and lower total health cost per cow than pure HO cows which means greater profitability for crossbreds. Furthermore, the calves resulted from the mating of Montbeliarde sires with Holstein dams had significantly greater birth weight than calves from pure HO with no significant increase in calving difficulty and stillbirth rate (Heins et al., 2010). Also, milk from MO × HO crossbred cows exhibits a lower somatic cell score compared with milk from pure HO cows (Heins and Hansen, 2012).

In Egypt, the performance of HO and its crosses with different breeds has been intensively studied (Ibrahim et al., 2009; Rushdi, 2015; El-Tarabany et al., 2016). But, no available studies were found concerning the milk yield or reproductive traits on MO × HO crosses. Therefore, this work is considered the first study on the Montbeliarde cows in Egypt. The objective was to compare the milk yield and reproductive performance of pure Holstein cows with those of first generation crossbreds of Montbeliarde with Holstein cows in four commercial dairy herds in Egypt.

MATERIALS AND METHODS

Ethical approval

The study was carried out with existing records from four commercial dairy herds and did not involve animal handling.

Data collection

Data of productive and reproductive traits used in the current study were collected from 2268 records for the first four lactations of 531 pure HO and 536 first generation crossbred MO × HO cows. These records covered the period from 2012 to 2020. Pure Ho cows were daughters of 42 sires, whereas, MO × HO crossbred cows were daughters of 23 Montbeliarde sires. Cows in this study either pure HO or MO × HO crossbred were generated from artificial insemination (AI) proven bulls from the United States for both HO and MO breeds mated to locally born pure Holstein dams. The locally born HO cows originated from European HO dams. Data used were provided from four commercial dairy herds where pure HO and MO × HO crossbred cows were kept together all the time. These herds were located at four different governorates namely (herd one: Helaly farm, Dakahlia governorate, herd two: Osama Nigm farm, Gharbia governorate, herd three: Elyosr farm, Ismailia governorate, and herd four: Shash farm, Sharkia governorate). Table 1 indicates the distribution of the two genotypes among the four herds. All the data for the four herds were entered using Dairy Live 0.3 software (Version, 5.208A, USA) manually by the managers and owners.

Table 1. Distribution of Holstein cows and Montbeliarde × Holstein F1 crossbred cows among the four herds

Herd	HO	MO × HO	Total
Helaly farm, Dakahlia governorate	25	64	89
Osama Nigm farm, Gharbia governorate	64	73	137
Elyosr farm, Ismailia governorate	412	270	682
Shash farm, Sharkia governorate	30	129	159
Total	531	536	106

HO: Holstein cows, MO × HO: Montbeliarde × Holstein F1 crossbred cows

Herd management

All cows in the four herds were fed an *ad libitum* total mixed ration (TMR) diet consisting of 50 % forage and 50 % concentrate and was adjusted monthly to account for dry matter. The ingredients consist of corn silage, Alfalfa hay or Egyptian clover, soybean meals, ground corn, vitamins and minerals, yeast, and other additives. Cows were fed 4-5 times a day by a TMR mixer. Cows were machine milked three times per day and housed in open yards equipped with a cooling system in groups depending on milk production level and lactation number. Automated recording of milk yield was conducted on daily basis to calculate the aggregated total milk production for each cow. Cows were dried off two months before the expected calving dates. The crossbred heifers have inseminated artificially for the first time when reached 12-13 months and 350-370 Kg. The pregnant cows were determined via ultrasound at 28-33 days after insemination, follow-up confirmation via rectal palpation at approximately 60 and 100 days after inseminated.

Studied traits

The milk yield traits were total milk yield (TMY, kg) calculated by the cumulated amount of milk yield in kilograms of a cow throughout the lactation period, 305-day milk yield (305-dMY, kg) calculated using the International Committee for Animal Recording equation (ICAR, 2000) as follows:

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

The 305-dMY = [(TMY+405) / (100+LP)]

where, TMY is the total milk yield and LP signifies lactation period, days in milk (DIM, days) is defined as a number of days in milk from calving to drying-off date, and daily milk yield (DMY, kg) is calculated by dividing total milk yield in kilograms by lactation period length in days. The reproductive traits included age at first calving (AFC, months) which is defined as the number of months from birth to first calving date of the cow, number of services per conception (NSPC) denotes the number of artificial insemination times required for each cow to be pregnant, and days open (DO, days) is estimated by the number of days from calving date to conception date. The descriptive statistics of the milk yield and reproductive studied traits dataset are presented in Table 2.

Table 2. Descriptive statistics of the milk yield and reproductive studied traits for Holstein cows and Montbeliarde × Holstein F1 crossbred cows in Egypt

Variable	N	Minimum	Maximum	Mean	SD
Milk yield traits					
Total milk yield (kg)	2268	2131	22491	9597	2226
305-day milk yield (kg)	2268	1833	21376	8544	2299
Days in milk (days)	2268	230	1071	358	99.3
Daily milk yield (kg)	2268	5.6	72.5	28.3	8.61
Reproductive traits					
Age at first calving (months)	1045	20	33	24	2.7
Number of services per conception	2268	1	18	3.2	2.7
Days open (days)	2268	23	866	151	99.1

N: Number of observations, SD: Standard deviation

Statistical analysis

Data were analyzed using the least square means technique as applied in XLSTAT 2020.3.1.27 program with the following two statistical models.

To analyze TMY, 305-dMY, DIM, DMY, NSPC, and DO, model 1 was used as follows:

$Y_{ijklmn} = \mu + G_i + H_j + P_k + S_l + Y_m + (G \times H)_{ij} + e_{ijklmn}$ (Model 1)

Where, Y_{ijklmn} is the observations on TMY, 305-MY, DIM, DMY, NSPC, and DO, μ refers to the overall mean, G_i stands for the fixed effect of the i^{th} genotype (i=1, 2), in which, 1: pure HO cows and 2: HO × MO F1 crossbred cows, H_j signifies the fixed effect of the j^{th} herd (j:1, 2, 3 and 4), where, 1: Helaly farm, Dakahlia governorate; 2: Osama Nigm farm, Gharbia governorate; 3: Elyosr farm, Ismailia governorate and 4: Shash farm, Sharkia governorate, P_k is the fixed effect of the k^{th} parity (k:1, 2, 3 and 4), S_l refers to the fixed effect of the l^{th} season of calving (l:1, 2, 3 and 4), where, 1: winter (December to February), 2: spring (March to May), 3: summer (June to August), and 4: autumn (September to November), Y_m signifies the fixed effect of the m^{th} year of calving, starting from 2012 and ending by 2020, $(G \times H)_{ij}$ is the effect of the interaction between i^{th} genotype and j^{th} herd, and e_{ijklmn} is residual error assumed to be N I D (0, $\sigma^2 e$).

To analyze AFC, model 2 was employed.

$Y_{ijkmn} = \mu + G_i + H_j + S_k + Y_m + e_{ijkmn}$ (Model 2)

Where, Y_{ijkmn} is the observations of AFC, μ refers to the overall mean, G_i stands for the fixed effect of the i^{th} genotype (i:1, 2), where, 1: pure HO cows and 2: HO × MO F1 crossbred cows, H_j signifies the fixed effect of the j^{th} herd (j:1, 2, 3 and 4), where, 1: Helaly farm, Dakahlia governorate; 2: Osama Nigm farm, Gharbia governorate; 3: Elyosr farm, Ismailia governorate and 4: Shash farm, Sharkia governorate, S_k is the fixed effect of the k^{th} season of birth (l:1, 2, 3 and 4), where, 1: winter (December to February), 2: spring (March to May), 3: summer (June to August), and 4: autumn (September to November), Y_m is the fixed effect of the m^{th} year of birth, starting from 2011 and ending by 2017, and e_{ijkmn} is residual error assumed to be N I D (0, $\sigma^2 e$).

RESULTS AND DISCUSSION

Milk yield

The least squares means (LSM) and standard errors (SE) for TMY, 305-dMY, DIM, and DMY are presented in Table 3. It could be observed that MO × HO crossbred cows have higher TMY (9827 ± 119 kg), compared with pure HO cows (9616 ± 138 kg) but the difference was not statistically significant ($p > 0.05$). On the other hand, MO × HO crossbred cows had significantly ($p < 0.05$) higher 305-dMY (9210 ± 96 kg) than pure HO (7987 ± 149 kg). Pure HO cows had significantly greater DIM (399 ± 6 days) than MO × HO crossbred ones (341 ± 5.2 days). Crossbred cows showed significantly superior milk yield performance, compared to pure HO cows due mainly to their higher DMY (30.0 ± 0.45 kg versus 25.9 ± 0.52 kg, respectively).

The herd had a significant effect on TMY, 305-dMY, and DMY ($p < 0.05$) with the highest values observed for herd one (Helaly farm, Dakahlia governorate, Egypt), scoring 10195 ± 179 kg, 9003 ± 191 kg, and 29.4 ± 0.67 kg for

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

TMY, 305-dMY, and DMY, respectively. While the lowest values were recorded for herd three (Elyosr farm, Ismailia governorate, Egypt), having 8933 ± 161 kg, 7910 ± 148 kg, and 25.6 ± 0.61 kg for TMY, 305-dMY, and DMY, respectively. However, the length of DIM was not significantly different across herds.

The interaction of the genotype and herd was significant for all productive traits ($p < 0.05$, Table 4). The LSM of TMY, 305-dMY, DIM, and DMY for genotype by herd revealed that MO × HO crossbred cows exhibited significant superiority than pure HO cows under the same management system for TMY, 305-dMY, and DMY across herds one, two, and four with significantly longer DIM for pure HO cows. Among the different herds, MO × HO crossbred cows located in herd one had a magnitude advantage for TMY and DMY over other crossbreds herds. However, pure HO cows exhibited a significant superiority over MO × HO crossbred cows for TMY in herd three with relatively higher LSM of 305-dMY and DMY for MO × HO cows, compared to HO cows without any significant differences for 305-dMY and DMY between the two genotypes.

Table 3. Least squares means and standard errors of milk yield traits for Holstein cows and Montbeliarde × Holstein F1 crossbred cows in Egypt

Classification	N	Traits			
		TMY (kg)	305-dMY (kg)	DIM (days)	DMY(kg)
		LSM±SE	LSM±SE	LSM±SE	LSM±SE
The overall mean	2268	9722 ± 47	8598 ± 48	370 ± 2.1	27.9 ± 0.18
Genotype		NS	*	*	*
HO	1096	9616^{a} ± 138	7987^{a} ± 149	399^{b} ± 6	25.9^{a} ± 0.52
MO × HO	1172	9827^{a} ± 119	9210^{b}± 96	341^{a} ± 5.2	30.0^{b} ± 0.45
Herd		*	*	NS	*
1	236	10195^{c} ± 179	9003^{b} ± 191	370^{a} ± 7.8	29.4^{b} ± 0.67
2	311	10094^{c} ± 153	8923^{b} ± 178	372^{a} ± 6.6	28.8^{b} ± 0.57
3	1338	8933^{a} ± 161	7910^{a} ± 148	369^{a} ± 7	25.6^{a} ± 0.61
4	383	9664^{b} ± 182	8557^{b} ± 193	368^{a} ± 7.9	28^{b} ± 0.68
Parity		*	*	NS	*
1	1052	9384^{a} ± 109	7999^{a} ± 106	377^{a} ± 4.7	26.6^{a} ± 0.41
2	768	9603^{b} ± 121	8735^{b} ± 108	367^{a} ± 5.2	27.8^{b} ± 0.45
3	344	9714^{bc} ± 152	8748^{b} ± 150	366^{a} ± 6.6	28.1^{b} ± 0.57
4	104	10185^{c} ± 237	8910^{b} ± 263	370^{a} ± 10.3	29.3^{b} ± 0.89
Season of calving		*	*	*	*
Winter	736	9522^{a} ± 123	8622^{b} ± 110	363^{b} ± 5.4	28.3^{b} ± 0.46
Spring	243	9783^{ab} ± 181	8221^{a} ± 166	397^{d} ± 7.8	26.5^{a} ± 0.68
Summer	427	9731^{ab} ± 145	8503^{ab} ± 128	375^{c} ± 6.3	27.1^{a} ± 0.54
Autumn	862	9850^{b} ± 119	9047^{c} ± 101	345^{a} ± 5.2	29.9^{c} ± 0.45
Year of calving		*	*	*	*
2012	13	8866^{a} ± 622	7223^{a} ± 589	348^{b} ± 27	26.1^{bc} ± 2.33
2013	69	8575^{a} ± 288	7112^{a} ± 214	417^{e} ± 12.5	22.5^{a} ± 1.08
2014	145	8907^{a} ± 212	7457^{a} ± 202	406^{de} ± 9.2	23.6^{ab} ± 0.80
2015	200	9282^{a} ± 181	7953^{b} ± 188	385^{cde} ± 7.9	26^{bc} ± 0.68
2016	317	10146^{b} ± 140	8569^{c} ± 146	382^{cd} ± 6.1	28.4^{cd} ± 0.53
2017	348	10656^{b} ± 137	9522^{d} ± 142	371^{bc} ± 6	30.7^{de} ± 0.51
2018	553	10382^{b} ± 137	9106^{d} ± 141	361^{bc} ± 5.9	30.5^{de} ± 0.51
2019	572	10185^{b} ± 141	9283^{d} ± 145	346^{b} ± 6.1	30.6^{de} ± 0.53
2020	51	10495^{b} ± 333	9323^{d} ± 374	313^{a} ± 14.5	33.5^{e} ± 1.25
Genotype and herd interaction	2268	*	*	*	*

Means followed by different superscripts within each column are significantly different (*$p < 0.05$), LSM±SE: Least squares means ± standard errors, N: Number of observations, NS: Non-significant, TMY: Total milk yield, 305-dMY: 305-day milk yield, DIM: Days in milk, DMY: Daily milk yield, HO: Holstein cows, MO × HO: Montbeliarde × Holstein F1 crossbred cows, herd 1: Helaly farm, Dakahlia governorate, herd 2: Osama Nigm farm, Gharbia governorate, herd 3: Elyosr farm, Ismailia governorate, herd 4: Shash farm, Sharkia governorate

Table 4. Least squares means and standard errors of interaction between genotypes and cow herds

Traits	Herd 1		Herd 2		Herd 3		Herd 4	
	HO (n=25)	MO × HO (n=64)	HO (n=64)	MO × HO (n=73)	HO (n=412)	MO × HO (n=270)	HO (n=30)	MO × HO (n=129)
TMY (kg)	10001^{cd} ± 283	10389^{d}±189	9800^{c} ± 200	10388^{d}±192	9166^{b} ± 163	8700^{a} ± 182	9498^{bc} ± 293	9830^{c} ± 158
305-dMY (kg)	8102^{a} ± 300	9904^{c} ± 196	7908^{a} ± 239	9938^{c} ± 207	7790^{a} ± 161	8030^{a} ± 183	8146^{a} ± 325	8967^{b} ± 151
DIM (days)	415^{de} ± 12.3	326^{a} ± 8.2	416^{e} ± 8.7	327^{a} ± 8.4	382^{c} ± 7.1	356^{b} ± 7.9	382^{cd} ± 12.7	354^{b} ± 6.8
DMY(kg)	26^{a} ± 1.06	32.9^{c} ± 0.71	25.2^{a} ± 0.75	32.4^{c} ± 0.72	25.4^{a} ± 0.61	25.7^{a} ± 0.68	26.9^{ab} ±1.10	29.1^{b} ± 0.59
NSPC	3.9^{cd} ± 0.34	2.1^{a} ± 0.23	3.8^{cd} ± 0.25	2.0^{a} ± 0.24	4.1^{d} ± 0.26	3.8^{cd} ± 0.28	3.2^{bc} ± 0.39	2.4^{ab} ± 0.20
DO (days)	204^{d} ± 12.3	116^{a} ± 8.2	205^{d} ± 8.7	116^{a} ± 8.4	176^{c} ± 7.1	149^{b} ± 7.9	175^{c} ± 12.7	145^{b} ± 6.8

Means within a row with different superscript are significantly different (*$p < 0.05$), Results are expressed as mean value ± standard error, HO: Holstein cows, MO × HO: Montbeliarde × Holstein F1 crossbred cows, TMY: Total milk yield, 305-dMY: 305-day milk yield, DIM: Days in milk, DMY: Daily milk yield, NSPC: Number of services per conception, DO: Days open, n: Number of cows, herd 1: Helaly farm, Dakahlia governorate, herd 2: Osama Nigm farm, Gharbia governorate, herd 3: Elyosr farm, Ismailia governorate, herd 4: Shash farm, Sharkia governorate

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

Parity had a significant effect ($p < 0.05$) on TMY, 305-dMY, and DMY. The three traits increased gradually with increasing parity order. The LSM of TMY, 305-dMY, and DMY were the highest (10185 ± 237 kg, 8910 ± 263 kg, and 29.3 ± 0.89 kg, respectively) at the fourth lactation. The length of DIM was not significantly different across parities.

Season of calving explained a significant variation for all productive traits ($p < 0.05$). The LSM highest estimates for TMY, 305-dMY, and DMY (9850 ± 119 kg, 9047 ± 101 kg, and 29.9 ± 0.45 kg, respectively) were observed in autumn. However, the lowest estimate for TMY (9522 ± 123 kg) was observed in winter, whereas the lowest estimates for 305-dMY (8221 ± 166 kg) and DMY (26.5 ± 0.68 kg) were observed in spring. The length of DIM was significantly different across seasons. The DIM was significantly longer in spring and shorter in autumn.

Year of calving significantly affected all the studied productive traits ($p < 0.05$). Cows calved in 2013 had significantly longer DIM (417 ± 12.5 days), compared with other years. On the contrary, the LSM for TMY (8575 ± 288 kg), 305-dMY (7112 ± 214 kg), and DMY (22.5 ± 1.08 kg) were significantly lower for cows calving in 2013. On the other hand, DIM (313 ± 14.5 days) was significantly shorter for dams that calved in 2020. However, cows that calved in 2017 had significantly greater TMY (10656 ± 137 kg), 305-dMY (9522 ± 142 kg), and DMY (30.7 ± 0.51 kg). It could be observed from Table 3 that the LSM of TMY for calving years (2012 to 2015) was significantly different from the other years.

In contrast to the findings of the present study, many studies have shown that pure Holstein cows tended to have higher total milk yield, and consequently higher daily milk yield than MO × HO crossbred cows during a uniformed lactation period of 305 days. Heins et al. (2006) reported that pure Holstein cows had significantly higher 305-days milk yield (9757 kg) than MO × HO cows (9161 kg) during the first lactation. Likewise, Heins and Hansen (2012) found that pure Holstein cows had significantly higher 305-day milk yield (11417 kg), compared to MO × HO crossbred cows (10744 kg) across the first five lactations. Hazel et al. (2014) observed higher 305-dMY for pure HO cows versus MO ×HO crossbreds without significant difference during their first five lactations. Furthermore, Hazel et al. (2013) reported that MO × HO crossbred cows had significantly lower total milk yield, compared to pure HO cows during the first 150 days of the first lactation. Likewise, 305-dMY was lower for MO × HO crossbred, compared to HO cows without significant difference.

The higher trend of TMY, 305-dMY, and DMY for MO × HO crossbred cows than pure HO cows in the current study could be attributed to the positive heterotic effect for these traits. According to Sørensen et al. (2008), the heterosis effect for yield traits was approximately 3% depending on the average production of the parental purebreds that were crossed as well as environmental conditions. Furthermore, the pure Montbeliarde breed is genetically adapted to hot climates and has a higher heat stress tolerance than pure HO cows (Allouche et al., 2018; Ouarfli and Chehma, 2018). Consequently, MO × HO crossbred cows were able to produce more efficiency under Egyptian subtropical environmental conditions than pure HO cows.

Reproductive performance

Table 5 shows the LSM of NSPC and DO. The effect of genotype on NSPC and DO was significant ($p < 0.05$). Crossbred cows (MO × HO) had significantly lower ($p < 0.05$) NSPC and DO than pure HO cows (2.6 ± 0.16 vs. 3.7 ± 0.18 for NSPC) and (132 ± 5.2 days vs. 190 ± 6 days for DO), respectively with significant advantages of approximately one dose fewer for NSPC and 58 days fewer for DO.

Herds differed significantly ($p < 0.05$) in NSPC which was significantly greater (3.9 ± 0.25) in herd three (Elyosr farm, Ismailia governorate, Egypt). However, DO estimates were not significantly different across herds. The interactions of genotype and herd were significant ($p < 0.05$) for NSPC and DO traits (Table 4). The MO × HO crossbred cows showed significantly lower NSPC and DO, compared to pure HO cows within the same herd with a magnitude advantage for crossbreds found in the first two herds than in the third and fourth herds. Parity had no significant effect on both NSPC and DO. Season of calving explained a significant variation for the two reproductive traits. The NSPC was significantly greater in winter and spring (3.4 ± 0.17 and 3.8 ± 0.27, respectively) compared to summer and autumn (2.7 for both). Dams calved in autumn had significantly shorter DO (136 ± 5.2 days), while cows calved in spring and summer had longer DO (188 ± 7.9 and 165 ± 6.3 days, respectively). Year of calving was a significant source of variation in both NSPC and DO. Cows calved in 2013 had significantly greater NSPC (3.7 ± 0.36) and longer DO (206 ± 12.5 days) compared with other years. Cows calved in 2012 scored the lowest NSPC (2.5 ± 0.74), and those calves in 2020 had significantly the shortest DO (105 ± 14.5 days).

Many studies arrived at the same results of the current study concerning the superiority of MO × HO crossbred cows compared to pure Holstein cows in reproductive traits. Hazel et al. (2017a) reported that the MO × HO cows had fewer bred times (2.07) than pure HO cows (2.30) during the first lactation in eight high-performance dairy herds in the United States. Furthermore, Malchiodi et al. (2014) found that the MO × HO cows were inseminated fewer times (2.02) than the first lactation pure HO cows (2.53). However, Walsh et al. (2008) observed no significant difference in the NSPC between MO × HO cows (1.97) and pure Holstein-Friesian cows (1.98) in Ireland. The results of DO were in

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

accordance with those of Hazel et al. (2014) who reported that MO × HO cows had significantly shorter DO (128 days), compared with that of pure HO cows (167 days) during their first five lactations. Similarly, Hazel et al. (2017a) found that MO × HO cows had fewer DO (113 days) than pure HO cows (125 days) during the first lactation. The same trend was observed by Malchiodi et al. (2014) who found that DO was 83.7 days for MO × HO cows versus 109.1 days for HO cows in the first lactation. Heins et al. (2012) also found that DO was 19 days longer for pure HO cows compared with MO × HO crossbred. Furthermore, the current findings agreed with those of Heins and Hansen (2012) who observed that the advance of parity in MO × HO crossbred cows led to a significantly fewer DO ranging from 131 days in the first lactation to 110 days in the fifth lactation. On the other hand, the authors of this study found that pure HO cows tended to have greater DO with the advance of parity with a range from 148 days in the first lactation to 157 days in the fifth lactation.

Table 5. Least squares means and standard errors of number of services per conception and days open for Holstein cows and Montbeliarde × Holstein F1 crossbred cows in Egypt

Classification	N	Traits	
		NSPC	DO (days)
		LSM±SE	LSM±SE
The overall mean	2268	3.2 ± 0.06	161 ± 2.1
Genotype		*	*
HO	1096	3.7^{b} ± 0.18	190^{b} ± 6
MO × HO	1172	2.6^{a} ± 0.16	132^{a} ± 5.2
Herd		*	NS
1	236	3.0^{a} ± 0.22	160^{a} ± 7.8
2	311	2.9^{a} ± 0.19	161^{a} ± 6.6
3	1338	3.9^{b} ± 0.25	162^{a} ± 7
4	383	2.8^{a} ± 0.24	160^{a} ± 7.9
Parity		NS	NS
1	1052	3.4^{bc} ± 0.15	168^{a} ± 4.7
2	768	3.4^{c} ± 0.17	158^{a} ± 5.2
3	344	3^{ab} ± 0.21	157^{a} ± 6.6
4	104	2.8^{a} ± 0.32	160^{a} ± 10.3
Season of calving		*	*
Winter	736	3.4^{b} ± 0.17	154^{b} ± 5.4
Spring	243	3.8^{b} ± 0.27	188^{d} ± 7.9
Summer	427	2.7^{a} ± 0.20	165^{c} ± 6.3
Autumn	862	2.7^{a} ± 0.16	136^{a} ± 5.2
Year of calving		*	*
2012	13	2.5^{a} ± 0.74	138^{b} ± 27
2013	69	3.7^{c} ± 0.36	206^{e} ± 12.5
2014	145	3.6^{bc} ± 0.27	196^{de} ± 9.2
2015	200	3.1^{abc} ± 0.23	174^{cde} ± 7.9
2016	317	3.0^{abc} ± 0.19	173^{bcde} ± 6.1
2017	348	2.7^{ab} ± 0.19	162^{bcd} ± 6
2018	553	3.4^{abc} ± 0.22	153^{bc} ± 6
2019	572	3.4^{abc} ± 0.23	138^{b} ± 6.1
2020	51	2.9^{abc} ± 0.53	105^{a} ± 14.5
Genotype and herd interaction	2268	*	*

Means followed by different superscripts within each column are significantly different (*p < 0.05), LSM±SE: Least squares means ± standard errors, N: number of observations, NS: Non-significant, NSPC: Number of services per conception, DO: Days open, HO: Holstein cows, MO × HO: Montbeliarde × Holstein F1 crossbred cows, herd 1: Helaly farm, Dakahlia governorate, herd 2: Osama Nigm farm, Gharbia governorate, herd 3: Elyosr farm, Ismailia governorate, herd 4: Shash farm, Sharkia governorate

Table 6 shows the LSM of age at first calving (AFC). The values of AFC for the two genotypes were very closed (22.9 ± 0.11 and 23.1 ± 0.15 months for MO × HO and pure HO cows, respectively) and the difference was not significant (p = 0.156). A similar trend was recorded by Hazel et al. (2017b) who found that there was no significant difference for AFC between MO × HO crossbred cows and pure HO cows (23.8 vs. 23.9 months, respectively). All herds could significantly affect AFC (p < 0.05) with the highest value observed for Shash farm, Sharkia governorate (25.7 ± 0.20 months). While heifers of herd two (Osama Nigm farm, Gharbia governorate, Egypt) had the lowest AFC with 21 ± 0.19 months. The effect of the season of birth was significant (p < 0.05) on AFC. Heifers born in spring had significantly lower AFC (22.6 ± 0.16 months) and calved for the first time 0.7 months earlier than those born in summer (23.3 ± 0.16 months). Also, the year of birth affected significantly AFC (p < 0.05). Heifers born in 2015 significantly showed the highest AFC of 24.1 ± 0.18 months, compared with other years.

In the current study, despite MO × HO crossbred cows scored a high mean of milk yield, they revealed better and significant reproductive performance than that of pure HO ones. This result showed the positive heterosis direction for

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

fertility along with milk yield traits for the first generation of MO × HO crossbred cows under Egyptian subtropical conditions.

Table 6. Least squares means and standard errors of age at first calving for Holstein cows and Montbeliarde × Holstein F1 crossbred cows in Egypt

Classification	N	Trait
		AFC (months)
		LSM±SE
The overall mean	1045	22.9 ± 0.08
Genotype		NS
HO	524	23.1^{a} ± 0.15
MO × HO	521	22.9^{a} ± 0.11
Herd		*
1	87	21.1^{a} ± 0.26
2	136	21^{a} ± 0.19
3	152	24.2^{b} ± 0.14
4	670	25.7^{c} ± 0.20
Season of birth		*
Winter	317	23.1^{bc} ± 0.13
Spring	172	22.6^{a} ± 0.16
Summer	177	23.3^{c} ± 0.16
Autumn	379	22.9^{ab} ± 0.13
Year of birth		*
2011	61	22.7^{a} ± 0.26
2012	105	22.7^{a} ± 0.21
2013	75	22.6^{a} ± 0.23
2014	172	23.1^{a} ± 0.17
2015	218	24.1^{b} ± 0.18
2016	271	23.1^{a} ± 0.19
2017	143	22.8^{a} ± 0.23

Means followed by different superscripts within each column are significantly different (*p < 0.05), LSM±SE: Least squares means ± standard errors, N: Number of observations, NS: Non-significant, AFC: Age at first calving, HO: Holstein cows, MO × HO: Montbeliarde × Holstein F1crossbred cows, herd 1: Helaly farm, Dakahlia governorate, herd 2: Osama Nigm farm, Gharbia governorate, herd 3: Elyosr farm, Ismailia governorate, herd 4: Shash farm, Sharkia governorate

The reasons behind the superiority of MO × HO crossbred cows, compared to pure Holstein cows, for fertility traits were explained in several previous studies. Hazel et al. (2013) and Hazel et al. (2014) found that the body traits of MO × HO crossbred cows were characterized by significantly greater body weight and body condition score (BCS) without consuming extra dry matter intake than pure HO cows across the first five lactations. The larger body characteristics for MO × HO crossbred cows were attributed to continuous selection for BCS and feed efficiency for pure MO breed that was in contrast to breeding goal for HO breed to enhance angularity features. Greater BCS at the time of calving or less loss in BCS after calving increases the reproductive ability for cows in the next parity (Hazel et al., 2014). Likewise, greater BCS for MO × HO crossbred cows was associated with enhancement of immunity and a lower rate of health problems, and consequently, improved the reproductive performance for MO × HO crossbred cows than pure HO cows (Walsh et al., 2008; Hazel et al., 2013; Hazel et al., 2017a).

Furthermore, Sørensen et al. (2008) stated that the heterosis estimate for fertility traits was at least 10% for the first generation crosses when unrelated two breeds are mating involving HO cows. The power of the heterosis for fertility traits could be generated from the new combinations of additive genetic effects in the F1 crosses. Moreover, crossing between unrelated breeds contributed to significant changes in genes interaction within and among loci in terms of non-additive genetic effects of dominance and epistasis (Sørensen et al., 2008). In addition, crossbreeding between genetically different breeds leads to an increase in the proportion of animals with heterozygous loci over the animals with homozygous loci especially when the two breeds had a greater genetic distance; and consequently, obtain crossbred with better performance compared with the average of the purebred parental breeds (Sørensen et al., 2008). According to Malchiodi et al. (2014), the intensive selection for high milk production that HO cows were subjected to, may alter their metabolic physiology to use the energy for production over reproduction; however, MO × HO crossbred cows may have a different metabolic mechanism that allows to better respond to the physiological process of production, reproduction, and survival at the same time in early lactation.

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

The magnitude of economic advantages for reproductive superiority of MO × HO crossbred cows is due to lower costs required for hormonal treatment for synchronization programs, lower rate of culling for fertility problems, lower replacement costs, and a faster return to peak production at second lactation (Hazel et al., 2017a).

CONCLUSION

Under Egyptian subtropical conditions, the first generation of MO × HO crossbred cows showed better performance compared to pure HO cows for milk yield and reproductive traits. Crossing pure Holstein with Montbeliarde may provide an effective strategic option for the genetic improvement of dairy cattle raised in hot subtropical climates. Further studies are required to define the best gene combination (s) for crossing and also, phenotypic, genetic, and molecular characterization of the crossbreds are needed.

DECLARATIONS

Authors' contribution

R. R. Sadek, S. Abou-Bakr and A. A. Nigm designed the study. M. A. A. Awad and M. M. Badr collected the data. M. A. M. Ibrahim and M. A. A. Awad performed the editing and statistical analysis of data. M. A. A. Awad interpreted the results and wrote the manuscript. R. R. Sadek, S. Abou-Bakr and A. A. Nigm edited and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

Ethical issues including plagiarism, consent to publish, data fabrication, and double publication have been checked by all the authors.

Acknowledgments

The authors express gratitude to the four commercial dairy farms (Helaly farm, Dakahlia governorate, Egypt; Osama Nigm farm, Gharbia governorate, Egypt; Elyosr farm, Ismailia Governorate, Egypt, and Shash farm, Sharkia governorate, Egypt) for their assistance in providing data on the cows in their herds.

REFERENCES

Allouche L, Madani T, Mechmeche M, and Bouchemal A (2018). Reproductive performance of Montbeliard cows reared under subtropical environment: Effects of heat stress and acclimatization duration. Livestock Research for Rural Development, 30(7): 128. Available at: http://www.lrrd.org/lrrd30/7/lyall30128.html

Blöttner S, Heins BJ, Wensch-Dorendorf M, Hansen LB, and Swalve HH (2011). Short communication: A comparison between purebred Holstein and Brown Swiss × Holstein cows for milk production, somatic cell score, milking speed, and udder measurements in the first 3 lactations. Journal of Dairy Science, 94: 5212-5216. DOI: https://www.doi.org/10.3168/jds.2011-4255

Buckley F, Lopez-Villalobos N, and Heins BJ (2014). Crossbreeding: Implications for dairy cow fertility and survival. Animal, 8: 122-133. DOI: https://www.doi.org/10.1017/S1751731114000901

Clasen JB, Fogh A, and Kargo M (2018). Differences between performance of F1 crossbreds and Holsteins at different production levels. Journal of Dairy Science, 102: 436-441. DOI: https://www.doi.org/10.3168/jds.2018-14975

Clasen JB, Kargo M, Fikse WF, Strandberg E, Wallenbeck A, Østergaard S, and Rydhmer L (2021). Conservation of a native dairy cattle breed through terminal crossbreeding with commercial dairy breeds. Acta Agriculturae Scandinavica, Section A- Animal Science, 70: 1-12. DOI: https://www.doi.org/10.1080/09064702.2020.1867632

Dezetter C, Bareille N, Billon D, Côrtes C, Lechartier C, and Seegers H (2017). Changes in animal performance and profitability of Holstein dairy operations after introduction of crossbreeding with Montbéliarde, Normande, and Scandinavian Red. Journal of Dairy Science, 100: 8239-8264. DOI: https://www.doi.org/10.3168/jds.2016-11436

El-Tarabany MS, Roushdy EM, and El-Tarabany AA (2016). Production and health performance of Holstein, Brown Swiss and their crosses under subtropical environmental conditions. Animal Production Science, 57(6): 1137-1143. DOI: https://www.doi.org/10.1071/AN15809

Hazel AR, Heins BJ, and Hansen LB (2017a). Fertility, survival, and conformation of Montbéliarde × Holstein and Viking Red × Holstein crossbred cows compared with pure Holstein cows during first lactation in 8 commercial dairy herds. Journal of Dairy Science, 100: 9447-9458. DOI: https://www.doi.org/10.3168/jds.2017-12824

Hazel AR, Heins BJ, and Hansen LB (2017b). Production and calving traits of Montbéliarde × Holstein and Viking Red × Holstein cows compared with pure Holstein cows during first lactation in 8 commercial dairy herds. Journal of Dairy Science, 100: 4139-4149. DOI: https://www.doi.org/10.3168/jds.2016-11860

Hazel AR, Heins BJ, Seykora AJ, and Hansen LB (2013). Montbéliarde-sired crossbreds compared with pure Holsteins for dry matter intake, production, and body traits during the first 150 days of first lactation. Journal of Dairy Science, 96: 1915-1923. DOI: https://www.doi.org/10.3168/ jds .2012 -5667

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

Heins BJ, and Hansen LB (2012). Short communication: Fertility, somatic cell score, and production of Normande × Holstein, Montbéliarde× Holstein, and Scandinavian Red × Holstein crossbreds versus pure Holsteins during their first 5 lactations. Journal of Dairy Science, 95: 918-924. DOI: https://www.doi.org/10 .3168/ jds .2011-4523

Hazel AR, Heins BJ, Seykora AJ, and Hansen LB (2014). Production, fertility, survival, and body measurements of Montbéliarde-sired crossbreds compared with pure Holsteins during their first 5 lactations. Journal of Dairy Science, 97: 2512-2525. DOI: https://www.doi.org/10.3168/jds .2013-7063

Heins BJ, Hansen LB, and De Vries A (2012). Survival, lifetime production, and profitability of crossbreds of Holstein with Normande, Montbéliarde, and Scandinavian Red compared to pure Holstein cows. Journal of Dairy Science, 95: 1011-1021. DOI: https://www.doi.org/10.3168/jds.2011-4525

Heins BJ, Hansen LB, and Seykora AJ (2006). Production of pure Holsteins versus crossbreds of Holstein with Normande, Montbeliarde, and Scandinavian Red. Journal of Dairy Science, 89: 2799-2804. DOI: https://www.doi.org/10.3168/jds.S0022-0302(06)72356-6

Heins BJ, Hansen LB, Hazel AR, Seykora AJ, Johnson DG, and Linn JG (2010). Birth traits of pure Holstein calves versus Montbeliarde-sired crossbred calves. Journal of Dairy Science, 93: 2293-2299. DOI: https://www.doi.org/10.3168/jds.2009-2911

Ibrahim MAM, Rushdi HE, Abdel-Salam SAM, and Abou-Bakr S (2009). Genetic and phenotypic trends of calving interval and age at first calving in a commercial Holstein herd. Egyptian Journal of Animal Production, 46(2): 103-112. Available at: https://ejap.journals.ekb.eg/article_94034_3490d6fe45b6cbc3d46cd0d7ee1d95ac.pdf

International Committee for Animal Recording (ICAR) (2000). Yearly enquiry on the situation and results of cows with recording in member countries. Available at: https://www.icar.org/index.php/publications-technical-materials/milk-yearly-surveys-cow-sheep-goats/

Knob DA, Scholz AM, Alessio DR M, Mendes BPB, Perazzoli L, Kappes R, and Neto AT (2020). Reproductive and productive performance, udder health, and conformation traits of purebred Holstein, F1, and R1 crossbred Holstein × Simmental cows. Tropical Animal Health and Production, 52: 1639-1647. DOI: https://www.doi.org/10.1007/s11250-019-02174-9

Knob DA, Thaler Neto A, Schweizer H, Weigand AC, Kappes R, and Scholz AM (2021). Energy balance indicators during the transition period and early lactation of purebred Holstein and Simmental cows and their crosses. Animals, 11: 309. DOI: https://www.doi.org/10.3390/ani11020309

Malchiodi F, Cecchinato A, and Bittante G (2014). Fertility traits of purebred Holsteins and 2- and 3-breed crossbred heifers and cows obtained from Swedish Red, Montbéliarde, and Brown Swiss sires. Journal of Dairy Science, 97: 7916-7926. DOI: https://www.doi.org/10 .3168/ jds .2014-8156

Ouarfli L, and Chehma A (2018). Effects of thermal stress on milk production of the European breeds Holstein and Montbéliarde in a saharian zone. Livestock Research for Rural Development, 30(12):211. Available at: http://www.lrrd.org/lrrd30/12/ouarf30211.html

Prendiville R, Pierce KM, and Buckley F (2010). A comparison between Holstein-Friesian and Jersey dairy cows and their F1 cross with regards to milk yield, somatic cell score, mastitis, and milking characteristics under grazing conditions. Journal of Dairy Science, 93: 2741-2750. DOI: https://www.doi.org/10.3168/jds.2009-2791

Puppel K, Bogusz E, Gołębiewski M, Nałęcz-Tarwacka T, Kuczyńska B, Slósarz J, Budziński A, Solarczyk P, Kunowska-Slósarz M, and Przysucha T (2018). Effect of dairy cow crossbreeding on selected performance traits and quality of milk in first generation crossbreds, Journal of Food Science, 83: 229-236. DOI: https://www.doi.org/10.1111/1750-3841.13988

Rushdi HE (2015). Genetic and phenotypic analyses of days open and 305-day milk yield in a commercial Holstein Friesian herd. Egyptian Journal of Animal Production, 52(1): 107-112. Available at: https://journals.ekb.eg/article_93627_05819e706767d8a4601b3d6edbe89e79.pdf

Shonka-Martin BN, Heins BJ, and Hansen LB (2019). Three -breed rotational crossbreds of Montbéliarde, Viking Red, and Holstein compared with Holstein cows for feed efficiency, income over feed cost, and residual feed intake. Journal of Dairy Science, 102: 3661-3673. DOI: https://www.doi.org/10.3168/jds.2018-15682

Sørensen MK, Norberg E, Pedersen J, and Christensen LG (2008). Invited review: Crossbreeding in dairy cattle: A Danish perspective. Journal of Dairy Science, 91: 4116-4128. DOI: https://www.doi.org/10.3168/jds.2008-1273

Walsh S, Buckley F, Pierce K, Bryne N, Patton J, and Dillon P (2008). Effects of breed and feeding system on milk production, body weight, body condition score, reproductive performance, and postpartum ovarian function. Journal of Dairy Science, 91: 4401-4413. DOI: https://www.doi.org/10.3168/jds.2007-0818

To cite this paper: Sadek RR, Abou-Bakr S, Nigm AA, Ibrahim MAM, Badr MM and Awad MAA (2021). Evaluation of Milk Yield and Reproductive Performance of Pure Holstein and Its F1 Crossbreds with Montbeliarde in Egypt. *World Vet. J.,* 11 (3): 489-497. DOI: https://dx.doi.org/10.54203/scil.2021.wvj63

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 498-503, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*)

Agung Wahyu Prasetya[1], Kismiyati[1*], and Sri Subekti[2]

[1]*Department of Fish Health and Management and Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya-60115, Indonesia*
[2]*Department of Fish Health and Management and Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya-60115, Indonesia*

*Correspondence author's Email: miyati.59@gmail.com; ORCID: 0000-0003-3627-2895

SHORT COMMUNICATION
pii: S232245682100064-11
Received: 27 July 2021
Accepted: 17 September 2021

ABSTRACT

Marine fish, particularly the red snappers, are often exposed to helminth parasitic infestation. As a result of the parasitic infestation, the fish population, the fish weight, and the morphological changes in the fish are shrinking. The present research aimed to find out the intensity and predilection of the helminth ectoparasites over the infection of the red snapper (*Lutjanus argentimaculatus*) and employed the survey method for this purpose. The sampling was carried out by the purposive sampling technique. The sample obtained consisted of 30 fish, 20% of the total red snapper population of 150 fish reared in the floating net cages of Balai Besar Perikanan Budidaya Laut Lampung. The intensity of the fish infestation by a mixture of *Haliotrema epinepheli* and *Benedenia epinepheli* was 132.5 individuals/fish. *Neobenedenia girellae* and *Haliotrema epinepheli* infected fish with an intensity of 149.41 individuals/fish. The 66.7% of *Benedenia epinepheli* had a predilection for the dorsal fin, and 33.3% for the anal fin. In *Neobenedenia girellae*, 57.1% had a predilection for the body surface, 37.2% for the head surface, and 5.7% for the dorsal fin. In conclusion, all sampled fish were positively infected with helminth ectoparasites, including *Neobenedenia girellae, Haliotrema epinepheli*, and *Benedenia epinepheli*.

Keywords: Ectoparasite, Helminths, Infestation, Red snapper

INTRODUCTION

One of the main problems and challenges in red snapper cultivation is disease control (Hussan et al., 2016; Oliveira et al., 2020). Diseases of red snapper could occur in water due to imbalances in the environment, host, and pathogenic organisms (Engering et al., 2013; Hussan et al., 2016; Oliveira et al., 2020). Parasitic infestation and infection might also be attributed to high fish density and inadequate nourishment in fish, as well as poor water and environmental quality (Yadollahi et al., 2013; Hussan et al., 2016).

In nature, red snappers might be susceptible to a number of diseases or parasites (Cable et al., 2017; Oliveira et al., 2020). In the culture environment, the parasites might even be present in far greater numbers and could be lethal to fish (Omeji et al., 2011). Marine fish, particularly the red snappers, are often exposed to parasitic helminth infestation (Montoya-Mendoza et al., 2014; Oliveira et al., 2020). Visual results indicated that the most prevalent ectoparasitic worm in the red snapper was *Benedenia epinepheli*, which was found on the fin and skin (Jithendran et al., 2005; Loh et al., 2020). Meanwhile, *Neobenedenia girellae* (Kishimori et al., 2015), *Hatschekia poche* (Justine et al., 2012), *Haliotrema* species, and *Diplectanum squamatum* N. (Santos et al., 2002) were encountered on the gill.

A parasitic infestation could lead to economic losses. The economic impacts of parasites in fish include reduced fish population for consumption, decreased fish weight, and changes to fish morphology (Iwanowiez, 2011). The prevention and treatment of diseases had thus developed into an important cultivation component. Diseases might be caused by stress, pathogens (e.g., protozoans, bacteria, and viruses), environmental changes (e.g., algae bloom), overdose, and malnutrition (Pikarsky et al., 2004). Different causes could produce distinctive external symptoms of illness in fish, including sudden death, changes in behavior, loss of appetite, and bleeding in places where scales have peeled off (Madhun et al., 2015). Parasites infest or infect the red snapper in a number of ways by, for instance, parasitic contamination of the water in which red snappers are cultivated, direct contact, or rubbing with red snappers contaminated with parasites. In this case, the spread of the sick red snappers would have negative effects, especially if the density is too high. Furthermore, the equipment previously used to hand or transport parasite-contaminated red snappers would also cause parasite infestation or infection (Melianawati and Aryati, 2012).

Helminths are classified according to the International code. Every parasite belongs to particular phylum, class, order, genus, and species. Helminths per se are assigned to four different phyla, namely Platyhelminthes, Nemathelminthes, Acanthocephala, and Annelida (Gibson et al., 2014; Morand et al., 2015).

To cite this paper: Prasetya AW, Kismiyati, and Subekti S (2021). Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*). *World Vet. J.,* 11 (3): 498-503. DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

The phylum Platyhelminthes is characterized by a dorsoventral flattening of the body and bilateral symmetry, whereby its organs are embedded within the parenchyma tissues, and there are no respiratory and circulatory systems. The phylum Platyhelminthes is composed of two classes, namely Trematode and Cestode (Hahn et al., 2014; Morand et al., 2015). The parasites of the Trematode class have an ovoid, leaf-like, unsegmented body, and an incomplete digestive tract (a sac with one or two suckers for attachment). Worms in this class have a hermaphrodite reproductive system. This class is divided into three subclasses of Monogenea, Aspidogastrea, and Digenea (Crotti, 2013). Parasites of the order Dactylogyridae of the class Trematode are parasites of aquatic vertebrates, for example, the Reptiles (crocodiles), Amphibians (frogs), and Pisces (fishes). The majority of the parasites in the order Dactylogyridea are ectoparasites with a direct life cycle. The order Dactylogyridea of the class Trematoda has a distinctive feature on its posterior part of the body, that is, a haptor with a pair of hooks to attach itself to its host (Gibson et al., 2014).

There are a number of ectoparasitic worm types that infect the red snapper, namely Benedenia, Neobenedenia, and Diplectanum. These three worms are described as follows. Benedenia is a colorless ectoparasite that attacks the surface of the body. In particular, Benedenia causes fish to lose their appetite and have abnormalities in the way they swim on the bottom or surface of the container or floating net cage. Severe infection with Benedenia could cause wounds or ulcers (a pus-like liquid) on the skin and eventually secondary bacterial and fungal infection (Zhang et al., 2017). *Neobenedenia girellae* shows a higher degree of pathogenicity than *Benedenia epinephel*ibecause *Neobenedenia girellae* infects not only the skin but also the eye and causes blindness. Severely infected fish lose their appetite, have dark body colors, and have irregular swimming patterns and movements (Zhang et al., 2017). *Neobenedenia melleni* shows clinical symptoms in the skin, such as hyperemia and bleeding, and often infects the eye, causing damages to the cornea and blindness of the fish (Trujillo-González et al., 2015). Diplectanum disrupts the respiratory function in fish. The resulting wounds would lead to secondary bacterial infection. The growth of fish might be delayed, and the body weight and appetite are reduced. The fish infected by Diplectanum had pale gills, which produced excessive amounts of mucous, and a pale body (Cecchini et al., 1998). These three worms (Benedenia, Neobenedenia, and Diplectanum) are responsible for the diseases that the red snappers suffer from. The present research was then conducted to figure out the intensity and predilection of the helminth ectoparasites of the red snappers.

MATERIALS AND METHODS

Ethical approval

The present research employed the survey method. The sample of red snappers (*L. argentimaculatus*) was obtained by the purposive sampling method according to specified criteria. The sample had sizes specified by the researchers from the location of the floating net cages for one week. In this case, the red snappers were found to be 25–35 centimeters long.

The red snappers enrolled as samples were taken from one of seven floating net cages at Balai Besar Perikanan Budidaya Laut (BBPBL) Lampung with the number 30, or accounting for 20% of the total population of 150 red snappers, which were reared in $1 \times 1 \times 1.5$ m^3 polyethylene nets. The extracted sample was transported to the Laboratory of Fish Health and Environment of BBPBL Lampung, Indonesia, for helminth parasites examination.

The current research used a fishing net, a plastic bucket for fish sampling, and a Kemmerer bottle for water sampling. The instruments used to calculate the intensity and predilection of the helminth parasites were a set of surgical instruments (scalpel, surgical scissors, forceps, object-glass, and cover glass), pipette, towel paper, ruler, digital scale, digital camera, label paper, microtube, and trinocular microscope. Instruments used to measure water quality included a pH meter for pH measurement, a refractometer for measuring salinity, a Dissolved Oxygen (DO) meter for measuring dissolved oxygen, and a thermometer for measuring temperature.

The main materials used for the present research were the red snappers (*L. argentiaculatus*) and the seawater sample from the floating net cage location. Meanwhile, 250 mL of 5% glycerin was used to study the helminth parasite. The current research was undertaken from February through March 2016 in the Laboratory of Fish Health and Environment, BBPBL Lampung, and the Education Laboratory of the Faculty of Fisheries and Marine Affairs, Universitas Airlangga, while the sampling was performed at the location of the floating net cages of BBPBL Lampung.

RESULTS

The results of the present research concerned the intensity of the helminth ectoparasites, the predilection of helminth ectoparasites, the distribution of predilection of helminth ectoparasites, and the water quality parameters.

Intensity of helminth ectoparasites

From the investigation of the 30 samples of red snappers (*Lutjanus argentimaculatus*) that were cultivated in a floating net cage of BBPBL Lampung, helminth ectoparasites were found. The results of the sample examination indicated that a single sample fish could be infested with more than one type of helminth ectoparasite. Of the 30 sample

To cite this paper: Prasetya AW, Kismiyati, and Subekti S (2021). Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*). *World Vet. J.*, 11 (3): 498-503. DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

fish examined, 12 were only infested by *Haliotrema epinepheli* (sole infestation), including 1534 worms found with an intensity of 127.83 individuals/fish. Aside from those fish, 6 were infected with a mixture of *Benedenia epinepheli* and *Haliotrema epinepheli*, with 795 worms found with an intensity of 132.5 individuals/fish, and 12 were infested with a mixture of *Neobenedenia girellae* and *Haliotrema epinepheli*, having 1793 worms with an intensity of 149.41 individuals/fish.

Predilection of helminth ectoparasites

Observations on the predilection of the helminth ectoparasites were performed in the Laboratory of Fish Health and Environment of BBPBL Lampung. The observations were carried out on macroscopic and microscopic bases. The predilection test procedure was performed by scraping off all body parts of the fish, followed by observations under a microscope at 100 × and 400 × magnifications. Predilection sites for the helminth ectoparasites were the body surface of the fish and the fins, including the dorsal, pectoral, anal, and pelvic fins. The predilection study on the gills was conducted by observing the histopathological preparations of the fins infested by the ectoparasitic worms. The examination included changes in the structure and appearance of the organs infested by the ectoparasitic worms.

According to the examination results of the worms in the red snappers, *Benedenia epinepheli* had a round, elongated body with a length of 2.06 to 3.05 mm, a width of 0.67 to 1.51 mm, and a diameter of 0.50 to 0.78 mm. *Benedenia epinepheli* had attachment organs 0.16-0.25 mm long and 0.12 to 0.27 mm wide, a pharynx of 0.16-0.35 mm long and 0.18-0.25 mm wide, accessory sclerites of 0.07 to 0.11 mm long, anterior hamuli of 0.08 to 1.21 mm long, and posterior hamuli of 0.04 to 0.07 mm long.

Neobenedenia girellae was found to have a round elongated body with a length of 4.6 to 5.7 mm, and a width of 1.5 to 2.5 mm. In addition to an opisthaptor of 1.2-1.5 mm in size, *Neobenedenia girellae* also had a pair of attachment organs with the length of 0.14 to 0.22 mm and width of 0.17 to 0.29 mm, a pharynx with a length from 0.51 to 0.71 and a width of 0.39 to 0.42 as well as accessory sclerites with a length of 0.25 to 0.31 mm, anterior hamuli of 0.28-0.39 mm long, and posterior hamuli of 0.11-0.18 mm long. Meanwhile, *Haliotrema epinepheli* was found to be 200.8 µm in length and 48.12 µm in width, and also had a pharynx 15.40 to18.71 µm in diameter.

Distribution of the predilection of helminth parasites

66.7% of the *Benedenia epinepheli* that infest the red snappers preferred the dorsal fin, and 33.3% preferred the anal fin. No *Benedenia epinepheli* infestation was found on the caudal, pectoral, and ventral fins, and head. For the *Neobenedenia girellae* infestation in the red snappers, a majority of 57.1% had a high predilection for the body surface, 37.2% a medium predilection for the head, and 5.7% a low predilection for the dorsal fin. No predilection was indicated for the caudal, pectoral, anal, and ventral fins. Meanwhile, a 100% predilection for *Haliotrema epinepheli* was indicated for the gills and none for other parts of the body. As for the histopathologic observations of the gills, the results indicated that *Haliotrema epinepheli* had a predilection for the lamellae part of the red snapper gills.

Water quality parameters

The measurement of the water quality in terms of pH, DO, and salinity was performed once a week in the morning. The results of the observation of some indicators of water quality in the floating net cages of BBPBL Lampung in 2016 indicated that the water was in a normal condition for the life of the cultivated fish. The data indicated that the values were within normal ranges, where the temperature was between 30°C and 31°C, salinity was reported as 5 ppt, pH was estimated at 7, DO was between 6 and 8.37 mg/L, and the ammonia was also between 0.044 and 0.221 mg/L.

DISCUSSION

Based on the results of the examination of the body surface and the gills of red snappers (*Lutjanus argentimaculatus*), it was found that three parasitic worm species infest the red snappers in the floating net cages of BBPBL Lampung, namely *Benedenia epinepheli*, *Neobenedenia girellae*, and *Haliotrema epinepheli*. The helminth parasites *Benedenia epinepheli* and *Neobenedenia girellae* were encountered on the body surface of the red snappers, while *Haliotrema epinepheli* were found in the gills. The present findings are similar to Kritsky's (2012) review in the Persian Gulf, the eastern, Indo-west Pacific Ocean, and Mexico gulfs snappers.

The helminth ectoparasite *Benedenia epinepheli*, which infested the body surface of the red snappers as estated previously by Ravi and Yahaya (2016), had a predilection, especially on the dorsal and anal fins and in the skin area. When examining the ectoparasitic worms in the red snappers, *Benedenia epinepheli* was found to infest the dorsal fin area of 4 out of 30 sample fish, and infestation on the anal fin was rare.

The present research also discovered an infestation of the ectoparasitic worm *Neobenedenia girellae*. During the investigation, *Neobenedenia girellae* was found on the body surface, especially in the head area, in 13 out of 30 sample red snappers. It can be said that the worms had the highest prevalence on the body surface and rarely showed up on the

To cite this paper: Prasetya AW, Kismiyati, and Subekti S (2021). Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*). *World Vet. J.*, 11 (3): 498-503. DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

dorsal fin. *Neobenedenia girellae* had a predilection for the skin around the head and would also cause blindness, but was not found on the dorsal fin and the remaining body parts such as the gills and the internal organs.

The ectoparasitic worm *Haliotrema epinepheli* was also encountered in the present research. The predilection of the worm was exclusively for the gills. All sample fish infested by *Haliotrema epinepheli* demonstrated clinical symptoms of excessive mucus and swimming on the water surface. A severe infection of *Haliotrema epinepheli* could damage the filaments of the gills and cause the infected fish to suffer respiratory disorders or, in the worst case, die.

Based on the visual results, all sampled fish were infested by *Haliotrema epinepheli* in the gills. The *Benedenia epinepheli* infestation was present on the dorsal and anal fins, while the *Neobenedenia girellae* infestation occurred on the body surface, head, and dorsal fin, suggesting that *Heliotrema epinepheli* predominantly infested fish gills, eliminating competition between worm species in the areas. The fish infested by the helminth ectoparasite *Haliotrema epinepheli* rarely suffered wounds in their gill lamellae.

Although many of the red snappers (*Lutjanus argentimaculatus*) cultivated in the floating net cages at BBPBL Lampung were infested by *Haliotrema epinepheli* in the gills, none of the fish died. *Benedenia epinepheli* and *Neobenedenia girellae* did not leave any serious impact on the red snappers' health in small numbers. However, if *Benedenia epinepheli* and *Neobenedenia girellae* were present in significant numbers, they could be fatal to the fish (Melianawati and Aryati, 2012).

Based on the results of the examination on the 30 sample red snappers, 100% of the fish were positively infested with helminth ectoparasites, suggesting that the infestation intensity in floating net cages was very high. The infestation with helminth ectoparasites in the cultivation of floating net cages occurred due to the poor fish rearing and uncontrolled water quality since the cultivation was weather-dependent. Another factor presumed to decrease the red snappers' physical endurance was the stress resulted from the flow of water and net cages spoilage from the attachment of parasites and other microorganisms which blocked water circulation, which was mentioned by Cooper (2015). In addition, stress could also be caused by high density, which allowed parasites to develop rapidly, with high density triggered competition for space, food, and oxygen. The mortality rate from infestation with helminth ectoparasites at this cultivation location (East Java) was categorized as so high that the red snapper population in these floating net cages was smaller compared to other populations.

CONCLUSION

All of the fish samples were positively infected by helminth ectoparasites, including *Neobenedenia girellae*, *Haliotrema epinepheli*, and *Benedenia epinepheli*. Therefore, in the East Java region, Ocean fish are most likely to be infected with these parasites. In fish inspections, parasite-related infections should be considered cautiously, especially in marine fish like Red Snapper (*Lutjanus argentimaculatus*).

DECLARATIONS

Authors' contribution

All the authors contributed equally to this study.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

Cable J, Barber I, Boag B, Ellison AR, Morgan ER, Murray K, and Booth M (2017). Global change, parasite transmission and disease control: lessons from ecology. Philosophical Transactions of the Royal Society B: Biological Sciences, 372(1719): 20160088. DOI: https://www.doi.org/10.1098/rstb.2016.0088

Cecchini S, Saroglia M, Berni P, and Cognetti-Varriale AM (1998). Influence of temperature on the life cycle of Diplectanum aequans (Monogenea, Diplectanidae), parasitic on sea bass, Dicentrarchus labrax (L.). Journal of Fish Diseases, 21(1): 73-75. DOI: https://www.doi.org/10.1046/j.1365-2761.1998.00068.x

Crotti M (2013). Digenetic trematodes: An existence as parasites. Brief General Overview. Microbiologia Medica, 28(2): 97-101. DOI: https://www.doi.org/10.4081/mm.2013.2256

Cooper J (2015). Does seagrass influence the behavioural and physiological response to flow in juvenile snapper (Pagrus auratus)?. Thesis, Master of Science,University of Waikato, Hamilton, New Zealand. Available at: https://hdl.handle.net/10289/9495

To cite this paper: Prasetya AW, Kismiyati, and Subekti S (2021). Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*). *World Vet. J.*, 11 (3): 498-503. DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

Engering A, Hogerwerf L, and Slingenbergh J (2013). Pathogen–host–environment interplay and disease emergence. Emerging microbes and infections, 2(1): 1-7. DOI: https://www.doi.org/10.1038/emi.2013.5

Gibson DI, Bray RA, Hunt D, Georgiev BB, Scholz T, Harris PD, Bakke TA, Pojmanska T, Niewiadomska K, Kostadinova A et al. (2014). Fauna Europea: Helminths (Animal Parasitic). Biodeversity Data Journal, (2): e1060. DOI: https://www.dx.doi.org/10.3897%2FBDJ.2.e1060

Hahn C, Fromm B, and Bachmann L (2014). Comparative genomics of flatworms (Platyhelminthes) reveals shared genomic features of ecto-and endoparastic neodermata. Genome Biology and Evolution, 6(5): 1105-1117. DOI: https://www.doi.org/10.1093/gbe/evu078

Hussan A, Vinay TN, Tanmoy Gon C, and Sanjay KG (2016). Common problems in aquaculture and their preventive measures. Matrix ANU Advanced Aquaculture Research Centre (Aquaculture Times), 2(5): 1-55.

Iwanowiez DD (2011). Overview on the effects of parasites on fish health. Conference paper, kearneysville: United States Geological Survey, Leetown Science Center, National Fish Health Research Laboratory, USA. Availabe at: https://pubs.er.usgs.gov/publication/70047221

Jithendran KP, Vijayan KK, Alavandi SV, and Kailasam M (2005). Benedenia epinepheli (Yamaguti 1937), a monogenean parasite in captive broodstock of grouper, epinephelustauvina (Forskal). Asian Fisheries Science, 18: 121-126. Available at: https://krishi.icar.gov.in/jspui/bitstream/123456789/1215/1/29-2005%20Jithendran%20AFS%20Monogenean%20Parasite%20Grouper.pdf

Justine JL, Beveridge I, Boxshall GA, Bray RA, Miller TL, Moravec F, and Whittington ID (2012). An annotated list of fish parasites (Isopoda, Copepoda, Monogenea, Digenea, Cestoda, Nematoda) collected from Snappers and Bream (Lutjanidae, Nemipteridae, Caesionidae) in New Caledonia confirms high parasite biodiversity on coral reef fish. Aquatic Biosystems, 8(1): 1-29. DOI: https://www.doi.org/10.1186/2046-9063-8-22

Kishimori JM., Takemura A, and Leong JAC (2015). Neobenedenia melleni-specific antibodies are associated with protection after continuous exposure in mozambique Tilapia. Journal of Immunology Research, Article ID: 635387. DOI: https://www.doi.org/10.1155/2015/635387

Kritsky DC (2012). Dactylogyrids (Monogenoidea: Polyonchoinea) parasitizing the gills of snappers (Perciformes: Lutjanidae): Revision of Euryhaliotrema with new and previously described species from the red sea, Persian Gulf, the eastern and Indo-west Pacific Ocean, and the Gulf of Mexico. Zoologia (Curitiba), 29: 227-276. DOI: https://www.doi.org/10.1590/S1984-46702012000300006

Madhun AS, Karlsbakk E, Isachsen CH, Omdal LM, Eide Sørvik AG, Skaala O, Barlaup BT, and Glover KA (2015). Potential disease interaction reinforced: Double-virus-infected escaped farmed A tlantic salmon, Salmo salar L., recaptured in a nearby river. Journal of Fish Diseases, 38(2): 209-219. https://www.doi.org/10.1111/jfd.12228

Melianawati R, and Aryati RW (2012). Budidaya ikan kakap merah (Lutjanus sebae). Jurnal Ilmu dan Teknologi Kelautan, 4(1): 80-88. Availabe at: https://media.neliti.com/media/publications/103901-ID-culture-of-emperor-snapper-lutjanus-seba.pdf

Montoya-Mendoza J, Jiménez-Badillo L, Salgado-Maldonado G, and Mendoza-Franco EF (2014). Helminth parasites of the red snapper, Lutjanus campechanus (Perciformes: Lutjanidae) from the reef Santiaguillo, Veracruz, Mexico. The Journal of Parasitology, 100(6): 868-872. DOI: https://www.doi.org/10.1111/jfd.12228

Loh JY, Chan HK, and Yam HC (2020). An overview of the immunomodulatory effects exerted by probiotics and prebiotics in grouper fish. Aquaculture International, 28: 729-750. https://www.doi.org/10.1007/s10499-019-00491-2

Morand S, Krasnov B, and Littlewood D (2015). The evolutionary history of parasite diversity. In Parasite Diversity and Diversification: Evolutionary Ecology Meets Phylogenetics, Cambridge University Press, Cambridge, pp. 91-334. Available at: https://www.cambridge.org/core/books/parasite-diversity-and-diversification/evolutionary-history-of-parasite-diversity/79E3BEC08B09A2D5F839A4974630B8DE

Oliveira BL, Souza RM, Gomes LC, and Fernandes LFL (2020). First record of Neobenedenia melleni (Monogenea: Capsalidae) in dog snapper (Lutjanus jocu) in the western South Atlantic. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 72(3): 1051-1055. DOI: https://www.dx.doi.org/10.1590/1678-4162-11600

Omeji S, Solomon SG, and Idoga ES (2011). A comparative study of the common protozoan parasites of clarias gariepinus from the wild and cultured environments in Benue State, Nigeria. Journal of Parasitology Research, Article ID: 916489. DOI: https://www.doi.org/10.1155/2011/916489

Pikarsky E, Ronen A, Abramowitz J, Levavi-Sivan B, Hutoran M, Shapira Y, Steinitz M, Perelberg A, Soffer D, and Kotler M (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. Journal of virology, 78(17): 9544-9551. DOI: https://www.doi.org/10.1128/JVI.78.17.9544-9551.2004

Ravi R, and Yahaya ZS (2016). Neobenedenia melleni parasite of red snapper, Lutjanus erythropterus, with regression statistical analysis between fish length, temperature, and parasitic intensity in infected fish, cultured at Jerejak Island, Penang, Malaysia. Journal of Parasitology Research, Article ID: 1946283. DOI: https://www.doi.org/10.1155/2016/1946283

Santos CP, Timi JT, and Gibson DI (2002). Diplectanum squamatum n. sp.(Monogenea: Diplectanidae) from the gills of Cynoscion guatucupa (Sciaenidae) in Southwest Atlantic waters. Systematic Parasitology, 52(3): 199-204. DOI: https://www.doi.org/10.1023/A:1015737719326

Trujillo-González A, Constantinoiu CC, Rowe R, and Hutson KS (2015). Tracking transparent monogenean parasites on fish from infection to maturity. International Journal for Parasitology: Parasites and Wildlife, 4(3): 316-322. DOI: https://www.doi.org/10.1016/j.ijppaw.2015.06.002

Yadollahi S, Momtaz H, Doudi M, and Taj bakhsh E (2013). The objective of this study was to isolation and characterization of listeria species and determines Listeria Monocytogenes Serotypes in fresh fish, shrimp, crab and lobster in Isfahan and

To cite this paper: Prasetya AW, Kismiyati, and Subekti S (2021). Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*). *World Vet. J.*, 11 (3): 498-503. DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

Shahrekord, Iran. International Journal of Advanced Biological and Biomedical Research, 1(5): 493-504. Available at: http://www.ijabbr.com/article_7739.html

Zhang D, Zou H, and Wu SG (2017). Sequencing of the complete mitochondrial genome of a fish-parasitic flatworm Paratetraonchoides inermis (Platyhelminthes: Monogenea): tRNA gene arrangement reshuffling and implications for phylogeny. Parasites Vectors, 10: 462. DOI: https://www.doi.org/10.1186/s13071-017-2404-1

To cite this paper: Prasetya AW, Kismiyati, and Subekti S (2021). Intensity and Predilection of Helminth Parasites of the Red Snapper (*Lutjanus argentimaculatus*). *World Vet. J.,* 11 (3): 498-503. DOI: https://dx.doi.org/10.54203/scil.2021.wvj64

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 504-509, September 25, 2021
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique

Rani Wilujeng Asmorowati[1], Kusnoto[1*], and Hana Eliyani[2]

[1] *Department of Veterinary Parasitology, Faculty of Veterinary, Universitas Airlangga, Surabaya-60115, Indonesia*
[2] *Department of Veterinary Anatomy, Faculty of Veterinary, Universitas Airlangga, Surabaya-60115, Indonesia*

*Corresponding author's Email: kk.kusnoto@yahoo.com; ORCID: 0000-0003-3915-654X

ORIGINAL ARTICLE
pii: S232245682100065-11
Received: 08 July 2021
Accepted: 26 August 2021

ABSTRACT

In the adult stage, *Haemonchus contortus* worms infect the abomasum host causing anemia and even death in animals. However, identifying the *H. contortus* protein can be used as a reference for the diagnosis of diseases. The diagnosis is performed by serological cross-reaction between *H. contortus* protein and anti-L2 *Toxocara vitulorum* (*T. vitulorum*) serum using the western blot technique. The main purpose of the current research was to identify the cross-reaction between *H. contortus* proteins and anti-L2 *T. vitulorum* serum using the western blot technique. *T. vitulorum* worms were collected from the intestine of cattle and *H. contortus* worms were collected from the abomasum of goats. The first step was making antibodies by oral infection of rats with infective eggs (L2) of *T. vitulorum*. The blood was taken 21 days after infection. Then, the blood was centrifuged at 1500 rpm for 10 minutes to get the serum. The second step was making homogenates from the whole worm extract of *H. contortus*. After crushing the worms, it was centrifuged at 5000 rpm for 15 minutes and the supernatant was taken. The supernatant was then analyzed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with coomassie brilliant blue staining. The third step was the analysis of *H. contortus* protein with serum anti-L2 *T. vitulorum* using the western blot technique. From the *H. contortus* homogenates analysis using SDS-PAGE, 16 protein bands were obtained. The cross-reactions were 141.3, 81.3, 64. 6, 51.3, 46.8, and 38 kDa. The data from cross-reactions suggested that the *H. contortus* protein cannot be used as a diagnostic material. It is serologically Haemonchosis because it caused false positives with diagnostic Toxocariasis.

Keywords: Cross reaction, *Haemonchus contortus*, SDS-PAGE, *Toxocara vitulorum*, Western blot

INTRODUCTION

Haemonchosis is a parasitic disease caused by a nematode worm infection from the family of Trichostrongylidae and *Haemonchus* genus. The main cause of haemonchosis is *Haemonchus contortus* (*H. contortus*) worm. In the adult stage, this worm infects the abomasum of the host, causing anemia that leads to death (Githigia et al., 2001). Meanwhile, *Toxocara vitulorum* (*T. vitulorum*) is a pathogenic worm in ruminants. Their definitive hosts are buffaloes and they live as an adult within the small intestine lumen of ruminants (Abdelrahman and El-Menyawe, 2015). They can also be found in the calves of bulls (Van der Steen et al., 2014).

The conventional diagnosis of nematodes shows positive results if the infection has passed the prenatal stage (13-16 weeks). The effort which can be done to overcome the problem in diagnosis is developing a sensitive diagnostic technique based on serological examination. Serological testing using ELISA can be used to diagnose the disease. However, this technique has a disadvantage; If the antigen used is not specific, then the test's specificity will be in the low range (Sackey et al., 2003).

The *H. contortus* is the most dangerous and most observed worm in Southeast Asia, including in Indonesia; In North Sumatra, the prevalence of livestock infected by *H. contortus* has reached 40% (Silva et al., 2008). In Nigeria, the prevalence of toxocariasis due to *T. vitulorum* infection in calves is 61.4-91.1% (Sackey et al., 2003). Haemonchosis is a significant threat in tropical, subtropical, and warm regions as warm and moist conditions are favorable for the free-living stages (Besier et al., 2016; Fentahun, 2020). Worm-infected cases result in economic losses because of the decrease in animal productivity, weight loss, diarrhea, and severe cases that even lead to death (Githigia et al., 2001). Conventional diagnosis by finding eggs in a patient's stool is not possible because eggs are only produced by adult worms. For diagnosis, cases of *H. contortus* infected, require an accurate test and one of them is serological testing. Worms are multicellular organisms that have various specific and non-specific proteins that can trigger the host's immune response to form various antibodies so that when a positive diagnosis is made with serological tests, it allows to

To cite this paper: Asmorowati RW, Kusnoto, and Eliyani H (2021). Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique. *World Vet. J.*, 11 (3): 504-509. DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

conduct cross-reaction (Sackey et al., 2003). Cross reaction between worm species can cause false positives, but proteins that experience the cross-reaction can be developed as vaccine candidates. In proteins that are not cross-reactions, further detentions and evaluations can be carried out on the specificity of the worm antigen (Abdel-Rahman and Abdel Megeed, 2000). Identification of specific proteins plays an important role, which can be used as a reference for the diagnosis of disease. One of the tests recommended to confirm the diagnosis is the serological test. The main principle of serological tests is to react to antigens with appropriate antibodies. In the case of infection, it will result in antibodies that are dissolved in the serum. The formed antibodies will circulate throughout the tissues and blood circulation. The body will mobilize B lymphocyte cells which will differentiate into plasma cells, during the time it deals with infectious agents. Plasma cells will produce antibodies. If the antibodies find an antigen, then the antigen will be bound by antibodies (Slifka and Amanna, 2019).

The present study evaluated *H. contortus* protein along with anti-L2 *T. vitulorum* serum using Western blot technique. This study was conducted to determine the cross-reactions that can occur in worms of the same class, but in different species that were *H. contortus* and *T. vitulorum*. This study aimed to detect blood mononuclear (BM) *H. contortus* proteins that could bind to anti-L2 *T. vitulorum* serum using Western blot techniques.

MATERIALS AND METHODS

The current study was a laboratory exploratory study aimed at describing BM *H. contortus* proteins undergoing cross-reaction with anti-L2 *T. vitulorum* serum. The study was conducted at the Parasitology Laboratory of Universitas Airlangga and the Institute of Tropical Disease (ITD), Surabaya, Indonesia, from July until November 2016.

The sample collection process from the intestine of cattle and the abomasum of goats was in accordance with animal ethic regulations announced by the Airlaanga University Ethical committee.

Collection of *Haemonchus contortus* and *Toxocara vitulorum*

The *H. contortus* worms were obtained from goat abomasum and *T. vitulorum* worms were taken from the small intestine of male cows. The collected worms were separated from the stool and immediately cleaned using Phosphate Buffer Saline (PBS). The cleaned worms were put in a petri dish containing PBS and then incubated in an incubator at 37ºC (Kusnoto et al., 2011).

Isolation of L2 *Toxocara vitulorum*

Adult *T. vitulorum* worms were incubated for three days in an incubator at 37ºC to produce eggs. After three days, the eggs of *T. vitulorum* worm were surgically taken from the reproductive tract. Afterwards, the worm eggs were identified, then fertilized in PBS medium at room temperature for 28 days to obtain *T. vitulorum* L2 (Kusnoto et al., 2011).

Calculation of worm eggs per gram stool

In the current study, egg counting was performed by modifying the calculation of worm eggs per gram stool using the Lucient Brumpt method. Based on the calculation, to identify the number of *T. vitulorum* eggs in each ml suspension of culture media, the following formula was used. The number of drops per ml (N) × number of worm eggs per drop (n) × number of dilutions (Lindstrom, 2003).

Treatment of experimental animals

The experimental animals used in this research were five male rats aged two months old. The rats were infected with L2 *T. vitulorum* at a dose of 10 grains per gram of body weight orally (Kusnoto et al., 2011). Before being infected, the rats were adapted for seven days. After adaptation, the rats were infected with L2 *T. vitulorum*. The purpose of this artificial infection was to obtain a serum containing L2 *T. vitulorum* antibodies.

Blood sampling of rats

The blood of the rats was taken 21 days after infection by cutting the rat's tail. The blood was inserted into a microtube, centrifuged at 1500 rpm for 10 minutes to get the serum (Lee and Goosens, 2015).

Preparation of *Haemonchus contortus* homogenate

Homogenate was made manually by grinding *H. contortus* worms with a mortar, then it was inserted into the test tube and suspended with 3 ml PBS. The suspension was then centrifuged at 5000 rpm for 15 minutes. Pellets and supernatants were separated. Supernatants were stored for protein analysis with SDS-PAGE and western blotting. Running was conducted on the results using the SDS-PAGE technique (Lindstrom, 2003).

Protein analysis using the SDS-PAGE technique

During SDS-PAGE technique, the protein electrophoresis was done in an ionic detergent which was Sodium Dodecyl Sulfate (SDS). One of the systems in SDS is discontinuation (Laemmli). In this system, protein migrates rapidly through ion solvents on stacking gel and separating gel. Protein is concentrated in a thin band line (Lindstrom, 2003).

To cite this paper: Asmorowati RW, Kusnoto, and Eliyani H (2021). Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique. *World Vet. J.*, 11 (3): 504-509. DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

Characterization of proteins using Western blot technique

From the homogenate of *H. contortus* worms which has gone by the running gel process using SDS-PAGE, gel with protein fragments was obtained based on BM. In this process, the protein can be directly transferred to the nitrocellulose membrane using the Bio-rad model. The transfer of protein from the gel to nitrocellulose membrane was carried out by the Electrophoresis technique (Lindstrom, 2003).

Calculation of protein molecular weight

The Relative Molecular mass (MR) of the antigen protein was determined by a regression equation method. It was based on the value of the Retardation factor (Rf) of each band formed by using the western blot technique according to the method of Miletić and Saracević (1984). The MR was determined based on the MR log of the standard protein and the Rf value. Then, the equation was obtained by determining the standard curve of Rf and log MR (Lindstrom, 2003).

The molecular weight of an antigen or antibody was obtained by calculating the Rf value of each band (band) with the following formula:

$$Rf = \frac{\text{Migration distance of the protein}}{\text{Migration distance of the dye front}}$$

Where, distance refers to the distance of protein movement from the initial place, and Length is the distance of color movement from the starting point (dye front).

The relative molecular mass of protein was determined by converting the Rf value data and the relative molecular mass of standard protein to an equation. The logarithmic value of the relative molecular mass of standard proteins indicated the function Y, while X is the value of Rf. After the Rf value of the sample was found, it was entered into the equation. Furthermore, based on the results of the equation, the anti-log value was obtained to get the relative molecular mass value of protein (Kusnoto et al., 2011).

RESULTS AND DISCUSSION

Collection and identification of *Haemonchus contortus* worms

The *H. contortus* worms were obtained from the goat abomasum. *H. contortus* worms have a very small body size. Male *H. contortus* worms are 10-20 mm in length and red color, while female worms are 18-30 mm in length and red and white colors (it is known as barber's pole). The color produced by the variation of ovaries and intestines is red because of the blood sucked from the host. Identification of *H. contortus* worms is based on their reproductive organs. Male *H. contortus* worms have dorsal rays that are shaped like "Y" and spicules with 0.46-0.506 mm length (Figure 1). In female worms, the vulvars are covered by large and prominent flaps (Vulva flap). Male and female *H. contortus* have been demonstared in Figure 2.

Collection and identification of *Toxocara vitulorum* worms

The *T. vitulorum* worms were collected from the small intestine of adult male cows. The male *T. vitulorum* worms are 15-26 cm in length and 3-5 mm diameter with a small tail and female *T. vitulorum* worms are 22-30 cm in length with 5-6 mm diameter. Female *T. vitulorum* worms have thin, soft, and transparent cuticles. The identification of the *T. vitulorum* worms is based on the length of the body and the presence of its reproductive organ (Figure 3).

Collection of L2 *Toxocara vitulorum*

The eggs of *T. vitulorum* are subglobular and brownish. The eggs have thick spotted walls. Stage II (L2) larvae were found in ineffective worm eggs. The details are presented in Figure 4.

Protein analysis using sodium dodecyl sulphate polyacrylamide gel electrophoresis technique

From the protein analysis of Whole Worm Extract (WWE) of *H. contortus* using SDS-PAGE technique, WWE of *H. contortus*, 16 protein bands were prepared. By running SDS-PAGE on WWE of *H. contortus*, 16 proteins bonds with the following BM 141.3, 117.5, 81.3, 64.6, 51.3, 46.8, 42.7, 38, 34.7, 29.5, 26.9, 23.4, 20.9, 15.5, 12.9, and 11.7 kDa were obtained.

Protein characterization using western blot technique

The results from WWE of *H. contortus* analyzed proteins which were reacted with anti-L2 *T. vitulorum* serum by western blot technique indicated that the WWE of *H. contortus* bounded to anti-L2 *T. vitulorum* serum on BM 141.3; 81.3; 64.6; 51.3; 46.8 and 38 kDa.

To cite this paper: Asmorowati RW, Kusnoto, and Eliyani H (2021). Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique. *World Vet. J.*, 11 (3): 504-509. DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

Results of protein analysis using* sodium *dodecyl sulphate polyacrylamide gel electrophoresis technique

In SDS-PAGE, the protein is electrophoresed in ionic detergent and SDS. In the discontinuous system (Laemmli), proteins migrate rapidly through ion solvents on stacking gel and separating gel. The protein will be concentrated on a thin line in the form of a thin band (Slifka and Amanna, 2019). In the current study from the results of WWE of *H. contortus* protein analysis, 16 kinds of protein bands with the BM of 141.3, 117.5, 81.3, 64.6, 51.3, 46.8, 42.7, 38, 34.7, 29.5, 26.9, 23.4, 20.9, 15.5, 12.9, and 11.7 kDa were obtained. Meanwhile, from the identification of the other WWE of *H. contortus*, 16 protein bands included the BM of 107.74, 64.68, 51.39, 47.1, 43.52, 38.55, 36.27, 32.97, 28.95, 25.8, 25.8, 23.25, 20.95, 15.65, 12.85, and 9.93 kDa. Protein bands that were similar to those obtained in this study included 64.6, 51.3, 38, 23.4, 20.9, 15.5, and 12.9 kDa. The difference was related to the calculation and possible relative differences in determining the distance of the protein band and the length, as well as the beginning of the measurement of the gel. It is possible that several protein bands had some differences with the results of other studies (Santos et al., 2014; Tak et al., 2015), but they referred to the same protein (Kusnoto et al., 2011).

The SDS-PAGE is the commonly used analytical method for resolving components of a protein mixture (Grabski and Novagen, 2001). Observations on protein analysis using the SDS-PAGE technique indicated that the success rate of the analysis was influenced by three factors, including homogenate cleanliness, homogenate purity level, and protein content in homogenates. Hygiene cleanliness affects the quality of protein bands formed in the gel where the ribbon could look sharply colored on a bright gel, thus allowing protein analysis and documentation. This technique is also a powerful tool for estimating the molecular weights of proteins (Grabski and Novagen, 2001).

Characterization of proteins using western blot technique

The *H. contortus* and *T. vitulorum* are worm pathogens because they attack internal organs and cause diarrhea in animals and in case the infected animal is not treated intensively, it will even die. One of the techniques that can determine the presence of antigen reactions with antibodies is blotting. Blotting is a technique for transferring macromolecules from the gel medium onto the membrane after the Electrophoresis process. Separate bands can be detected by coloring. The blotting technique is used to detect proteins, carbohydrates, and enzyme activity. One of the used blotting techniques is Western blot.

The basic principle of the Western blot technique is to separate proteins by SDS-PAGE, then transfer the proteins to the appropriate nitrocellulose membrane, followed by labeling the proteins with antibodies and visualizing them by the desired coloring, such as coomassie blue (Slifka and Amanna, 2019). Cross-reactions occurred between *H. contortus* protein and anti-L2 *T. vitulorum* serum on BM 141.3, 81.3, 64.6, 51.3, 46.8, and 38 kDa. The presence of a cross-reaction indicated that the protein is not a good diagnostic agent for haemonchosis because it causes false positives with diagnostic Toxocariasis.

Figure 1. Adult stage of *Haemonchus contortus*

To cite this paper: Asmorowati RW, Kusnoto, and Eliyani H (2021). Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique. *World Vet. J.*, 11 (3): 504-509. DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

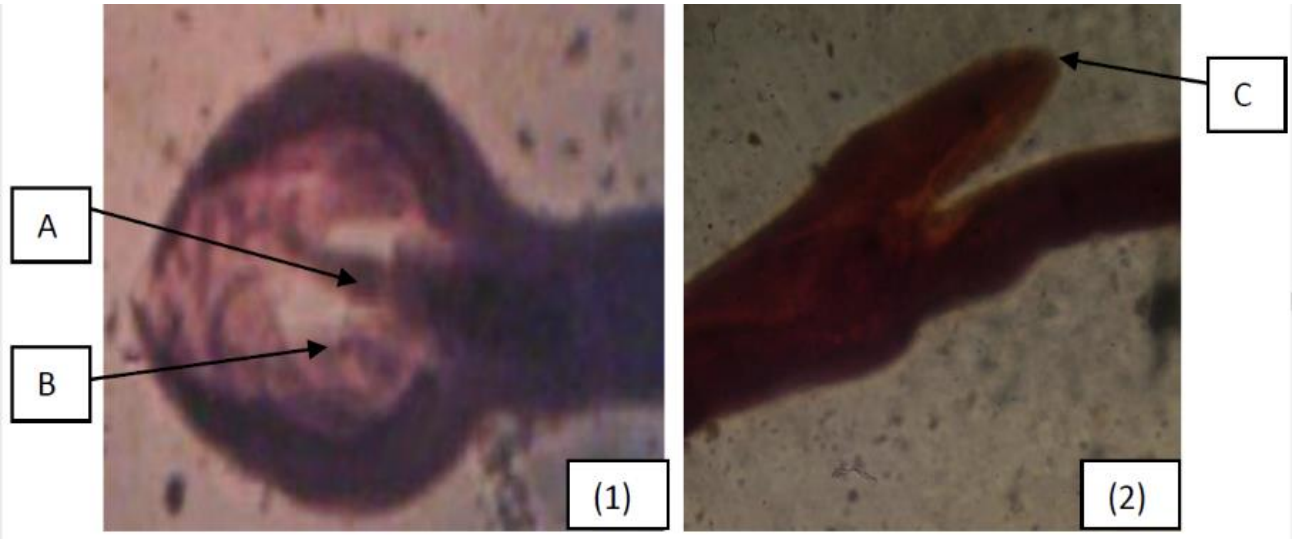


Figure 2. Identification of male and female *H. contortus* worms. Male worms (**1**), A is Spikula and B is dorsal rays. Female worms (**2**), part of C is vulva flap. The observation was done by using 40× magnification.

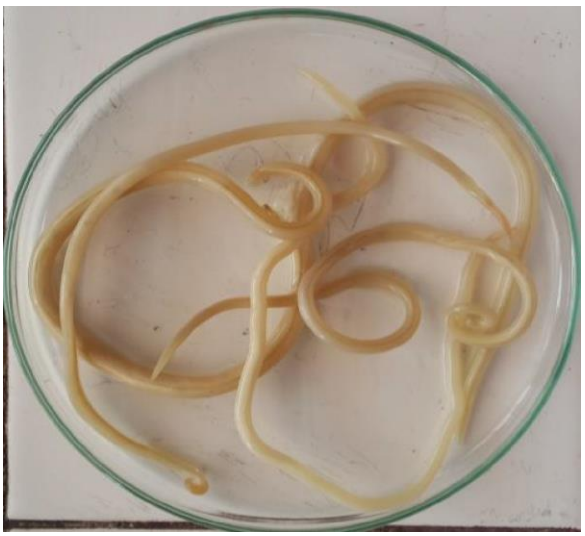


Figure 3. Adult *Toxocara vitulorum* worms

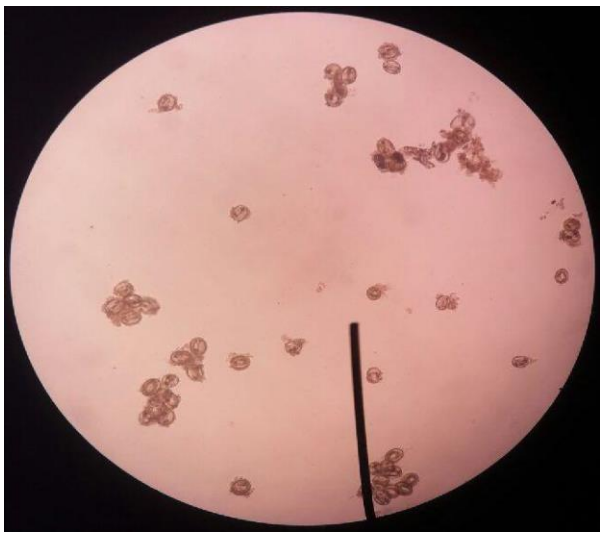


Figure 4. L2 *Toxocara vitulorum* (100× magnification).

CONCLUSION

In this research, cross-reactions between *Haemonchus contortus* protein and anti-L2 *Toxocara vitulorum* serum on BM of 141.3, 81.3, 64.6, 51.3, 46.8, and 38 kDa were obtained. The anti-L2 *T. vitulorum* serum can recognize antigens from *Haemonchus contortus*, *Moniezia benedeni*, and *Toxocara canis* on proteins with the same BM of 38 kDa. The presence of a cross-reaction indicates that the protein is not a good diagnostic agent for haemonchosis because it causes false positives with diagnostic toxocariasis.

DECLARATIONS

Authors' contribution

R. Wilujeng Asmorowati, Kusnoto, and H. Eliyani contribute equally in conduction, analysis, and writing of the manuscript. The authors checked and confirmed the final version of article.

Competing interests

The authors have no conflict of interests

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

To cite this paper: Asmorowati RW, Kusnoto, and Eliyani H (2021). Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique. *World Vet. J.*, 11 (3): 504-509. DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

REFERENCES

Abdel-Rahman EH, and Abdel Megeed KN (2000). Molecular identity of major cross-reactive adult antigens in *Fasciola gigantica*, *Toxocara vitulorum* and *Moniezia expansa*. Journal of Egyptian Society of Parasitology, 30(2): 561-572. Available at: https://pubmed.ncbi.nlm.nih.gov/10946516/

Abdelrahman M, and El-Menyawe SM (2015). A Review of Toxocariasis in man and animal. Egyptian Journal of Chemistry Environment and Health, 1(1): 315-330. Available at: http://cehea.org/wp-content/uploads/2016/08/23-.pdf

Besier RB, Kahn LP, Sargison ND, and van Wyk JA (2016). Diagnosis, treatment and management of Haemonchus contortus in small ruminants. Advances in Parasitology, 93: 181-238. DOI: https://www.doi.org/10.1016/bs.apar.2016.02.024

Fentahun T (2020). Systematic review on gastrointestinal helminthes of domestic ruminants in Ethiopia. Online Journal of Animal and Feed Research, 10(5): 216-230. DOI: https://www.dx.doi.org/10.51227/ojafr.2020.31

Githigia SM, Thamsborg SM, Munyua WK, and Maingi (2001). Impact of gastrointestinal helminths on production in goats in Kenya. Small Ruminant Research, 42(1): 21-29. DOI: https://www.doi.org/10.1016/S0921-4488(01)00240-1

Grabski AC, and Novagen RRB (2001). Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: Procedures and Tip. Novations 13, Madison- Wisconsin, pp. 10-12. Available at: http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.378.613

Kusnoto SS, Sudiana IK, and Koesdarto S (2011). Karakteristik dan isolasi protein spesifik dari material excretory-secretory (ES) toxocara cati untuk pengembangan diagnostik toxocariasis dengan teknik ELISA. Jurnal Biosains Pascasarjana, 13(1): 56-65. Available at: http://journal.unair.ac.id/download-fullpapers-Vol%2013%20No%201%20Jan%202011-7.pdf

Lee G, and Goosens KA (2015). Sampling blood from the lateral tail vein of the rat. Journal of Visualized Experiments, (99): e52766. DOI: https://www.doi.org/10.3791/52766

Lindstrom R (2003). Epidemiology of Brucellosis. Office Internationale des Epizooticae, Universitas Airlangga Press, Surabaya, Indonesia, pp. 112-120.

Miletić VD, and Saracević M (1984). Isolation and determination of the molecular mass of the RHoD antigen in human erythrocytes. Bilten za Hematologiju i Transfuziju, 12(2): 51-58. Available at: https://pubmed.ncbi.nlm.nih.gov/6441566/

Sackey AKB, George JBD, and Sale M (2003). Observation on the age at initial infection of Zebu calves by *Toxocara vitulorum* in Northern Nigeria. Tropical Veterinarian, 21(3): 124-128. DOI: https://www.doi.org/10.4314/tv.v21i3.4531

Santos MC, Amarante MR, Silva MR, and Amarante AF (2014). Differentiation of Haemonchus placei from Haemonchus contortus by PCR and by morphometrics of adult parasites and third stage larvae. Brazilian Journal of Veterinary Parasitology, 23(4): 495–500. DOI: https://www.doi.org/10.1590/S1984-29612014085

Silva BF, Amarante MRV, Kadri SM, Carrijo-Mauad JR, and Amarante AFTD (2008). Vertical migration of Haemonchus contortus third stage larvae on Brachiaria decumbens grass. Veterinary Parasitology, 158: 85-92. DOI: https://www.doi.org/10.1016/j.vetpar.2008.08.009

Slifka MK, and Amanna IJ (2019). Role of multivalency and antigenic threshold in generating protective antibody responses. Frontiers in immunology 10: 956. DOI: https://www.doi.org/10.3389/fimmu.2019.00956

Tak IU, Chishti MZ, and Ahmad F (2015). Protein profiling of Haemonchus contortus found in sheep of Kashmir valley. Journal of parasitic diseases: official organ of the Indian Society for Parasitology, 39(4): 639-644. DOI: https://www.doi.org/10.1007/s12639-014-0433-7

Van der Steen L, Pardon B, Sarre C, Valgaeren B, van Hende D, Vlaminck L, and Deprez P (2014). Intestinal obstruction by *Toxocara vitulorum* in a calf. Vlaams Diergeneeskundig Tijdschrift, 83(6): 299-305. DOI: https://www.doi.org/10.21825/vdt.v83i6.16628

To cite this paper: Asmorowati RW, Kusnoto, and Eliyani H (2021). Cross Reaction of *Haemonchus contortus* Protein with *Toxocara vitulorum* Anti-L2 Serum Using Western Blot Technique. *World Vet. J.*, 11 (3): 504-509. DOI: https://dx.doi.org/10.54203/scil.2021.wvj65

2021, Scienceline Publication
World's Veterinary Journal
World Vet J, 11(3): 510-520, September 25, 2021
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation

Wafaa A. Abd El-Ghany

Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
*Corresponding author's E-mail: wafaa.ghany@yahoo.com; ORCID: 0000-0003-1686-3831

REVIEW ARTICLE
pii: S232245682100066-11
Received: 10 July 2021
Accepted: 29 August 2021

ABSTRACT

This review article was developed to the infection of avian species with *Toxoplasma gondii* (*T. gondii*), diagnosis, pet bird and human infection, and control methods with a special reference to the current status of infection among the Egyptian poultry farms and population. Toxoplasmosis is a zoonotic disease caused by a unicellular, protozoan parasite *T. gondii*. Different domesticated and wild animals, as well as birds can harbor *T. gondii* and may be a potential source of infection to humans. Avian species could be infected with *T. gondii* through the ingestion of contaminated food, soil, and water with oocysts shed in the excreta of infected animals, especially cats. Poor sanitation and hygienic conditions increase the risk of infection. Consumption of food or water, as well as undercooked poultry meat or meat products containing the oocysts of the parasite, are the main sources of human infection with *T. gondi*. Diagnosis of *T. gondii* in the infected host depends on the serological detection of specific antibodies and molecular detection of the parasite. Microscopic demonstration of the oocysts and other developmental stages of the parasite in the intestine, liver, brain, and skeletal muscles tissues is another means for rapid diagnosis. Generally, a high prevalence of the disease is also reported in pet birds. Toxoplasmosis in humans is associated with abortion, congenital disorders, stillbirth, and other complications, especially in immunocompromised patients. Application of hygienic measures, as well as public awareness, are essential for the prevention and control of toxoplasmosis. In different Egyptian governorates, a high prevalence of *T. gondii* has been detected in animals, birds, and humans. High incidence of infection was recorded due to the contact with *Toxoplasma* oocysts shed mainly from infected cats or other carriers. Egyptian chicken and turkey flocks and backyard birds revealed the presence of different developmental stages of the parasite and even its antibodies. In addition, human populations showed signs of toxoplasmosis with severe complications.

Keywords: Bird, Egypt, Human, *Toxoplasma gondii*, Zoonosis

INTRODUCTION

Toxoplasmosis is an important zoonotic parasitic disease of public health importance (CDC, 2004) and it is caused by an obligatory intracellular protozoan parasite, *Toxoplasma gondii* (*T. gondii*) (Tenter et al., 2000). The disease is of widespread nature and causes significant economic and reproductive losses in animals and serious public health problems in humans (Sukthana, 2006; Pan et al., 2017). Toxoplasmosis is a cosmopolitan zoonotic parasitic disease of nearly all warm-blooded mammals and birds. The infection rate with *T. gondii* in humans, animals, and birds is influenced by some epidemiological risk factors, such as age, sex, feeding pattern, and geographical distribution (Wilking et al., 2016; Zhang et al., 2016). Strains of *T. gondii* are transferred through continents mainly by stray cats and other animals, including migratory birds (Can et al., 2014). Most domestic and wild avian species showed infection with *T. gondii* (Ammar et al., 2020; Mikaeel and Al-Saeed, 2020; Lv et al., 2021). Avian species become infected with *T. gondii* through ingestion of the infective oocysts contaminated soil, food, and water (Ruiz and Frenkel, 1980; Dubey et al., 2008) as these oocysts may be shed in the feces of host animal's like cats (Yan et al., 2009). The oocysts of the parasite were detected in the liver, brain, and muscles of broiler chickens (Deyab and Hassanein, 2005; Amin et al., 2012). On the other hand, humans get infected with *T. gondii* through ingestion of undercooked chicken meat containing infective oocysts or other stages of the parasite (Zhang et al., 2016). However, spontaneous abortion, mental, and congenital disorders, as well as stillbirth and other complications were observed in people infected with *T. gondii* (Krueger et al., 2014; Egorov et al., 2018). Generally, the diagnosis of toxoplasmosis depends mainly on the detection of specific antibodies using serological tests (Li et al., 2020). Microscopic examination and molecular techniques such as Polymerase Chain Reaction (PCR) are other methods used for the diagnosis of toxoplasmosis (Barakat et al., 2012; Ibrahim et al., 2016a).

Egyptian environment enhances the infection and transmission of *T. gondii* due to the presence abundant number of domestic or stray cats that shed oocysts and contaminate the environment. Animals such as sheep, goats, and chickens

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.,* 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

are regarded as important intermediate hosts for *T. gondii* and their meat is the main source of the parasite to humans in case consumed unhygienically (El-Massey et al., 1990). Serological tests reveal the presence of *T. gondii* infections with a high prevalence rate either in animal or human populations. In addition, definitive diagnostic procedures of clinical cases with toxoplasmosis in Egypt are still required (Abbas et al., 2020). Regarding the infection of avian species with *T. gondii*, insufficient data are available. Therefore, this review article was developed to highlight the infection of avian species with *T. gondii*, diagnosis, pet birds and human infection, and control methods with a special reference to the current status of infection among the Egyptian poultry farms and population.

The protozoon parasite

There are three morphological stages of toxoplasmosis infection, namely an active multiplying tachyzoite stage, slowly multiplying semi-dormant bradyzoite stage in tissue cysts, and a sporozoite stage within oocysts that present in the environment (Ferguson, 2004; Al-Ammash et al., 2018). It has been reported that sporulated oocysts of *Toxoplasma* spp. can persist and survive in the environment for a long time and can infect all warm-blooded animals, birds, and humans (Frenkel, 2000; Sibley et al., 2009; Abdel-Shafy et al., 2015). Oocysts require 1 to 5 days to sporulate and become infective. Environmental conditions such as warm temperature or high humidity are essential for the survival of oocysts. It has been found that the incidence of toxoplasmosis is higher in humid tropical areas than in arctic areas (Meerburg and Kijlstra, 2009; Simon et al., 2013). Suitable environmental conditions, such as high temperature especially in spring, summer, and early autumn enhance the sporulation of *T. gondii* oocysts.

Genetic polymorphic classification of *T. gondii* strains is based on detection of three major clonal lineage types I, II, and III, additional lineage, and recombinant or atypical genotypes (Shwab et al., 2014; Lorenzi et al., 2016). Type II strains of *T. gondii* were common in Europe (Ajzenberg et al., 2002), while type II and III strains were detected in sub-Saharan and North Africa, the Middle East, and the Peninsula (Al-Kappany et al., 2010a; Dubey et al., 2010; Mercier et al., 2010). In addition, types I, II, and III of *T. gondii* and the recombinant strains were found in North and Central, and South America (Pena et al., 2008; Khan et al., 2011; Rajendran et al., 2012). In some Asian countries such as Iran, the three major clonal lineages of *T. gondii* were also detected (Zia-Ali et al., 2007; Chaichan et al., 2017).

Susceptibility of avian species to toxoplasmosis

Various domestic and farm animals, including birds, showed infection with *T. gondii* (Mose et al., 2016). Direct detection of *T. gondii* oocysts in the environment is difficult, so infected broiler chickens are regarded as an important indicator for severe environmental contamination and poor hygienic conditions in poultry farms (Dubey, 2010; Mahmood et al., 2014). World-wide infection of domestic avian species, such as chickens, turkeys, ducks, and pigeons, with *T. gondii* has been recorded and they are considered as important sources of humans' infection (Dubey, 2010; Guo et al., 2015).

The prevalence rate of *T. gondii* in backyard chickens was 65.1% in Rio de Janeiro (da Silva et al., 2003), 64% in Ghana (Dubey et al., 2008), 11.4% in China (Yan et al., 2009), and 38.4% in Ethiopia (Tilahun et al., 2013). In Iraq, Mohammed and Abdullah (2013) detected *T. gondii* in domestic chickens kept in the house in Sulaimania Province, while Mikaeel and Al-Saeed (2020) found a high prevalence rate of the parasite among free-range local chickens in Duhok Province, and this was indicative for heavy environmental contamination with the parasite. In the same context, some researchers indicated that the infection rate of *T. gondii* was more in free-range chickens when compared with cages reared birds in Nanjing region, China (Liu et al., 2017) and in Egypt (Ibrahim et al., 2009). Moreover, in Pakistan, it has been observed that domesticated chickens could harbor more *T. gondii* than broiler chickens kept in farms which may be attributed to frequent contact of domesticated birds with cats and ground feeding habits (Khan et al., 2020). Besides, broiler chickens kept in farms are reared under a controlled environment, have fast growth, and have low chances of contact with reservoir animals, such as cats (Krueger et al., 2014). The experimental challenge of turkeys with *T. gondii* was successful (Dubey et al., 1993b).

Some reports investigated the presence of toxoplasmosis in pigeons as well as their relation to public health (Dubey, 2002; Tsai et al., 2006). Free-flying birds such as pigeons may act as another reservoir or source of *Toxoplasma* infection for contact birds, animals, or humans (Biancifiori et al., 1986). Besides, successful experimental infection of pigeons with *T. gondii* has been conducted with induction of signs and death (Simitch et al., 1965).

Water fowls, including ducks and geese, are also susceptible to *T. gondii* infection. The first detection of fetal toxoplasmosis in domesticated ducks was in Argentina (Boehringer et al., 1962), then the disease was recorded worldwide in different countries (Zardi et al., 1967; Literák and Hejlícek, 1993; El-Massry et al., 2000). The prevalence's rates of *T. gondii* in ducks were recorded as 56% in Italy (Zardi et al., 1967) and 6% in Florida (Burridge et al., 1979), as well as 20% (Chen et al., 1986), 32.19% (Zhai et al., 1987), 23.33% (Zhang, 1989), and 3.93% (Lv, 1993) in China. Moreover, ToxoDB#9 genotype (Chinese I strain) was found to be predominant in ducks (Including 115 duck muscle samples, Zou et al., 2017). In China, *T. gondii* has been detected molecularly and serologically either in ducks (Lv et al., 2021) or geese (Rong et al., 2014).

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

Successful oral infection of bobwhite quails (Dubey et al., 1993b) and Japanese quails (Dubey et al., 1994) with 49 strain of *T. gondii* oocysts has been reported. From the previous studies, the oocysts were re-isolated from the brain, heart, and muscles of the inoculated quails, moreover, antibodies to *T. gondii* were detected in quails for 63 days post-infection (PI) using an agglutination test. Experimental inoculation of quails with *T. gondii* tachyzoites revealed distribution and presence of this stage in the liver, lungs, and spleen at the day 7 PI, while the oocysts were detected in the brain and the heart of the birds on day 70 PI (Albuquerque et al., 2001).

Antibodies against *T. gondii* have been demonstrated serologically in ostriches in different localities of the world like Ghana (Dubey et al., 2000), Spain (Martínez-Díaz et al., 2002), Zimbabwe (Hove and Mukaratirwa, 2005), Brazil (Contente et al., 2009) and Egypt (El-Madawy and Metawea, 2013).

Wild birds are regarded as an important source and a reservoir for *T. gondii* for carnivores, besides, some of these birds are migratory and can spread the parasite worldwide (Nardoni et al., 2019). For instance, the prevalence rates of the parasite were 1% in doves, and 6.9% in the wild pigeon (Ammar et al., 2020), as well as 26.5% and 17.5% in sparrows of Iran (Khademvatan et al., 2013) and Brazil (Gondim et al., 2010), respectively. Moreover, the seroprevalences of *T. gondii* in broiler and layer chickens, pigeons, and sparrows using a random-effect model in Iran were 20%, 8%, and 15%, respectively (Shokri et al., 2017). In the same context, Amouei et al. (2018) serologically detected *T. gondii* in 51.4% of 385 free-ranging birds (chicken, ducks, and geese) and migratory birds (*Anas crecca*, *Anas platyrhynchos*, and *Fulica atra*).

Regarding the age's susceptibility, it has been reported that older hens were more susceptible to *T. gondii* than younger ones in the Thika Region of Kenya (Mose et al., 2016). A recent study by Lv et al. (2021) in China showed that ducks older than one year were more susceptible to the parasite than ducks younger than one year old.

Pathogenicity of parasite

Infection with *T. gondii* is mainly induced lesions in the liver and small intestine. The lesions are represented as congestion of the blood vessels and degenerative changes of the tissues. Congestion of the blood vessels is related to the ability of the parasite to pass from the intestine to the bloodstream and release some protein substances that destruct the blood platelets leading to increased vascular permeability and hemorrhages (Burney et al., 1999). The hyperplasia of the liver and the small intestine's cells were also reported (Amin et al., 2012). Infiltration of the parasite in the intestinal cells leads to apoptosis and deaths of some cells, while other cells show rapid regeneration and hyperplasia (Liesenfeld, 2002).

Diagnosis of toxoplasmosis

Rapid detection of the stages of *T. gondii* infection in avian hosts can be carried out through finding the parasites in stained impression smears or through histopathological sections of the affected organs (Dubey et al., 2007a; Dubey et al., 2007b; Ibrahim et al., 2016a). Moreover, *T. gondii* antigen could be detected using immunohistochemical staining techniques with polyclonal rabbit antibodies (Dubey et al., 2001). Intraperitoneal mouse inoculation (mouse bioassay) of *T. gondii* positive tissues should be done to obtain the tachyzoites stage (Dubey, 2010).

Diagnosis of *T. gondii* infection especially in birds is mainly based on the detection of specific antibodies using serological tests (Cabezón et al., 2011; Li et al., 2020). It has been reported that antibodies to *Toxoplasma* infection in cats could be detected within 3 weeks of infection and persisted in high titers for 5 years even in the absence of re-infection (Dubey, 1995). Enzyme-linked immunosorbent assay (ELISA), competitive-inhibition ELISA, indirect fluorescent antibody test (Nardoni et al., 2019), modified agglutination test (MAT) (Dubey, 2010; Alvarado-Esquivel et al., 2012; Rong et al., 2014), latex agglutination test (Raafat et al., 2011), Sabin-Feldman dye test (Literák and Hejlícek, 1993) and Western blotting are commonly used as serological techniques for the diagnosis of the disease (Huang et al., 2004). The most useful and commonly used serological test for the detection of *T. gondii* infection is MAT as it is specific, sensitive, does not require special equipment, and can be used for all avian species (Dubey, 2002). Tachyzoites of *T. gondii* could be maintained on monkey kidney adherent fibroblasts (Vero cells) cultures supplemented with 8% heat-inactivated fetal bovine serum for further serological detection (Ibrahim et al., 2016a).

The seroprevalences of *T. gondii* in domestic birds vary from one country to another according to the method used in testing, the number of the examined birds, and the type and the hygiene of breeding (Dubey, 2010). Many factor such as the locality, the number of birds, and the type and the hygiene of breeding affect the incidence or seroprevalences of *T. gondii* in domestic avian species (Dubey, 2010). For example, the seroprevalence of *T. gondii* antibodies among free-ranging chickens was 27.1% in Southern Iran (Asgari et al., 2008) and 40.4% in Giza Province of Egypt (Dubey et al., 2003a),

The molecular techniques for the diagnosis of *T. gondii* infection are based on the identification of immunodominant antigens using sera of animals infected with geographically distant isolates and from acute and chronically infected animals. In this regard, the surface antigen 2 of *T. gondii* (TgSAG2) which is expressed in *Escherichia coli* or the insect cells can be used as a useful, highly sensitive, and specific antigen for ELISA (Huang et

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

al., 2002). Moreover, PCR is a specific, rapid, sensitive, and cost-effective technique that could be used for the detection of *T. gondii* DNA in chickens (Barakat et al., 2012). Howe et al. (1997) and Dubey et al. (2005) a fragment of 94 bp from the B1 gene of the parasite as a target to PCR amplification.

Toxoplasmosis in pet birds

Pet birds are usually kept in close contact with a human for companionship and entertainment. These birds play an important epidemiological role in the transmission and maintenance of many pathogens with public health significance for humans. Pet birds are bred in a semi-free-range system, so the birds have opportunities to contact food or water contaminated with *T. gondii* when they gather together. In addition, wild pet birds could transmit the protozoon in a long distance during flying and migration, and this transmission accelerated the spread of *T. gondii* diffusion. The role of pet birds in the transmission of *T. gondii* should be given more concern because they can serve as an important source of infection for cats (Ruiz and Frenkel, 1980; Dubey and Hamir, 2002). For instance, dead *T. gondii* infected pet birds from parks, pet shops or households are often un-hygienically disposed of and may be eaten by cats, and consequently, cats may become infected with the parasite and shed millions of oocysts. However, the transmission of *T. gondii* from pet birds to humans is not common as they are not bred for meat production (Boseret et al., 2013).

Up to now, some studies have been carried out to investigate the prevalence of *T. gondii* in psittacines and passerines species (Dubey, 2002; Hartley et al., 2008; Gazzonis et al., 2021). Fetal toxoplasmosis has been reported in parrots in Australia and New Zealand, psittacines in America, and budgerigars in Switzerland and the Netherlands (Dubey et al., 2004; Ferreira et al., 2012; Howe et al., 2014). In Brazil, the anti-*T. gondii* antibodies (IgY) were found in the serum of 71 adult blue-fronted Amazon parrots with a seropositivity rate of 9.8% (Marietto-Gonçalves et al., 2013). Besides, Andrade et al. (2016) serologically examined 67 different psittacine species and found anti-*T. gondii* antibodies in 1.3% of the examined birds. However, the recent investigation of Sato et al. (2020) revealed the absence of antibodies against *T. gondii* in wild red-tailed Amazon parrots. For the first time in China, the anti- *T. gondii* antibodies were found in 13.63% of Cockatiels and 3.85% of Lovebirds (Zhang et al., 2014). Further Chinese study of Cong et al. (2014) demonstrated that the seroprevalences of *T. gondii* were 11.65%, 11.39%, and 5.26% in Eurasian Siskin, Oriental Skylark, and Black-tailed Grosbeak, respectively, and these birds molecularly showed the presence of *T. gondii* B1 gene and type II variant (ToxoDB genotype #3). A virulent type II *T. gondii* strain has been isolated from a black-winged lory in North America (Dubey et al., 2004; Dubey et al., 2011), while type I/III variant *T. gondii* strain was demonstrated in Valley quail in Brazil (Casagrande et al., 2015). In Australia, based on histopathology, immunohistochemistry, and multilocus DNA typing findings, atypical type II genotype *T. gondii* strain was found in a pet peach-faced lovebird with nervous signs and lesions in the brain, spleen, liver, and heart (Cooper et al., 2015). Budgerigars are relatively resistant to clinical toxoplasmosis (Dubey and Hamir, 2002; Zhang et al., 2014). However, successful experimental infection of Budgerigars with *T. gondii* has been carried out (Kajerová et al., 2003). Before the identification of *Toxoplasma* species in 1908, the *Toxoplasma*-like parasite was detected in Java sparrows in the 1900s (Tenter et al., 2000), however, antibodies against *T. gondii* were detected in 34.29% of Java sparrows (Huang et al., 2019).

Toxoplasmosis in human

Recently, there is increasing attention to understand the main sources for human infection with toxoplasmosis (Dubey et al., 2008). Humans could be infected with *T. gondii* through ingestion of contaminated water or consumption of undercooked or raw poultry meat products containing infective oocysts or other stages of the parasite (Dubey and Jones, 2008; Dubey et al., 2010; Zhang et al., 2016). In addition, other problems such as congenital infection as well as blood transfusions and organs transplantation transmission methods have been reported (Tenter et al., 2000). Several factors are associated with infection of humans with *T. gondii* such as food handling and preparation hygiene, eating habits, levels of natural immunity, the oocysts contamination of the environment, and the level of contact with infected animals (del-Castillo and Herruzo, 1998; Swai and Schoonman, 2009).

It has been estimated that one-third of humans could be infected by *T. gondii* (Shokri et al., 2017) leading to different mental and congenital disorders, spontaneous abortion, and stillbirth (Krueger et al., 2014). In the United State of America, about 400-4000 infants are born with congenital toxoplasmosis having some complications like schizophrenia and obsessive-compulsive disorder (Egorov et al., 2018).

Toxoplasmosis is not commonly a significant problem for healthy people, however, it can be a life-threatening problem for congenitally infected young immuno-deficient patients, and primary infected pregnant women resulting in an acute or reactivated infection or even death (Pinard et al., 2003; Montoya and Liesenfeld, 2004; Remington et al., 2006). In Pakistan, the reports showed that the prevalence of *T. gondii* in the human population ranged from 12% to 28% (Majid et al., 2016; Latif et al., 2017; Nazir et al., 2017).

Toxoplasmosis in Egypt

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

Identification of *T. gondii* infections either in humans, animals, and birds in Egypt is commonly based on serological and molecular techniques. The high prevalence of *T. gondii* infection in Egypt may be related to the presence of an abundant number of homeless cats that live on scraps of garbage to hunt for their food (Abbas et al., 2020). These cats are the main host and source of the parasite that heavily contaminate the environment with oocysts. Animals and birds can get the infection with *T. gondii* from this contaminated environment (Al-Kappany et al., 2010b). The main risk factor associated with *T. gondii* seropositive free-range and wild birds may be the contact with soil-harboring oocysts from street cats (Ibrahim et al., 2009). The high seroprevalence rate of *T. gondii* in free-range and cage chickens may be owing to the contact with soil-harboring oocysts shed from street cats (Ibrahim et al., 2009). Consumption of improperly cooked or grilled meat and meat products of domesticated animals (rabbits and poultry) is a major risk factor for Egyptians, especially those living in rural areas (Abou Elez et al., 2017).

From 2000 until 2020, the seroprevalences of *T. gondii* in birds were variable in different provinces of Egypt based on the geographical location, type of the collected samples, season, and the bird's species, age, and sex. Table 1 shows the incidences and the prevalence rates of *T. gondii* infections in different avian species from different provinces in Egypt in the period from 2000-2020.

Table 1. The incidences and the prevalence rates of *Toxoplasma gondii* infections in different avian species from different provinces in Egypt in the period from 2000-2020.

Species of birds	The findings	References
Commercial turkeys, chickens, and ducks	The MAT has been done to detect the presence of antibodies against *T. gondii* in the sera of 173 turkeys, 108 chickens, and 48 ducks from Giza, Egypt. The prevalence rateS of anti-*T. gondii* antibodies among turkeys, chickens, and ducks were 59.5%, 47.2%, and 50%, respectively.	El-Massry et al. (2000)
Commercial chickens	A high prevalence rate (40.4 %) of *T. gondii* in chickens from the rural area surrounding Giza (South of Cairo), Egypt was detected using MAT.	Dubey et al. (2003)
House-bred and farm-bred chickens	The seroprevalence of *Toxoplasma* antibodies was 30.0% (18 out of 60) in house-bred chickens, while it was 11.1% (10 out of 90) in farm-bred chickens by MAT in different Egyptian governorates. The histopathological examination of the tissues revealed lesions induced by *T. gondii* and the oocysts were detected in the liver, brain, heart, and skeletal muscles of 22 (78.6%) out of 28 positive chickens.	Deyab and Hassanein (2005)
Free-range and caged chickens	Serological detection of *T. gondii* specific antibodies, as well as tissue oocysts, showed positive percentages of 16.49 and 11.34 % in the free-range chickens and 8.69 and 4.83 % in the caged chickens, respectively in Delta provinces, Egypt.	Ibrahim et al. (2009)
Ducks	The prevalence of *T. gondii* was detected in ducks from Behera governorate, Egypt using MAT. The prevalence rate of the parasite was 13.9%. The highest prevalence was in the native breed (17.65%) and the 6-8-months age group (19.4%).	AbouLaila et al. (2011)
Quails	The presence of anti-*T. gondii* antibodies in the fecal and serum samples of native quails in Giza province, Egypt has been detected. It has been found that *T. gondii* antibodies prevalence rates were 29.8 and 25.5%, using MAT and LAT, respectively.	Raafat et al. (2011)
Free-range and commercial chickens	The seroprevalence rates of *T. gondii* in chickens of six Egyptian governorates were compared using ELISA. In addition, the presence of local *T. gondii* chicken strain was confirmed by PCR. The total prevalence rate was 68.8% comprised of 59.5%, 82.3%, 67.1%, 62.2%, 75%, and 50% in El Sharkia, El Gharbia, Kafr El sheikh, Cairo, Quena, and Sohag governorates, respectively. Moreover, the prevalence rates were higher among free-range (69.5%) than commercial farm chickens (68.5%); while the prevalence rate was less in upper Egypt than lower Egypt governorates and Cairo.	Barakat et al. (2012)
Free-range and farmed chickens	The seroprevalence of *T. gondii* infection in domestic chickens and humans in Beni-Suef province, Egypt was demonstrated. Serum samples of 215 (90 free-range and 125 farmed) chickens were examined using MAT. In addition, 250 sera samples were collected and examined for IgG using ELISA. The results showed 20% and 9.6% of antibodies of *T. gondii* in free-range and farmed chickens, respectively. However, antibodies to *T. gondii* were detected in 37.5% of poultry contact workers and in 30.5% of non-poultry contacts persons. Seroprevalence of 45.0 and 41.66% were observed among persons of ages 41-50 years and >50 years, respectively.	Aboelhadid et al. (2013)
Chickens	A total of 304 blood and brain samples were collected from chickens in the Delta provinces of Egypt. The prevalence rates of *T. gondii* infection were 11.18%, 6.91%, 6.91% using ELISA, histopathology, and immunohistochemistry methods, respectively. Moreover, significant differences in the prevalence of *T. gondii* were detected on the basis of season, sex and habitat.	Ibrahim et al. (2016a)
Ostriches	One hundred and twenty serum samples from ostriches in Ismailia province, Egypt were tested for anti- *T. gondii* antibodies using ELISA and MAT tests. Using enzyme immunoassay, 5 out of 120 birds (4.2%) were positive to IgM, while 11 birds (9.2%) were positive to IgG. However, MAT detected IgG in 15 birds (12.5%). The results of PCR revealed the presence of *T. gondii* DNA in the blood of 9 birds (7.5%). The results of PCR of the tissues showed positive *T. gondii* DNA in 5 dead birds either in the heart, brain, and thigh muscles.	El-Madawy and Metawea (2013)
Quails	One hundred samples were collected from 7-37 days old diseased quails in Assiut and El-Menia governorates, Egypt. The results of Giemsa-stained smears revealed the presence of *Toxoplasma's* tachyzoites in 7% of the examined samples.	Hassan et al. (2020)

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

The seroprevalences of *Toxoplasma* infections in humans varied from 27 % to 68% in asymptomatic pregnant women (Ghoneim et al., 2010; El Deeb et al., 2012; Ibrahim et al., 2016b), 26% in cerebrospinal fluid of patients with meningoencephalitis (Mabrouk and Dahawi, 1991), and 59.6% in blood donors without signs (Elsheikha et al., 2009). It has been reported a higher percentage of toxoplasmosis among Egyptians with liver cirrhosis (El-Henawy et al., 2015; El-Sayed et al., 2016). In the Province of Sharkia, Egypt, Mostafa et al. (2018) demonstrated a significant correlation between the seroprevalence of *T. gondii* in patients and the presence of different types of tumors as breast cancer, bone's squamous cell carcinoma, and brain tumors. Anti-*Toxoplasma* IgG was found in patients with liver tumors and bladder cancer, while IgM was detected in patients with benign uterine tumors, bone carcinoma, and breast cancer. In addition, *T. gondii* oocysts were detected in immuno-stained brain sections. In another study revealed a significantly higher proportion of seropositive anti-*Toxoplasma* IgG antibodies among type I diabetes mellitus patients (45%) compared with the control group (23.3%) (Khattab et al., 2019).

Prevention and control of toxoplasmosis

Estimation of zoonotic diseases in animals and birds is helpful for monitoring and improving public health in humans. Therefore, a definitive diagnosis of *Toxoplasma* infection in animals is necessary to prevent human zoonose infection (AbouLaila et al., 2011). Definitive diagnosis of zoonotic diseases with public health concerns, such as toxoplasmosis is very helpful for the prevention of human infection (AbouLaila et al., 2011).

Consumption of insufficiently cooked meat of birds should be avoided as well as fecal contamination from birds should be controlled (Raafat et al., 2011). Nardoni et al. (2019) demonstrated the wide distribution of *T. gondii* in game and wild birds and advised accurate estimation of the human infection risks in handling, managing, and eating wild bird species with regard to domestic carnivores and the impact of viscera or offal's in the environment.

Free-ranged and farmed chickens are regarded as a potential risk to the contact resident persons, so, application of strict preventive hygiene measures is a must to avoid transmission of *T. gondii* infection from birds to humans (Aboelhadid et al., 2013; Mikaeel and Al-Saeed, 2020).

It is very essential to implement a high level of education and awareness among populations to reduce the prevalence of toxoplasmosis (Jones et al., 2001). Consumption of undercooked animal products or unwashed raw vegetable/fruit, poor hands hygiene, and un-carful contact with soil, farm animals, and birds should be avoided (El Deeb et al., 2012). Furthermore, periodic exclusion diagnosis of toxoplasmosis in patients with chronic diseases should be done to avoid the possibility of increasing the disease's severity and for possible management of these malignancies.

CONCLUSION

Toxoplasmosis represents a complex problem, not only for animals and birds but also for human health, especially under Egyptian conditions. However, the current situation of avian toxoplasmosis in Egypt still needs more investigation. Therefore, periodical detection of toxoplasmosis prevalence's in living birds or in their products as well as application of hygienic measures in poultry farms are important to avoid the possibility of zoonotic transmission. In addition, periodical testing and examination, as well as public health awareness of human populations, are very critical.

DECLARATIONS

Competing interests

The author has not declared any conflict of interest.

Ethical considerations

Plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by the author.

REFERENCES

Abbas I, Villena I, and Dubey J (2020). A review on toxoplasmosis in humans and animals from Egypt. Parasitology, 147(2): 135-159. DOI: https://www.doi.org/10.1017/s0031182019001367

Abdel-Shafy S, Shaapan RM, Abdelrahman KA, El-Namaky AH, Abo-Aziza FAM, and Zeina HAA (2015). Detection of *Toxoplasma gondii* Apicomplexa: Sarcocystidae in the brown dog tick *Rhipicephalus sanguineus* Acari: Ixodidae fed on infected rabbits. Research Journal of Parasitology, 10: 142-150. DOI: https://www.dx.doi.org/10.3923/jp.2015.142.150

Aboelhadid SM, Abdel-Ghany AE, Ibrahim MA, and Mahran HA (2013). Seroprevalence of *Toxoplasma gondii* infection in chickens and humans in Beni Suef, Egypt. Global Veterineria, 11: 139-144. Available at: https://www.idosi.org/gv/gv11(2)13/3.pdf

Abou Elez R, Hassanen E, Tolba H, and Elsohaby I (2017). Seroprevalence and risk factors associated with *Toxoplasma gondii* infection in domestic rabbits and humans. Veterinary Parasitology: Regional Studies and Reports, 8: 133-137. DOI: https://www.doi.org/10.1016/j.vprsr.2017.02.009

AbouLaila M, El-Bahy N, Hilali M, Yokoyama N, and Igarashi I (2011). Serodiagnosis of *Toxoplasma gondii* in ducks from Behera governorate, Egypt. The Journal of protozoology Research, 21: 45-49. Available at: https://obihiro.repo.nii.ac.jp/?action=repository_action_common_download&item_id=14

Ajzenberg D, Cogné N, Paris L, Bessières MH, Thulliez P, Filisetti D, Pelloux H, Marty P, and Dardé ML (2002). Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. Journal of Infectious Diseases, 186(5): 684-689. DOI: https://www.doi.org/10.1086/342663

Al-Ammash MSJ, Al-Shaibani KTM, and Al-Abodi HRJ (2018). Investigating the prevalence of infection with *Toxoplasma gondii* in men and women in Samaraa city, Iraq. Plant Archives, 18(2): 2501-2508. Available at: http://www.plantarchives.org/18-

Albuquerque GR, Munhoz AD, Oliveira FCR, and Lopes CWG (2001). Biological behavior of P and Sero-47 strain of Toxoplasma gondii in an experimental infection in Japanese quail. British Journal of Veterinary Parasitology, 10: 55-59. Available at: http://rbpv.org.br/download/1022001/c10255_59

Al-Kappany YM, Rajendran C, Abu-Elwafa SA, Hilali M, Su C, and Dubey JP (2010a). Genetic diversity of *Toxoplasma gondii* isolates in Egyptian feral cats reveals new genotypes. Journal of Parasitology, 96(6): 1112-1114. DOI: https://www.doi.org/10.1645/ge-2608.1

Al-Kappany YM, Rajendran C, Ferreira LR, Kwok OC, Abu-Elwafa SA, Hilali M, and Dubey JP (2010b). High prevalence of toxoplasmosis in cats from Egypt: Isolation of viable *Toxoplasma gondii*, tissue distribution, and isolate designation. Journal of Parasitology, 96: 1115-1118. DOI: https://www.doi.org/10.1645/ge-2554.1

Alvarado-Esquivel C, González-Salazar AM, Alvarado-Esquivel D, OntiverosVázquez F, Vitela-Corrales J, Villena I, and Dubey JP (2012). Seroprevalence of *Toxoplasma gondii* infection in chickens in Durango State, Mexico. Journal of Parasitology, 98(2): 431-432. DOI: https://www.doi.org/10.1645/ge-2979.1

Amin D, GholamReza H, Koorosh S, and Mohammad M (2012). Clinical, serological and histopathological signs of toxoplasmosis in broiler chicken (*Gallus domesticus*) after experimental infection. Comparative Clinical Pathology, 21: 1379-1382. DOI: https://www.doi.org/10.1007/s00580-011-1300-x

Ammar S, Purple K, and Gerhold R (2020). *Toxoplasma gondii* prevalence in hunter-killed mourning doves (*Zenaida macroura*) and rock pigeons (*Columba livia*) from East Tennessee. Journal of Wildlife Diseases, 56: 479-481. DOI: https://www.doi.org/10.7589/2019-06-155

Amouei A, Sharif M, Hosseini SA, Sarvi S, Mizani A, Salehi S, Gholami S, Jafar-Ramaji T, and Daryani A (2018). Prevalence of *Toxoplasma gondii* infection in domestic and migrating birds from Mazandaran province, northern Iran. Avian Biology Research, 11(1): 12-15. DOI: https://www.doi.org/10.3184%2F175815617X15105842200290

Andrade LHM, Lugarini C, Oliveira RAS, Silva LTR, Marvullo MFV, Garcia JE, Dubey JP, and Silva JCR (2016). Occurrence of antibodies to *toxoplasma gondii* in wild birds from three federal conservation units in Paraíba and Bahia / Occurrence of antibodies to *toxoplasma gondii* in wild birds from three federal conservation units in Paraíba and Bahia, Brazil. The Brazilian Journal of Veterinary Parasitology, 36(2): 103-107. DOI: http://www.dx.doi.org/10.1590/S0100-736X2016000200007

Asgari Q, Akrami Mohajeri F, Kalantari M, Esmaeilzadeh B, Farzaneh A, Moazeni M, Ghalebi SR, Saremi F, Zarifi Kalyani M, and Motazedian MH (2008). Chicken toxoplasmosis in different types of breeding: A seroprevalence survey in Southern Iran. International Journal of Poultry Science, 7: 1247-1250. DOI: https://www.dx.doi.org/10.3923/ijps.2008.1247.1250

Barakat AM, Salem LM, El-Newishy AM, Shaapan RM, and El-Mahllawy EKZ (2012). Zoonotic chicken toxoplasmosis in some Egyptians governorates. Pakistan Journal of Biological Sciences, 15: 821-826. DOI: https://www.dx.doi.org/10.3923/pjbs.2012.821.826

Biancifiori F, Rondini C, Grelloni V, and Frescura T (1986). Avian toxoplasmosis: Experimental infection of chicken and pigeon. Comparative Immunology, Microbiology and Infectious Diseases, 9(4): 337-346. DOI: https://www.doi.org/10.1016/0147-9571(86)90046-9

Boehringer EG, Fornari OE, and Boehringer OK (1962). The first case of fatal toxoplasmosis in domestic ducks in Argentina. Avian Diseases, 6(4): 391-396. DOI: https://www.doi.org/10.2307/1587913

Boseret G, Losson B, Mainil JG, Thiry E, and Saegerman C (2013). Zoonoses in pet birds: Review and perspectives. Veterinary Research, 44: 36. DOI: https://www.doi.org/10.1186/1297-9716-44-36

Burney DP, Lappin MR, and Spiker M (1999). Detection of *Toxoplasma gondii* parasitemia in experimentally inoculated cats. Journal of Parasitology, 65(5): 947-951. Available at: https://pubmed.ncbi.nlm.nih.gov/10577732/

Burridge MJ, Bigler WJ, Forrester DJ, and Hennemann JM (1979). Serologic survey for *Toxoplasma gondii* in wild animals in Florida. Journal of the American Veterinary Medical Association, 175: 964-967. Available at: https://pubmed.ncbi.nlm.nih.gov/521384/

Cabezón O, García-Bocanegra I, Molina-López R, Marco I, Blanco JM, Höfle U, Margalida A, Bach-Raich E, Darwich L, Echeverría I et al. (2011). Seropositivity and risk factors associated with *Toxoplasma gondii* infection in wild birds from Spain. PLoS One, 6(12): e29549. DOI: https://www.doi.org/10.1371/journal.pone.0029549

Can H, Döşkaya M, Ajzenberg D, Özdemir HG, Caner A, İz SG, Döşkaya AD, Atalay E, Çetinkaya Ç, Ürgen S et al. (2014). Genetic characterization of *Toxoplasma gondii* isolates and toxoplasmosis seroprevalence in stray cats of İzmir, Turkey. PLoS One, 9(8): e104930. DOI: https://www.doi.org/10.1371/journal.pone.0104930

Casagrande RA, Pena HF, Cabral AD, Rolim VM, de Oliveira LG, Boabaid FM, Wouters AT, Wouters F, Cruz CE, and Driemeier D (2015). Fatal systemic toxoplasmosis in Valley quail (*Callipepla californica*). International Journal for Parasitology: Parasites and Wildlife, 4(2):264-267. DOI: https://www.dx.doi.org/10.1016%2Fj.ijppaw.2015.04.003

Centers of Disease Control and Prevention (CDC) (2004). Toxoplasmosis. pp. 11-22. Available at: https://www.cdc.gov/parasites/toxoplasmosis/index.html

Chaichan P, Mercier A, Galal L, Mahittikorn A, Ariey F, Morand S, Boumédiène F, Udonsom R, Hamidovic A, Murat JB et al. (2017). Geographical distribution of *Toxoplasma gondii* genotypes in Asia: A link with neighboring continents. Infection, Genetics and Evolution, 53: 227-238. DOI: https://www.doi.org/10.1016/j.meegid.2017.06.002

Chen GL, Zhang RT, Xu YW, and Ma JH (1986). A preliminary report for antibodies to *Toxoplasma gondii* in domestic livestock in Changchun area. Journal of Veterinary College, University of PLA, 6: 66-67. Available at: https://europepmc.org/article/MED/20707861

Cong W, Meng QF, Song HQ, Zhou DH, Huang SY, Qian AD, Su C, and Zhu XQ (2014). Seroprevalence and genetic characterization of *Toxoplasma gondii* in three species of pet birds in China. Parasites Vectors, 7: 152. DOI: https://www.doi.org/10.1186/1756-3305-7-152

Contente APA, Domingues PF, and Silva RC (2009). Prevalence of *T. gondii* antibodies in-ostriches (*Struthio camelus*) from commercial breeding facilities in the state of São Paulo, Brazil. Brasilian Journal of Veterinary Research and Animal Science, 46(3): 175-180. DOI: https://www.doi.org/10.11606/issn.1678-4456.bjvras.2009.26764

Cooper MK, Šlapeta J, Donahoe SL, and Phalen DN (2015). Toxoplasmosis in a pet peach-faced lovebird (*Agapornis roseicollis*). Korean Journal of Parasitology, 53(6): 749-753. DOI: https://www.doi.org/10.3347/kjp.2015.53.6.749

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

da Silva DS, Bahia-Oliveira LM, Shen S, Kwok OCH, Lehman T, and Dubey J (2003). Prevalence of *Toxoplasma gondii* in chickens from an area in southern Brazil highly endemic to humans. Journal of Parasitology, 89: 394-396. DOI: https://www.doi.org/10.1645/0022-

Del-Castillo F, and Herruzo R (1998). Risk factors for toxoplasmosis in children. Enfermedades Infecciosas y Microbiologia Clinica, 16: 224-222. Available at: https://pubmed.ncbi.nlm.nih.gov/9666585/

Deyab AK, and Hassanein R (2005). Zoonotic toxoplasmosis in chicken. Journal of the Egyptian Society of Parasitology, 35: 341-350. Available at: https://pubmed.ncbi.nlm.nih.gov/15881017/

Dubey JP (1995). Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. Journal of Parasitology, 81(3): 410-415. DOI: https://www.doi.org/10.2307/3283823

Dubey JP (2002). A review of toxoplamosis in wild birds. Veterinary Parasitology, 106(2): 121-153. DOI: https://www.doi.org/10.1016/s0304-4017(02)00034-1

Dubey JP (2010). *Toxoplasma gondii* infections in chickens *Gallus domesticus*: Prevalence, clinical disease, diagnosis and public health significance. Zoonoses Public Health, 57: 60-73. DOI: https://www.doi.org/10.1111/j.1863-2378.2009.01274.x

Dubey JP, and Hamir AN (2002). Experimental toxoplasmosis in budgerigars (*Melopsittacus undulatus*). Journal of Parasitology, 88: 514-519. DOI: https://www.doi.org/10.1645/0022-3395(2002)088[0514:etibmu]2.0.co;2

Dubey JP, and Jones JL (2008). *Toxoplasma gondii* infection in humans and animals in the United States. International Journal of Parasitology, 38(11): 1257-1278. DOI: https://www.doi.org/10.1016/j.ijpara.2008.03.007

Dubey JP, Camargo ME, Ruff MD, Shen SK, Wilkins GL, Kwok OCH, and Thulliez P (1993a). Experimental toxoplasmosis in turkeys. Journal of Parasitology, 79(6): 949-952. DOI: https://www.doi.org/10.2307/3283736

Dubey JP, Ruff MD, Camargo ME, Shen SK, Kwok OCH, Wilkins GL, and Thulliez P (1993b). Experimental toxoplasmosis in Bob-white quail (*Colinus virginianus*). Journal of Parasitology, 79(6): 935-939. DOI: https://www.doi.org/10.2307/3283733

Dubey JP, Goodwin MA, Ruff MD, Kwok OCH, Shen SK, Wilkins GL, and Thulliez P (1994). Experimental toxoplasmosis in Japanese quail. Journal of Veterinary Diagnostic Investigation, 6(2): 216-221. DOI: https://www.doi.org/10.1177%2F104063879400600213

Dubey JP, Scandrett WB, Kwok OCH, and Gajadhar AA (2000). Prevalence of antibodies to *T. gondii* in ostriches *Struthio camelus*). Journal of Parasitology, 86(3): 623-624. DOI: https://www.doi.org/10.1645/0022-3395(2000)086[0623:poattg]2.0.co;2

Dubey JP, Garner MM, Willette MM, Batey KL, and Gardiner CH (2001). Disseminated toxoplasmosis in magpie geese (*Anseranas sempalmata*) with large numbers of tissue cysts in livers. Journal of Parasitology, 87(1): 219-223. DOI: https://www.doi.org/10.1645/0022-

Dubey JP, Graham DH, Dahl E, Hilali M, El-Ghaysh A, Sreekumar C, Kwok OC, Shen SK, and Lehmann T (2003). Isolation and molecular characterization of *Toxoplasma gondii* from chickens and ducks from Egypt. Veterinary Parasitology, 114(2): 89-95. DOI: https://www.doi.org/10.1016/s0304-4017(03)00133-x

Dubey JP, Parnell PG, Sreekumar C, Vianna MC, De Young RW, Dahl E, and Lehmann T (2004). Biologic and molecular characteristics of *Toxoplasma gondii* isolates from striped skunk (*Mephitis mephitis*), Canada goose (*Branta canadensis*), black-winged lory (*Eos cyanogenia*), and cats (*Felis catus*). Journal of Parasitology, 90(5): 1171-1174. DOI: https://www.doi.org/10.1645/ge-340r

Dubey JP, Lopez B, Alvarez M, Mendoza C, and Lehmann T (2005). Isolation, tissue distribution and molecular characterization of *Toxoplasma gondii* from free-range chickens from Guatemala. Journal of Parasitology, 91: 955-957. DOI: https://www.doi.org/10.1645/ge-493r.1

Dubey JP, Sundar N, Gennari SM, Minervino AH, Farias NA, Ruas JL, dos Santos TR, Cavalcante GT, Kwok OC, and Su C (2007a). Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Pará state and the southern state Rio Grande do

Sul, Brazil revealed highly diverse and distinct parasite populations. Veterinary Parasitology, 143(2): 182-188. DOI: https://www.doi.org/10.1016/j.vetpar.2006.08.024

Dubey JP, Webb DM, Sundar N, Velmurugan GV, Bandini LA, Kwok OCH, Su C (2007b). Endemic avian toxoplasmosis on a farm in Illinois: clinical disease, diagnosis, biologic and genetic characteristics of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*), and a goose (*Anser anser*). Veterinary Parasitology, 148: 207-212. DOI: https://www.doi.org/10.1016/j.vetpar.2007.06.033

Dubey JP, Huong LTT, Lawson BWL, Subekti DT, Tassi P, Cabaj W, Sundar N, Velmurugan GV, Kwok OC, and Su C (2008). Seroprevalence and isolation of *Toxoplasma gondii* from free-range chickens in Ghana, Indonesia, Italy, Poland, and Vietnam. Journal of Parasitology, 94(1): 68-71. DOI: https://www.doi.org/10.1645/ge-1362.1

Dubey JP, Pas A, Rajendran C, Kwok OC, Ferreira LR, Martins J, Hebel C, Hammer S, and Su C (2010). Toxoplasmosis in Sand cats (*Felis margarita*) and other animals in the breeding centre for endangered Arabian wildlife in the United Arab Emirates and Al Wabra wildlife preservation, the State of Qatar. Veterinary Parasitology, 172: 195-203. DOI: https://www.doi.org/10.1016/j.vetpar.2010.05.013

Dubey JP, Velmurugan GV, Rajendran C, Yabsley MJ, Thomas NJ, Beckmen KB, Sinnett D, Ruid D, Hart J, Fair PA et al. (2011). Genetic characterisation of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. International Journal of Parasitology, 41(11): 1139-1147. DOI: https://www.doi.org/10.1016/j.ijpara.2011.06.005

Egorov AI, Converse R, Griffin SM, Styles J, Klein E, Sams E, Hudgens E, and Wade TJ (2018). Environmental risk factors for *Toxoplasma gondii* infections and the impact of latent infections on allostatic load in residents of Central North Carolina. BMC Infectious Diseases, 18(1): 421. DOI: https://www.doi.org/10.1186/s12879-018-3343-y

El Deeb HK, Salah-Eldin H, Khodeer S, and Allah AA (2012). Prevalence of *Toxoplasma gondii* infection in antenatal population in Menoufia governorate, Egypt. Acta Tropica, 124(3): 185-191. DOI: https://www.doi.org/10.1016/j.actatropica.2012.08.005

El-Henawy A, Abdel-Razik A, Zakaria S, Elhammady D, Saudy N, and Azab MS (2015). Is toxoplasmosis a potential risk factor for liver cirrhosis? Asian Pacific Journal of Tropical Medicine, 8(10): 784-791. DOI: https://www.doi.org/10.1016/j.apjtm.2015.09.003

El-Madawy SR, and Metawea FY (2013). Serological assays and PCR for detection of *Toxoplasma gondii* infection in an ostrich farm at Ismailia Province, Egypt. IOSR Journal of Agriculture Veterinary Science, 2(3): 56-60. Available at: https://www.bu.edu.eg/portal/uploads/Veterinary%20Medicine/Animal%20Hygiene,%20

El-Massey AA, Abdel-Gawad AM, and Nassar AM (1990). Isolation of *Toxoplasma gondii*, *Isospora felis* and *Isospora revolta* from (sheep, goats, chicken) in Egypt. Journal of the Egyptian Veterinary Medical Association, 50: 275-284. Available at: https://scialert.net/fulltext/?doi=pjbs.2012.821.826&org=11

El-Massry A, Mahdy OA, El-Ghaysh A, and Dubey JP (2000). Prevalence of *Toxoplasma gondii* antibodies in sera of turkeys, chickens, and ducks from Egypt. Journal of Parasitology, 86(3): 627-628. DOI: https://www.doi.org/10.1645/0022-3395(2000)086[0627:potgai]2.0.co;2

El-Sayed NM, Ramadan ME, and Ramadan ME (2016). *Toxoplasma gondii* infection and chronic liver diseases: Evidence of an association. Tropical Medicine and Infectious Diseases, 1(1): 7. DOI: https://www.dx.doi.org/10.3390%2Ftropicalmed1010007

Elsheikha HM, Azab MS, Abousamra NK, Rahbar MH, Elghannam DM, and Raafat D (2009). Seroprevalence of and risk factors for *Toxoplasma gondii* antibodies among asymptomatic blood donors in Egypt. Parasitology Research, 104(6): 1471-1476. DOI: https://www.doi.org/10.1007/s00436-009-1350-z

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

Ferguson DJ (2004). Use of molecular and ultra-structural markers to evaluate stage conversion of *Toxoplasma gondii* in both the intermediate and definitive host. International Journal of Parasitology, 34: 347-360. DOI: https://www.doi.org/10.1016/j.ijpara.2003.11.024

Ferreira FC Jr, Donatti RV, Marques MV, Ecco R, Preis IS, Shivaprasad HL, Vilela DA, and Martins NR (2012). Fatal toxoplasmosis in a vinaceous Amazon parrot (*Amazona vinacea*). Avian Diseases, 56(4):774-777. DOI: https://www.doi.org/10.1637/10063-011912-case.1

Frenkel JK (2000). Biology of *Toxoplasma gondii*. In: Ambroise-Thomas P., Petersen P.E. (eds) Congenital toxoplasmosis. Springer, Paris, pp. 9-25. DOI: https://www.doi.org/10.1007/978-2-8178-0847-5_2

Gazzonis AL, Villa L, Lubian E, Ressegotti S, Grilli G, Raimondi S, Zanzani SA, and Manfredi MT (2021). Molecular survey on *Toxoplasma gondii* and *Neospora caninum* infection in wild birds of prey admitted to Recovery Centers in Northern Italy. Microorganisms, 9(4): 736. DOI: https://www.doi.org/10.3390/microorganisms9040736

Ghoneim NH, Shalaby SI, Hassanain NA, Zeedan GS, Soliman YA, and Abdalhamed AM (2010). Comparative study between serological and molecular methods for diagnosis of toxoplasmosis in women and small ruminants in Egypt. Foodborne Pathogens and Disease, 7(1): 17-22. DOI: https://www.doi.org/10.1089/fpd.2008.0223

Gondim LSQ, Abe-Sandes K, Uzêda RS, Silva MSA, Santos SL, Mota RA, Vilela SM, and Gondim LF (2010). *Toxoplasma gondii* and *Neospora caninum* in sparrows (*Passer domesticus*) in the Northeast of Brazil. Veterinary Parasitology, 168: 121-124. DOI: https://www.doi.org/10.1016/j.vetpar.2009.09.055

Guo M, Duney J, Tender P, Hill D, Buchanan RL, Gamble HR, Jones JL, and Pradhan AK (2015). Prevalence and risk factors for *Toxoplasma gondii* infection in meat animals and meat products destined for human consumption. Journal of Food Protection, 78(2): 457-476. DOI: https://www.doi.org/10.4315/0362-028x.jfp-14-328

Hartley J, Booth R, Slocombe RF, and Dubey JP (2008). Lethal toxoplasmosis in an aviary of kakarikis (*Cyanoramphus* spp.) in Australia. Journal of Parasitology, 94(6): 1424-1425. DOI: https://www.doi.org/10.1645/ge-1619.1

Hassan AK, Naeem EV, and Soliman MA (2020). Investigation the prevalence of common parasitic infections in farmed quails in Upper Egypt. SVU-International Journal of Veterinary Sciences, 3(2): 38-50. DOI: https://www.dx.doi.org/10.21608/svu.2020.31915.1058

Hove T, and Mukaratirwa S (2005). Seroprevalence of *T. gondii* in farm-reared ostriches and wild game species from Zimbabwe, Acta Tropica, 94(1): 49-53. DOI: https://www.doi.org/10.1016/j.actatropica.2004.10.010

Howe DK, Honore S, Derouin F, and Sibley LD (1997). Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. Journal of Clinical Microbiology, 35(6): 1411-1414. Available at: https://www.ncbi.nlm.nih.gov/pubmed/9163454

Howe L, Hunter S, Burrows E, and Roe W (2014). Four cases of fatal toxoplasmosis in three species of endemic New Zealand birds. Avian Diseases, 58(1): 171-175. DOI: https://www.doi.org/10.1637/10625-080413-case.1

Huang X, Xuan X, Suzuki H, Sugimoto C, Nagasawa H, Fujisaki K, Mikami T, and Igarashi I (2002). Characterization of *Toxoplasma gondii* SAG2 expressed in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. Clinical and Diagnostic Laboratory Immunology, 9(6): 1343-1347. DOI: https://www.dx.doi.org/10.1128%2FCDLI.9.6.1343-1347.2002

Huang X, Xuan X, Hirata H, Yokoyama N, Xu L, Suzuki N, and Igarashi I (2004). Rapid immunochromatographic test using recombinant SAG2 for detection of antibodies against *Toxoplasma gondii* in cats. Journal of Clinical Microbiology, 42(1): 351-353. DOI: https://www.dx.doi.org/10.1128%2FJCM.42.1.351-353.2004

Huang SY, Fan YM, Chen K, Yao QX, and Yang B (2019). Seroprevalence and risk assessment of *Toxoplasma gondii* in Java sparrows (*Lonchura oryzivora*) in China. BMC Veterinary Research, 15: 129. DOI: https://www.doi.org/10.1186/s12917-019-1888-7

Ibrahim HM, Huang P, Salem TA, Talaat RM, Nasr MI, Xuan X, and Nishikawa Y (2009). Prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in northern Egypt. American Journal of Tropical Medicine and Hygiene, 80(2): 263-267. DOI: https://www.doi.org/10.4269/ajtmh.2009.80.263

Ibrahim HM, Abdel-Ghaffar F, Osman GY, El-Shourbagy SH, Nishikawa Y, and Khattab RA (2016a). Prevalence of *Toxoplasma gondii* in chicken samples from delta of Egypt using ELISA, histopathology and immunohistochemistry. Journal of Parasitic Diseases, 40(2): 485-490. DOI: https://www.dx.doi.org/10.1007%2Fs12639-014-0530-7

Ibrahim I, Salah H, El Sayed H, Mansour H, Eissa A, Wood J, Fathi W, Tobar S, Gur RC, Gur RE et al. (2016b). Hepatitis C virus antibody titers associated with cognitive dysfunction in an asymptomatic community-based sample. Journal of Clinical and Experimental Neuropsychology, 38: 861-868. DOI: https://www.doi.org/10.1080/13803395.2016.1168780

Jones JL, Kruszon-Moran D, Wilson M, McQuillan G, Navin T, and McAuley JB (2001). *Toxoplasma gondii* infection in the United States: seroprevalence and risk factors. American Journal of Epidemiology, 154(4): 357-365. DOI: https://www.doi.org/10.1093/aje/154.4.357

Kajerová V, Literák I, Bártová E, and Sedlák K (2003). Experimental infection of budgerigars (*Melopsittacus undulatus*) with a low virulent K21 strain of *Toxoplasma gondii*. Veterinary Parasitology, 116(4): 297-304. DOI: https://www.doi.org/10.1016/j.vetpar.2003.07.008

Khademvatan S, Saki J, Yousefi E, and Abdizadeh R (2013). Detection and genotyping of *Toxoplasma gondii* strains isolated from birds in the southwest of Iran. British Poultry Science, 54(1):76-80. DOI: https://www.doi.org/10.1080/00071668.2013.763899

Khan A, Dubey JP, Su C, SibleyL D, Ajioka JW, and Rosenthal BM (2011). Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. International Journal of Parasitology, 41(6): 645-655. DOI: https://www.doi.org/10.1016/j.ijpara.2011.01.005

Khan MB, Khan S, Rafiq K, Khan SN, Attaullah S, and Ali I (2020). Molecular identification of *Toxoplasma gondii* in domesticated and broiler chickens (*Gallus domesticus*) that possibly augment the pool of human toxoplasmosis. PLoS One, 15(4): e0232026. DOI: https://www.doi.org/10.1371/journal.pone.0232026

Khattab HM, El Bassiouni SO, Abuelela MH, and Abd Elsalam DO (2018). Seroprevalence of *Toxoplasma gondii* among a group of Egyptian patients with type I diabetes mellitus. Bulletin of the National Research Centre, 43: 20. DOI: https://www.doi.org/10.1186/s42269-019-0059-0

Krueger WS, Hilborn ED, Converse RR, and Wade TJ (2014). Drinking water source and human *Toxoplasma gondii* infection in the United States: a cross sectional analysis of NHANCES data. BMC Public Health, 14: 711. DOI: https://www.doi.org/10.1186/1471-2458-14-711

Latif AA, Mushtaq S, Fazal S, Mansha M, and Yaqub A (2017). Seroprevalence of *Toxoplasma gondii* among pregnant women in Lahore, Pakistan. Biologia, 63(2): 141-146. Available at: https://www.semanticscholar.org/paper/Seroprevalence-of-Toxoplasma-gondii-among-

Li MH, Yang BT, Yin ZW, Wang W, Zhao Q, and Jiang J (2020). A seroepidemiological survey of *Toxoplasma gondii* and *Chlamydia* infection in chickens, ducks, and geese in Jilin province, northeastern China. Vector-Borne and Zoonotic Diseases, 20(11): 825-830. DOI: https://www.doi.org/10.1089/vbz.2020.2614

Liesenfeld O (2002). Oral infection of C57BL/6 mice with *Toxoplasma gondii*: A new model of inflammatory. Journal of Infectious Diseases, 185(1): 96-101. DOI: https://www.doi.org/10.1086/338006

Literák I, and Hejlícek K (1993). Incidence of *Toxoplasma gondii* in populations of domestic birds in the Czech republic. Avian Pathology, 22(2): 275-281. DOI: https://www.doi.org/10.1080/03079459308418920

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

Liu XC, He Y, Han DG, Zhang ZC, Li K, Wang S, Xu LX, Yan RF, and Li XR (2017). Detection of *Toxoplasma gondii* in chicken and soil of chicken farms in Nanjing region, China. Infectious Diseases of Poverty, 6(1): 62. DOI: https://www.doi.org/10.1186/s40249-017-0277-3

Lorenzi H, Khan A, Behnke MS, Namasivayam S, Swapna LS, Hadjithomas M, Karamycheva S, Pinney D, Brunk BP, Ajioka JW et al. (2016). Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nature Communications, 7: 10147. DOI: https://www.doi.org/10.1038/ncomms10147

Lv YC (1993). A surveillance report of toxoplasmosis in domestic birds. Journal of Animal Health Inspection, 10: 31-32. Available at: https://pubag.nal.usda.gov/download/37140/PDF

Lv QY, Zheng HL, Yang WH, and Liu GH (2021). Molecular detection of *Toxoplasma gondii* and *Neospora caninum* in domestic ducks in Hunan province, China. Frontier in Veterinary Science, 8: 649603. DOI: https://www.doi.org/10.3389/fvets.2021.649603

Mabrouk MA, and Dahawi HS (1991). *Toxoplasma* antibodies in patients with meningoencephalitis. Journal of the Egyptian Society and Parasitology, 21(2): 547-551. Available at: https://pubmed.ncbi.nlm.nih.gov/1875079/

Majid A, Khan S, Jan AH, Taib M, Adnan M, Ali I, and Khan SN (2016). Chronic toxoplasmosis and possible risk factors associated with pregnant women in Khyber Pakhtunkhwa. Biotechnology and Biotechnological Equipment, 304: 733-736. DOI: https://www.doi.org/10.1080/13102818.2016.1175966

Mahmood Z, Zahid M, Sthanadar AA, Shah M, and Hussain A (2014). Seroprevalence of *Toxoplasma gondii* infection in *Gallus domesticatedus* of district Mardan, Khyber Pakhtunkhwa, Pakistan. Pakistan Journal of Zoology, 46(6): 1705-1710. Available at: https://www.zsp.com.pk/pdf46/1705-1710%20(30)%20PJZ-2007-14%2024-9-

Marietto-Gonçalves GA, Almeida SM, Camossi LG, Langoni H, and Andreatti Filho RL (2013). Serological evaluation of Parainfluenzavirus Type 1, *Salmonella* spp., *Mycoplasma* spp., and *Toxoplasma gondii* in wild birds. A revista Ciência Animal Brasileira, 14(4): 473-480. DOI: http://www.dx.doi.org/10.5216/cab.v14i4.16576

Martínez-Díaz RA, Simmons B, and Ponee-Gordo F (2002). Serologic screening to detect *T. gondii* antibodies in farmed ostriches (*Struthio camelus*) in Spain. Revista Ibérica de Parasitología, 62: 69-71. Available at: https://bibliotecavirtual.ranf.com/es/catalogo_imagenes/grupo.cmd?path=1001921

Meerburg BG, and Kijlstra A (2009). Changing climate-changing pathogens: *Toxoplasma gondii* in North-Western Europe. Parasitology Research, 105(1): 17-24. DOI: https://www.doi.org/10.1007/s00436-009-

Mercier A, Devillard S, Ngoubangoye B, Bonnabau H, Bañuls AL, Durand P, Salle B, Ajzenberg D, and Dardé ML (2010). Additional haplogroups of *Toxoplasma gondii* out of Africa: Population structure and mouse-virulence of strains from Gabon. PLoS Neglected Tropical Diseases, 4(11): e876. DOI: https://www.doi.org/10.1371/journal.pntd.0000876

Mikaeel FB, and Al-Saeed AT (2020). Molecular detection and seroprevalence of toxoplasmosis in free range local chickens (*Gallus domesticus*) in Duhok province, Iraq. Iraqi Journal of Veterinary Sciences, 34(2): 247-252. DOI: http://www.dx.doi.org/10.33899/ijvs.2019.125885.1173

Mohammed A, and Abdullah SH (2013). Diagnostic study of toxoplasmosis in domestic chickens in Sulaimani province. Al-Qadisiyah Journal of Veterinary Medicine and Science, 12(2): 63-69. DOI: https://www.doi.org/10.29079/vol12iss2art259

Montoya JG, and Liesenfeld O (2004). Toxoplasmosis. Lancet, 363: 1965-1976. DOI: https://www.doi.org/10.1016/s0140-

Mose JM, Kagira JM, Karanja SM, Ngotho M, Kamau DM, Njuguna AN, and Maina NW (2016). Detection of natural *Toxoplasma gondii* infection in chicken in Thika region of Kenya using nested polymerase chain reaction. BioMed Research International, pp. 1-5. DOI: https://www.doi.org/10.1155/2016/7589278

Mostafa NE, Abdel Hamed EF, Rahed HE, Mohamed SY, Abdelgawad M, and Elasbali AM (2018). The relationship between toxoplasmosis and different types of human tumors. Journal of Infection in Developing Countries, 12(2): 137-141. DOI: https://www.doi.org/10.3855/jidc.9672

Nazir MM, Akhtar M, Maqbool A, Waheed A, Sajid MA, Ali MA, Oneeb M, Alam MA, Ahmad AN, Nazir N et al. (2017). Antibody prevalence and risk factors for *Toxoplasma gondii* infection in women from Multan, Pakistan. Zoonoses and Public Health, 647: 537-542. DOI: https://www.doi.org/10.1111/zph.12336

Nardoni S, Rocchigiani G, Varvaro I, Altomonte I, Ceccherelli R, and Mancianti F (2019). Serological and molecular investigation on *Toxoplasma gondii* infection in wild birds. Pathogens, 8(2): 58. DOI: https://www.dx.doi.org/10.3390%2Fpathogens8020058

Pan M, Lyu C, Zhao J, and Shen B (2017). Sixty years (1957-2017) of research on toxoplasmosis in China-an overview. Frontier in Microbiology, 8: 1825. DOI: https://www.doi.org/10.3389/fmicb.2017.01825

Pena HF, Gennari SM, Dubey JP, and Su C (2008). Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. International Journal of Parasitology, 38(5): 561-569. DOI: https://www.doi.org/10.1016/j.ijpara.2007.09.004

Pinard JA, Leslie NS, and Irvine PJ (2003). Maternal serologic screening for toxoplasmosis. Journal of Midwifery Womens Health, 48: 308-316. DOI: https:// www.doi.org/10.1016/s1526-9523(03)00279-4

Raafat MS, Khalil FAM, and Abu El Ezz NMT (2011). Cryptosporidiosis and toxoplasmosis in native quails of Egypt. Research Journal of Veterinary Sciences, 4: 30-36. DOI: https://www.dx.doi.org/10.3923/rjvs.2011.30.36

Rajendran C, Su C, and Dubey JP (2012). Molecular genotyping of *Toxoplasma gondii* from Central and South America revealed high diversity within and between populations. Infection, Genetics and Evolution, 12(2): 359-368. DOI: https://www.doi.org/10.1016/j.meegid.2011.12.010

Remington JS, McLeod R, Thulliez P, and Desmonts G (2006). Toxoplasmosis. In Infectious Diseases of Fetus and Newborn Infant, 6th ed.; Remington JS, Klein JO, Wilson CB, Baker CJ., Eds.; Elsevier-Saunders Company: Philadelphia, PA, USA. pp. 947-1091. DOI: http://www.dx.doi.org/10.1016/B0-72-160537-0/50033-5

Rong G, Zhou HL, Hou GY, Zhao JM, Xu TS, and Guan S (2014). Seroprevalence, risk factors and genotyping of *Toxoplasma gondii* in domestic geese (*Anser domestica*) in tropical China. Parasite Vector, 7: 459. DOI: https://www.doi.org/10.1186/s13071-014-0459-9

Ruiz A, and Frenkel JK (1980). Intermediate and transport hosts of *Toxoplasma gondii* in Costa Rica. American Journal of Tropical Medicine and Hygiene, 29: 1161-1166. DOI: https://www.doi.org/10.4269/ajtmh.1980.29.1161

Sato AP, Vaz FF, Konell AL, Koch MO, Ferreira RF, Sipinski EAB, and Dittrich RL (2020). Survey of *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis neurona* antibodies in wild red-tailed Amazon parrots (*Amazona brasiliensis*). Revista Brasileira de Parasitologia Veterinaria, 29(1): e017519. DOI: https://www.doi.org/10.1590/s1984-29612019107

Shokri A, Sharif M, Teshnizi SH, Sarvi S, Rahimi MT, Mizani A, Ahmadpour E, Montazeri M, and Daryani A (2017). Birds and poultries toxoplasmosis in Iran: A systematic review and meta-analysis. Asian Pacific Journal of Tropical Medicine, 10(7): 635-642. DOI: https://www.doi.org/10.1016/j.apjtm.2017.07.013

Shwab EK, Zhu XQ, Majumdar D, Pena HF, Gennari SM, Dubey JP, and Su C (2014). Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. Parasitology, 141(4): 453-461. DOI: https://www.doi.org/10.1017/s0031182013001844

Sibley LD, Khan A, Ajioka JW, and Rosenthal BM (2009). Genetic diversity of *Toxoplasma gondii* in animals and humans. Philosophical Transactions of the Royal Society B: Biological Sciences, 364: 2749-2761. DOI: https://www.dx.doi.org/10.1098%2Frstb.2009.0087

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

Simitch T, Bordjochki A, Savin Z, and Mikovitch Z (1965). Experimental oral infection of the pigeon with the vegetative form and the cystic form of *Toxoplasma gondii*. Bulletin de l'Académie Vétérinaire de France, 38(8): 333-336. Available at: https://pubmed.ncbi.nlm.nih.gov/5858567/

Simon A, Poulin MB, Rousseau AN, and Ogden NH (2013). Fate and transport of *Toxoplasma gondii* oocysts in seasonally snow covered watersheds: A conceptual framework from a melting snowpack to the Canadian Arctic Coasts. International Journal of Environmental Research and Public Health, 10(3): 994-1005. DOI: https://www.dx.doi.org/10.3390%2Fijerph10030994

Sukthana Y (2006). Toxoplasmosis: beyond animals to humans. Trends in Parasitology, 22: 137-142. DOI: https://www.doi.org/10.1016/j.pt.2006.01.007

Swai ES, and Schoonman L (2009). Seroprevalence of *Toxoplasma gondii* infection amongst residents of Tanga District in north-east Tanzania, Tanzania. Journal of Health Research, 11(4): 205-209. DOI: https://www.doi.org/10.4314/thrb.v11i4.50178

Tenter AM, Heckeroth AR, and Weiss LM (2000). *Toxoplasma gondii*: From animals to humans. International Journal of Parasitology, 30: 1217-1258. DOI: https://www.doi.org/10.1016/s0020-7519(00)00124-7

Tilahun G, Tiao N, Ferreira L, Choudhary S, Oliveira S, Verma S, Kwok O, Molla B, Saville W, and Medhin G (2013). Prevalence of *Toxoplasma gondii* from free-range chickens (*Gallus domesticus*) from Addis Ababa, Ethiopia. Journal of Parasitology, 99: 740-741. DOI: https://www.doi.org/10.1645/12-25.1

Tsai YJ, Chung WC, Lei HH, and Wu YI (2006). Prevalence of antibodies to *Toxoplasma gondii* in pigeons (*Columba livia*) in Taiwan. Journal of Parasitology, 92(4): 871. DOI: https://www.doi.org/10.1645/ge-716r2.1

Wilking H, Thamm M, Stark K, Aebischer T, and Seeber F (2016). Prevalence, incidence estimations, and risk factors of *Toxoplasma gondii* infection in Germany: A representative, cross-sectional, serological study. Scientific Report, 6: 22551. DOI: https://www.doi.org/10.1038/srep22551

Yan C, Yue CL, Yuan ZG, He Y, Yin CC, Lin RQ, Dubey JP, and Zhua XQ (2009). *Toxoplasma gondii* infection in domestic ducks, free-range and caged chickens in southern China. Veterinary Parasitology, 165: 337-340. DOI: https://www.doi.org/10.1016/j.vetpar.2009.07.015

Zardi O, Giorgi G, Del Vecchio R, Venditti GE, and Drisaldi D (1967). Serological studies on *Toxoplasma gondii* infection in Amite risteto fauna of Lazio. Zoo Prophylaxis, 22: 223-237. Available at: https://core.ac.uk/download/pdf/59051783.pdf

Zhai CS, Jiang LX, and Shi ZL (1987). A survey on toxoplasmosis in human and domestic animals in the Nanjing area. Journal of Veterinary College, University of PLA, 7: 76-77. Available at: https://pubag.nal.usda.gov/download/37140/PDF

Zhang GN (1989). Epidemiological study on *Toxoplasma* infection in human beings and animals in Shandong Province. Zhonghua Liu Xing Bing Xue Za Zhi, 10: 30-33. Available at: https://pubmed.ncbi.nlm.nih.gov/2736611/

Zhang XX, Zhang NZ, Tian WP, Zhou DH, Xu YT, and Zhu XQ (2014). First report of *Toxoplasma gondii* seroprevalence in pet parrots in China. Vector Borne Zoonotic Diseases, 14(6): 394-398. DOI: https://www.doi.org/10.1089/vbz.2013.1522

Zhang XX, Zhao Q, Shi CW, Yang WT, Jiang YL, Wei ZT, Wang CF, and Yang GL (2016). Seroprevalence and associated risk factors of *Toxoplasma gondii* infection in the Korean, Manchu, Mongol and Han ethnic groups in eastern and northeastern China. Epidemiology and Infection, 144(9): 2018-2024. DOI: https:// www.doi.org/10.1017/s0950268815003337

Zia-Ali N, Fazaeli A, Khoramizadeh M, Ajzenberg D, Darde´ M, and Keshavarz-Valian H (2007). Isolation and molecular characterization of *Toxoplasma gondii* strains from different hosts in Iran. Parasitological Research, 101(1): 111-115. DOI: https://www.doi.org/10.1007/s00436-007-0461-7

Zou Y, Nie LB, Zhang NZ, Zou FC, Zhu XQ, and Cong W (2017). First genetic characterization of *Toxoplasma gondii* infection in poultry meat intended for human consumption in eastern China. Infections, Genetics and Evolution, 55: 172-174. DOI: https://www.doi.org/10.1016/j.meegid.2017.08.022

To cite this paper: Abd El-Ghany WA (2021). A Highlight on Avian Toxoplasmosis: One Health Disease with a Special Reference to the Current Egyptian Situation. *World Vet. J.*, 11 (3): 510-520. DOI: https://dx.doi.org/10.54203/scil.2021.wvj66

Instructions for Authors

Manuscript as Original Research Paper, Short Communication, Case Reports and Review or Mini-Review are invited for rapid peer-review publishing in the *World's Veterinary Journal* (WVJ). Considered subject areas include: Behavior; environment and welfare; animal reproduction and production; parasitology, endocrinology, microbiology, immunology, pathology, pharmacology, epidemiology, molecular biology, immunogenetics, surgery, radiology, ophthalmology, dermatology, chronic disease, anatomy, and non-surgical pathology issues of small to large animals, cardiology and oncology are sub-specialties of veterinary internal medicine. ... view full aims and scope

WVJ EndNote Style
Manuscript Template (.doc)
Sample Articles
Declaration form
Policies and Publication Ethics

Submission

The manuscript and other correspondence should be submit online preferentially. Please embed all figures and tables in the manuscript to become one single file for submission. Once submission is complete, the system will generate a manuscript ID and password sent to author's contact emails**:** editor@wvj.science-line.com. All manuscripts must be checked (by English native speaker) and submitted in English for evaluation (in totally confidential and impartial way).

Supplementary information

The online submission form allows supplementary information to be submitted together with the main manuscript file and covering letter. If you have more than one supplementary files, you can submit the extra ones by email after the initial submission. Author guidelines are specific for each journal. Our Word template can assist you by modifying your page layout, text formatting, headings, title page, image placement, and citations/references such that they agree with the guidelines of journal. If you believe your article is fully edited per journal style, please use our Word template before submission.
Supplementary materials may include figures, tables, methods, videos, and other materials. They are available online linked to the original published article. Supplementary tables and figures should be labeled with a "S", e.g. "Table S1" and "Figure S1". The maximum file size for supplementary materials is 10MB each. Please keep the files as small possible to avoid the frustrations experienced by readers with downloading large files.

Submission to the Journal is on the understanding that

1.The article has not been previously published in any other form and is not under consideration for publication elsewhere;
2.All authors have approved the submission and have obtained permission for publish work.
3.Researchers have proper regard for conservation and animal welfare considerations (see IAVE-Author Guidelines on Animal Ethics and Welfare). Attention is drawn to the '*Guidelines for the Treatment of Animals in Research and Teaching*'. Any possible adverse consequences of the work for populations or individual organisms must be weighed against the possible gains in knowledge and its practical applications. If the approval of an ethics committee is required, please provide the name of the committee and the approval number obtained.

Ethics Committee Approval

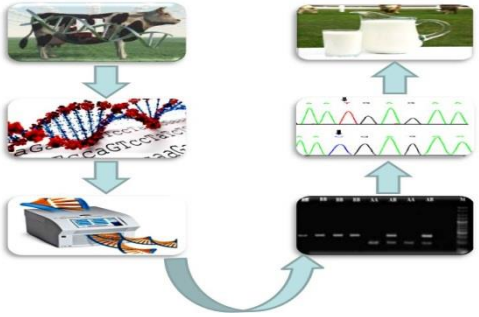
Experimental research involving human or animals should have been approved by author's institutional review board or ethics committee. This information can be mentioned in the manuscript including the name of the board/committee that gave the approval. Investigations involving humans will have been performed in accordance with the principles of *Declaration of Helsinki*. And the use of animals in experiments will have observed the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education by the New York Academy of Sciences, Ad Hoc Animal Research Committee. If the manuscript contains photos or parts of photos of patients, informed consent from each patient should be obtained. Patient's identities and privacy should be carefully protected in the manuscript.

Competing Interests

Competing interests that might interfere with the objective presentation of the research findings contained in the manuscript should be declared in a paragraph heading "Competing interests" (after Acknowledgment section and before References). Examples of competing interests are ownership of stock in a company, commercial grants, board membership, etc. If there is no competing interest, please use the statement "The authors have declared that no competing interest exists.

Graphical Abstract

Authors should provide a graphical abstract (a beautifully designed feature figure) to represent the paper aiming to catch the attention and interest of readers. Graphical abstract will be published online in the table of content. The graphical abstract should be colored, and kept within an area of 12 cm (width) x 6 cm (height) or with similar format. Image should have a minimum resolution of 300 dpi and line art 1200dpi. **Note:** Height of the image should be no more than the width. Please avoid putting too much information into the graphical abstract as it occupies only a small space. Authors can provide the graphical abstract in the format of PDF, Word, PowerPoint, jpg, or png, after a manuscript is accepted for publication. If you have decided to provide a Professional Graphical Abstract, please click **here**.



Presentation of the article

Main Format

First page of the manuscripts must be properly identified by the title and the name(s) of the author(s). It should be typed in Times New Roman (font sizes: 17pt in capitalization for the title, 10pt for the section headings in the body of the text and the main text, 9pt for References, double spaced, in A4 format with 2cm margins. All pages and lines of the main text should be numbered consecutively throughout the manuscript. The manuscript must be saved in a .doc format, (not .docx files). Abbreviations in the article title are not allowed.

Manuscripts should be arranged in the following order:

a. TITLE (brief, attractive and targeted)
b. Name(s) and Affiliation(s) of author(s) (including post code) and corresponding E-mail
c. ABSTRACT
d. Key words (separate by semicolons; or comma,)
e. Abbreviations (used in the manuscript)
f. INTRODUCTION
g. MATERIALS AND METHODS
h. RESULTS
i. DISCUSSION
j. CONCLUSION
k. DECLARATIONS
1. REFERENCES
m. Tables
n. Figure captions
o. Figures

Results and Discussion can be presented jointly if preferred.
Discussion and Conclusion can be presented jointly if preferred.

Article Sections Format

Title should be a brief phrase describing the contents of the paper. The first letter of each word in title should use upper case. The Title Page should include the author(s)'s full names and affiliations, the name of the corresponding author along with phone and e-mail information. Present address (es) of author(s) should appear as a footnote.

Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The abstract should be 150 to 300 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and Methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the author(s)'s experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the results but should be put into the discussion section.

Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

Conclusion can be presented jointly if preferred.

Declarations section

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph forms or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or PowerPoint before pasting in the Microsoft Word manuscript file. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

Declarations section - Please include declarations heading

Please ensure that the sections:
-Ethics (and consent to participate)
-Authors' contributions
-Competing interests
-Availability of data and materials
are included at the end of your manuscript in a Declarations section.

Authors' Contributions

For manuscripts with more than one author, WVJ require an Authors' Contributions section to be placed after the Competing Interests section.
An 'author' is generally considered to be someone who has made substantive intellectual contributions to a published study. To qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; and 3) have given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.
We suggest the following format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
For authors that equally participated in a study please write 'All/Both authors contributed equally to this work.'
Contributors who do not meet the criteria for authorship should be listed in an acknowledgements section.

Competing Interests

Competing interests that might interfere with the objective presentation of the research findings contained in the manuscript should be declared in a paragraph heading "Competing interests" (after Acknowledgment section and before References). Examples of competing interests are ownership of stock in a company, commercial grants, board membership, etc. If there is no competing interest, please use the statement "The authors declare that they have no competing interests.".
World's Veterinary Journal adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.
It is a requirement that all authors have been accredited as appropriate upon submission of the manuscript. Contributors who do not qualify as authors should be mentioned under Acknowledgements.

Change in authorship

We do not allow any change in authorship after provisional acceptance. We cannot allow any addition, deletion or change in sequence of author name. We have this policy to prevent the fraud.

Acknowledgements

We strongly encourage you to include an Acknowledgements section between the Authors' contributions section and Reference list. Please acknowledge anyone who contributed towards the study by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include their source(s) of funding. Please also acknowledge anyone who contributed materials essential for the study.
Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements. Please list the source(s) of funding for the study, for each author, and for the manuscript preparation in the acknowledgements section. Authors must describe the role of the funding body, if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Data Deposition

Nucleic acid sequences, protein sequences, and atomic coordinates should be deposited in an appropriate database in time for the accession number to be included in the published article. In computational studies where the sequence information is unacceptable for inclusion in databases because of lack of experimental validation, the sequences must be published as an additional file with the article.

References

1. A WVJ reference style for **EndNote** may be found **here**.
2. All references to publications made in the text should be presented in a list with their full bibliographical description.
3. In the text, a reference identified by means of an author's name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author's surname should be mentioned, followed by 'et al'. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like 'a' and 'b' after the date to distinguish the works.
4. References in the text should be arranged chronologically (e.g. Kelebeni, 1983; Usman and Smith, 1992 and Agindotan et al., 2003). The list of references should be arranged alphabetically on author's surnames, and chronologically per author. If an author's name in the list is also mentioned with co-authors, the following order should be used: Publications of the single author, arranged according to publication dates - publications of the same author with one co-author - publications of the author with more than one co-author. Publications by the same author(s) in the same year should be listed as 1992a, l992b, etc.
5. Names of authors and title of journals, published in non-latin alphabets should be transliterated in English.
6. A sample of standard reference is " 1th Author surname A, 2th Author surname B , 3th Author surname C. 2013. Article title should be regular and 7 pt . *World Vet. J.,* Add No. of Volume (Issue No.): 00-00."
7. The color of references in the text of article is dark blue. Example: (Preziosi et al., 2002; Mills et al., 2015).
8. At least 35% of the references of any submitted manuscript (for all types of article) should include scientific results published in the last five years.

-Examples (at the text- blue highlighted)
Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001).

--Examples (at References section)

a) For journal:
Lucy MC (2000). Regulation of ovarian follicular growth by somatotropin and insulin- like growth factors in cattle. Journal of Dairy Science, 83: 1635-1647. DOI: XXX
Kareem SK (2001). Response of albino rats to dietary level of mango cake. Journal of Agricultural Research and Development. pp 31-38. DOI: XXX
Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. African Journal of Biotechnology. 7: 3535-3539. DOI: XX

b) For symposia reports and abstracts:
Cruz EM, Almatar S, Aludul EK and Al-Yaqout A (2000). Preliminary Studies on the Performance and Feeding Behaviour of Silver Pomfret (Pampus argentens euphrasen) Fingerlings fed with Commercial Feed and Reared in Fibreglass Tanks. Asian Fisheries Society Manila, Philippine 13: 191-199. Link

c) For edited symposia, special issues, etc., published in a journal:
Korevaar H (1992). The nitrogen balance on intensive Dutch dairy farms: a review. In: A. A. Jongebreur et al. (Editors), Effects of Cattle and Pig Production Systems on the Environment: Livestock Production Science, 31: 17-27. Link

d) For books:
AOAC (1990). Association of Official Analytical Chemists. Official Methods of Analysis, 15th Edition. Washington D.C. pp. 69-88. Link
Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603. Link

e) Books, containing sections written by different authors:
Kunev M (1979). Pig Fattening. In: A. Alexiev (Editor), Farm Animal Feeding. Vol. III. Feeding of Different Animal Species, Zemizdat, Sofia, p. 233-243 (Bg). Link
In referring to a personal communication the two words are followed by the year, e.g. (Brown, J. M., personal communication, 1982). In this case initials are given in the text.

Nomenclature and Abbreviations

Nomenclature should follow that given in NCBI web page and Chemical Abstracts. Standard abbreviations are preferable. If a new abbreviation is used, it should be defined at its first usage. Abbreviations should be presented in one paragraph, in the format: "term: definition". Please separate the items by ";".
E.g. ANN: artificial neural network; CFS: closed form solution...

Abbreviations of units should conform to those shown below:

Decilitre	dl	**Kilogram**	kg
Milligram	mg	**hours**	h
Micrometer	mm	**Minutes**	min
Molar	mol/L	**Mililitre**	ml
Percent	%		

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (The Royal Society of Medicine London 1977).
Papers that have not been published should be cited as "unpublished". Papers that have been accepted for publication, but not yet specified for an issue should be cited as "to be published". Papers that have been submitted for publication should be cited as "submitted for publication".

Formulae, numbers and symbols

1. Typewritten formulae are preferred. Subscripts and superscripts are important. Check disparities between zero (0) and the letter 0, and between one (1) and the letter I.
2. Describe all symbols immediately after the equation in which they are first used.
3. For simple fractions, use the solidus (/), e.g. 10 /38.
4. Equations should be presented into parentheses on the right-hand side, in tandem.
5. Levels of statistical significance which can be used without further explanations are *P < 0.05, **P < 0.01, and ***P < 0.001
6. In the English articles, a decimal point should be used instead of a decimal comma.
7. In chemical formulae, valence of ions should be given, e.g. Ca2+ and CO32-, not as Ca++ or CO3.
8. Numbers up to 10 should be written in the text by words. Numbers above 1000 are recommended to be given as 10 powered x.
9. Greek letters should be explained in the margins with their names as follows: Aα - alpha, Bβ - beta, Γγ - gamma, Δδ - delta, Eε - epsilon, Zζ - zeta, Hη - eta, Θθ - theta, Iι - iota, Kκ - kappa, Λλ - lambda, Mμ - mu, Nν - nu, Ξξ - xi, Oo - omicron, Ππ - pi, Pρ - rho, Σσ - sigma, Tτ - tau, Yυ - ipsilon, Φφ - phi, Χχ - chi, Ψψ - psi, Ωω - omega.

Review/Decisions/Processing

Firstly, all manuscripts will be checked by Docol©c, a plagiarism finding tool. A single blind reviewing model is used by WVJ for non-plagiarized papers. The manuscript is edited and reviewed by the English language editor and three reviewers selected by section editor of WVJ respectively. Also, a reviewer result form is filled by reviewer to guide authors. Possible decisions are: accept as is, minor revision, major revision, or reject. See sample of evaluation form. Authors should submit back their revisions within 14 days in the case of minor revision, or 30 days in the case of major revision. To submit a revision please sign in here, fill out the form, and mark "Revised" attach the revision (MS word) and submit when completed. After review and editing the article, a final formatted proof is sent to the corresponding author once again to apply all suggested corrections during the article process. The editor who received the final revisions from the corresponding authors shall not be hold responsible for any mistakes shown in the final publication. Manuscripts with significant results are typically reviewed and published at the highest priority.

Plagiarism: There is a zero-tolerance policy towards plagiarism (including self-plagiarism) in our journals. Manuscripts are screened for plagiarism by Docol©c a plagiarism finding tool, before or during publication, and if found they will be rejected at any stage of processing. See sample of Docol©c-Report.

Declaration

After manuscript accepted for publication, a declaration form will be sent to the corresponding author who that is responsible to coauthors' agreements to publication of submitted work in WVJ after any amendments arising from the peer review.

Date of issue

The journal will be issued on 25th of March, June, September and December, each year.

Publication charges

No peer-reviewing charges are required. However, the publication costs are covered through article processing charges (APCs). There is a modest APC of 150 Euro(€) editor fee for the processing of each primary accepted paper (1000-4000 words) to encourage high-quality submissions. APCs are only charged for articles that pass the pre-publication checks and are published. A surcharge will be placed on any article that is over 4000 words in length to cover the considerable additional processing costs. Payment can be made by credit card, bank transfer, money order or check. Instruction for payment is sent during publication process as soon as manuscript is accepted. Meanwhile, this journal encourages the academic institutions in low-income countries to publish high quality scientific results, free of charges.

WORD COUNT	PRICE*
1000-4000 words	€150
over 4000 words	€230

* The prices are valid until 30th December 2021.

The Waiver policy

The publication fee will be waived for invited authors, authors of hot papers, and corresponding authors who are editorial board members of the *World's Veterinary Journal* (WVJ). The Journal will consider requests to waive the fee for cases of financial hardship (for high quality manuscripts and upon acceptance for publication). Requests for waiver of the submission fee must be submitted via individual cover letter by the corresponding author and cosigned by an appropriate institutional official to verify that no institutional or grant funds are available for the payment of the fee. Letters including the manuscript title and manuscript ID number should be sent to: editor.wvj@gmail.com. It is expected that waiver requests will be processed and authors will be notified within one business day.

Submission Preparation Checklist

- Authors are required to check off their submission's compliance with all of the following items, and submissions may be returned to authors that do not adhere to the following guidelines.
- The submission has not been previously published, nor is it before another journal for consideration (or an explanation has been provided in Comments to the Editor).
- The submission file is in Microsoft Word, RTF, or PDF document file format.
- Where available, URLs for the references have been provided.
- The text is single-spaced; uses a 12-point font; and all illustrations, figures, and tables are placed within the text at the appropriate points, rather than at the end.
- The text adheres to the stylistic and bibliographic requirements outlined in the Author Guidelines.

Editorial Offices:
Atatürk University, Erzurum 25100, Turkey
University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
University of Maragheh, East Azerbaijan, Maragheh 55136, Iran
Homepage: www.science-line.com
Phone: +98 914 420 7713 (Iran); +90 538 770 8824 (Turkey); +1 204 8982464 (Canada)
Emails: administrator@science-line.com ; saeid.azar@atauni.edu.tr

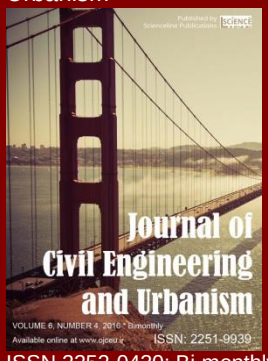
SCIENCELINE PUBLISHING CORPORATION

Scienceline Publication Ltd is a limited liability non-profit non-stock corporation incorporated in Turkey (Company No. 0757086921600001). Scienceline journals that concurrently belong to many societies, universities and research institutes, publishes internationally peer-reviewed open access articles and believe in sharing of new scientific knowledge and vital research in the fields of life and natural sciences, animal sciences, engineering, art, linguistic, management, social and economic sciences all over the world. Scienceline journals include:

Online Journal of Animal and Feed Research

ISSN 2228-7701; Bi-monthly
View Journal | Editorial Board
Email: editors@ojafr.ir
Submit Online >>

Journal of Civil Engineering and Urbanism

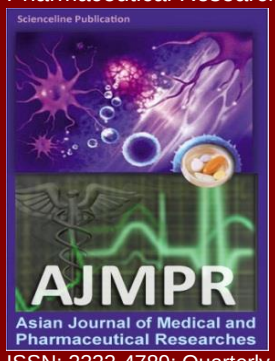


ISSN 2252-0430; Bi-monthly
View Journal | Editorial Board
Email: ojceu@ojceu.ir
Submit Online >>

Journal of Life Sciences and Biomedicine

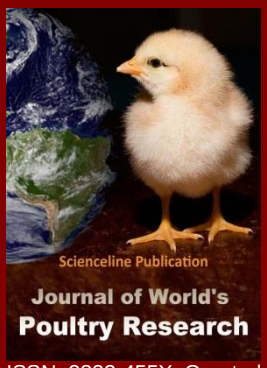
ISSN: 2251-9939; Bi-monthly
View Journal | Editorial Board
Email: editors@jlsb.science-line.com
Submit Online >>

Asian Journal of Medical and Pharmaceutical Researches



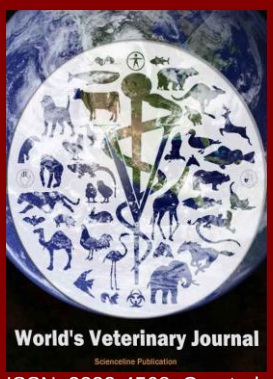
ISSN: 2322-4789; Quarterly
View Journal | Editorial Board
Email: editor@ajmpr.science-line.com
Submit Online >>

Journal of World's Poultry Research



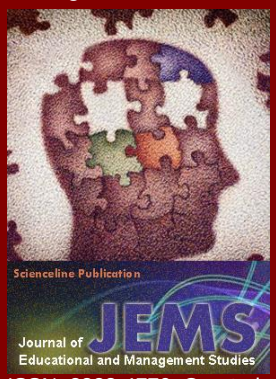
ISSN: 2322-455X; Quarterly
View Journal | Editorial Board
Email: editor@jwpr.science-line.com
Submit Online >>

World's Veterinary Journal



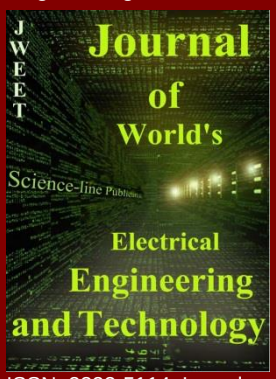
ISSN: 2322-4568; Quarterly
View Journal | Editorial Board
Email: editor@wvj.science-line.com
Submit Online >>

Journal of Educational and Management Studies



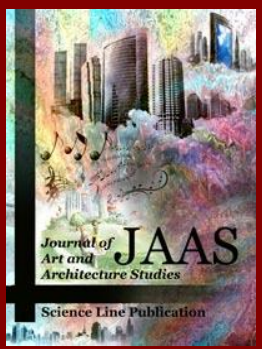
ISSN: 2322-4770; Quarterly
View Journal | Editorial Board
Email: info@jems.science-line.com
Submit Online >>

Journal of World's Electrical Engineering and Technology



ISSN: 2322-5114; Irregular
View Journal | Editorial Board
Email: editor@jweet.science-line.com
Submit Online >>

Journal of Art and Architecture Studies



ISSN: 2383-1553; Irregular
View Journal | Editorial Board
Email: jaas@science-line.com
Submit Online >>

Asian Journal of Social and Economic Sciences

ISSN: 2383-0948; Quarterly
View Journal | Editorial Board
Email: ajses@science-line.com
Submit Online >>

Journal of Applied Business and Finance Researches

ISSN: 2382-9907; Quarterly
View Journal | Editorial Board
Email: jabfr@science-line.com
Submit Online >>

Scientific Journal of Mechanical and Industrial Engineering

ISSN: 2383-0980; Quarterly
View Journal | Editorial Board
Email: sjmie@science-line.com
Submit Online >>

ABOUT
AIMS AND SCOPE
LEADERSHIP TEAM
WHO WE WORK WITH
POLICIES AND PUBLICATION ETHICS
TERMS AND CONDITIONS
CONTACT

Scienceline is a non-profit organisation inspired by research funders and led by scholars. Our mission is to help researchers accelerate discovery and innovation by operating a platform for research communication that encourages and recognises the most responsible behaviours in science.

Scienceline Publications, Ltd is a limited liability non-profit non-stock corporation registered in the State of Erzurum, Turkey, with company number 0757086921600001, and branch number 18677/25379 at the address: Scienceline Publications, Ltd., Ömer Nasuhi Bilmen Road, Dönmez Apart., G1/6, Yakutiye, Erzurum 25100, Turkey