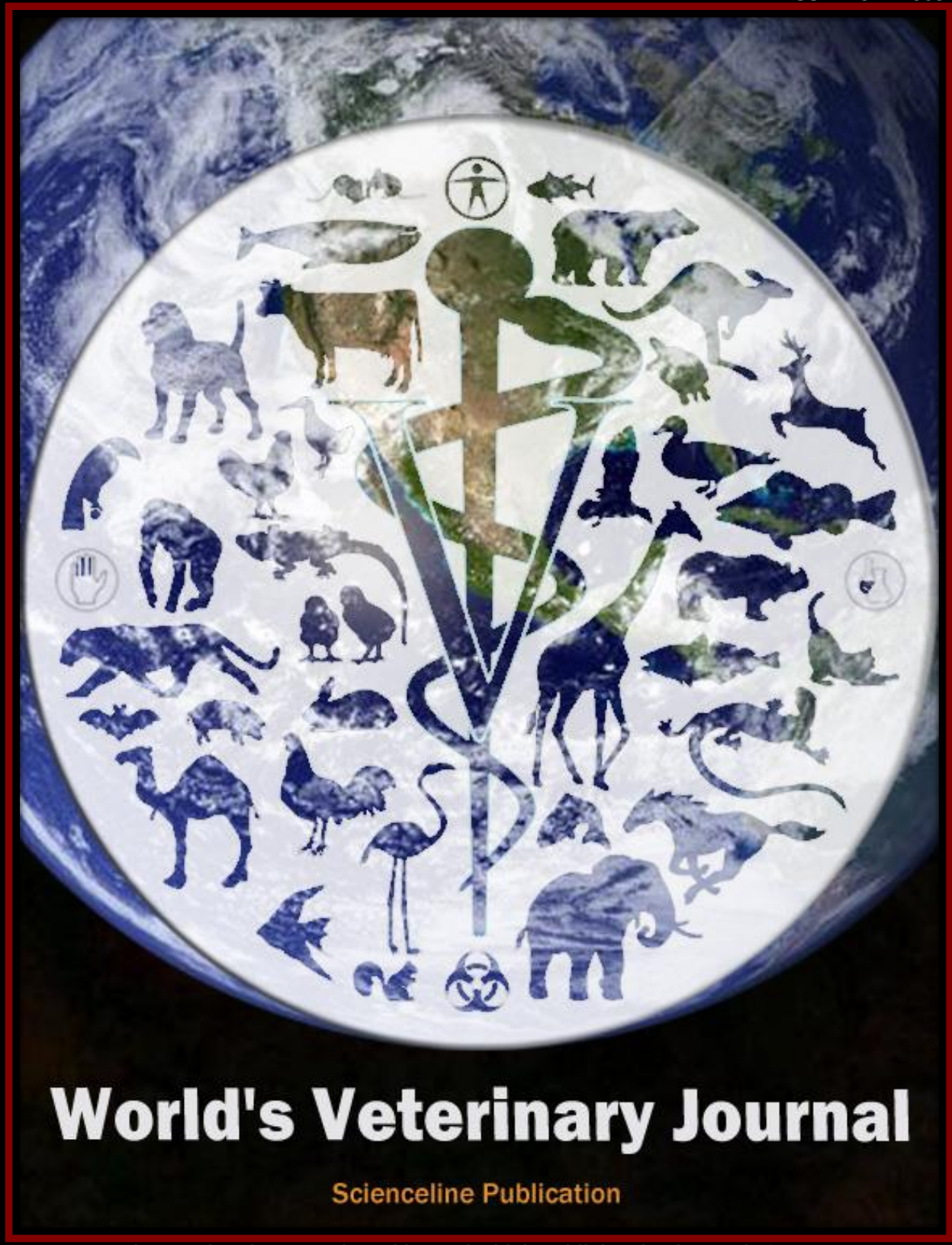
In the Name of God

ISSN: 2322-4568



World's Veterinary Journal

Scienceline Publication

An international peer-reviewed journal which publishes in electronic format

Volume 10, Issue 3, September 2020

WORLD'S VETERINARY JOURNAL

ISSN: 2322-4568

World Vet. J., 10 (3): 260-464; September 25, 2020

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Volume 10 (3); September 25, 2020

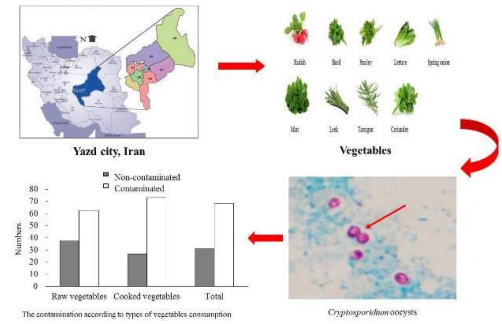
Research Paper

Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran.

Fattahi Bafghi A, Yavari MR, Mirzaei F, Siyadatpanah A, Mitsuwan W, Pereira ML, Nissapatorn V, Norouzi R, and Hosseini SA.

World Vet. J. 10(3): 260-266, 2020; pii:S232245682000034-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj34

ABSTRACT



Fattahi Bafghi A, Yavari MR, Mirzaei F, Siyadatpanah A, Mitsuwan W, Pereira ML, Nissapatorn V, Norouzi R, and Hosseini SA (2020). **Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran.** *World Vet. J.*, 10 (3): 260-266. DOI: https://dx.doi.org/10.36380/scil.2020.wvj34

Consumption of raw vegetables is an important route of parasites transmission. It is an important source for foodborne outbreaks in both developed and developing countries, and outbreaks of parasitic diseases in humans. The objective of the present study was to detect the presence of *Cryptosporidium* oocysts in raw fresh vegetables in Yazd city, Iran, from 2017 to 2018. A total of 275 fresh vegetable samples were collected and tested using a sucrose flotation medium of 1.21 specific gravity and a Modified Ziehl-Nielsen staining procedure. Of the 275 vegetables examined, 85 (31.5%) samples were positive for *Cryptosporidium* oocysts. Lettuce had the highest rate (n= 16, 47.1%) of contamination with *Cryptosporidium* oocysts while basil and parsley showed the lowest rates of contamination (n= 6, 20%). There was a significant association between the occurrence of *Cryptosporidium* oocysts and the investigated vegetable types. According to the locations of the vegetable field, Amir Abad and Bahaman Hospital area had the highest (n: 16, 59.3%) and lowest (n= 5, 18.5%) rates of *Cryptosporidium* oocysts contamination, respectively. The plant part showed that the root vegetables had the highest contamination rates (n= 41, 45.6%), followed by leafy vegetables (n= 44, 24.4%). The analysis further indicated a significant association between the occurrence of *Cryptosporidium* oocysts and the route of vegetable consumption. Based on these results, the edible vegetables in Yazd city are one of the potential sources of *Cryptosporidium* infections in humans. Moreover, the vegetable fields within the city of Yazd are contaminated with *Cryptosporidium* oocysts which can pose public health problems.

Keywords: *Cryptosporidium,* Oocysts, Raw vegetables, Yazd city, Iran.

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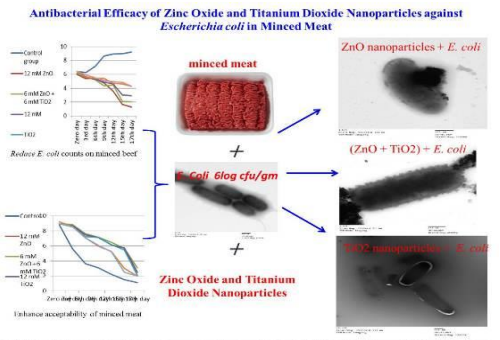
Research Paper

Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against Escherichia coli in Minced Meat.

Abd El-Aziz M.A., Ibrahim H.M., EL-Roos NA, Anis B and Elsabagh R.
World Vet. J. 10(3): 267-275, 2020; pii:S232245682000035-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj35

ABSTRACT



Abd El-Aziz M.A., Ibrahim H.M., EL-Roos NA, Anis B and Elsabagh R (2020). **Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against *Escherichia coli* in Minced Meat.** *World Vet. J.*, 10 (3): 267-275. DOI: https://dx.doi.org/10.36380/scil.2020.wvj35

Antibacterial nanoparticles are a new approach to control the safety of meat and meat products. This work aimed to investigate the antibacterial effect of zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles, alone or together, against *Escherichia coli*. Antibacterial activity of these nanomaterials was evaluated using the disc diffusion method. In this regard, minced meat samples were inoculated with *E. coli* and treated with different concentrations of two nanomaterials (approximately 20 nm), including 6 mM and 12 mM ZnO, 6 mM and 12 mM TiO2, and a combination of 6 mM ZnO and 6 mM TiO2, then stored at 4°C for 17 days. The results indicated that ZnO (12 mM) had a significant reduction effect on *E. coli* count in minced meat, followed by the combination of ZnO and TiO2, and 12 mM TiO2 alone. The antibacterial activity of ZnO, TiO2, and combination of ZnO and TiO2 was also examined using a transmission electron microscope and it was found that 12Mm ZnO had a higher destructive effect on bacterial cell than the mixture of ZnO + TiO2, and 12Mm TiO2 alone. The disc diffusion method showed that ZnO (12 mM) was the most effective concentration used against *E. coli*. It is concluded that 12 mM ZnO nanoparticles have the best antibacterial effect against *E. coli* in minced meat stored at 4 °C for 17 days.

Keywords: *E. coli,* Minced meat, Nanoparticles, TEM, Titanium dioxide, Zinc oxide.

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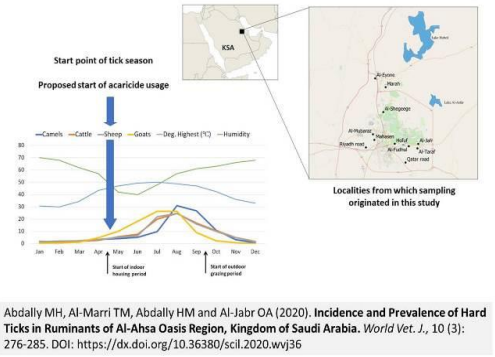
Research Paper

Incidence and Prevalence of Hard Ticks in Ruminants of Al-Ahsa Oasis Region, Kingdom of Saudi Arabia.

Abdally MH, Al-Marri TM, Abdally HM and Al-Jabr OA.
World Vet. J. 10(3): 276-285, 2020; pii:S232245682000036-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj36

ABSTRACT
In the present study, a number of camels, cattle, sheep, and goat herds have been examined and followed up to record the incidence of tick infestation in Al-Ahsa Oasis in the Eastern Region of the Kingdom of Saudi Arabia. From 24 herds distributed in eleven localities, a total of 4068 animals (123 camels, 60 cattle, 1780 sheep, and 2105 goats) were individually examined every second week during the period from January to December 2010. In total, 5320 ticks were collected from 1125 infested animals (27.65%). The overall prevalence rate of identified Ixodide ticks was: *Hyalomma anatolicum excavatum* (18.33%), *Hyalomma dromedarii* (17.63%), *Hyalomma anatolicum anatolicum* (14.29%), *Rhipicephalus turanicus* (14.04%), *Hyalomma impeltatum* (11.28%), *Rhipicephalus praetextatus* (8.56%), *Hyalomma marginatum turanicum* (6.20%), *Haemaphysalis sulcata* (3.57%), *Rhipicephalus kohlsi* (2.33%), *Hyalomma marginatum rufipes* (2.09%), *Hyalomma schulzei* (1.03%), *Amblyomma variegatum* (0.47%), and *Amblyomma gemma* (0.18%). Tick infestation in terms of mean intensity, abundance, and prevalence rates was highest in camels (41 tick/infested camel, 30 ticks/camel, and 73.17%, respectively). This was followed by cattle (15 tick/infested cow, 5 ticks/cow, and 33.33%, respectively), goats (1.35 tick/infested goat, 0.33 tick/goat, and 23.52%, respectively), and sheep (1.27 tick/infested sheep, 0.3 tick/sheep, and 29.21%, respectively). The incidence rate of ticks significantly increased during the warmest summer months of the year (highest recorded temperature ranged between 47℃ and 50℃), mainly due to the management practices of farmers. During this period animals were housed and water was supplied *ad libitum* during hot months, thereby increasing animal density and humidity in the shaded farms. According to the obtained results of the current study, tick control can be started in Al-Ahsa area in early May for all animals with the focus on camels as they are the harbor of the tick biomass, or on goats as they had initially increased in incidence rates.
Keywords: Kingdom of Saudi Arabia, Prevalence, Ruminants, Ticks.



Abdally MH, Al-Marri TM, Abdally HM and Al-Jabr OA (2020). **Incidence and Prevalence of Hard Ticks in Ruminants of Al-Ahsa Oasis Region, Kingdom of Saudi Arabia.** *World Vet. J.*, 10 (3): 276-285. DOI: https://dx.doi.org/10.36380/scil.2020.wvj36

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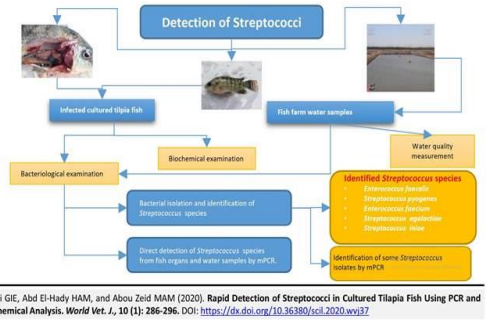
Research Paper

Rapid Detection of Streptococci in Cultured Tilapia Fish Using PCR and Chemical Analysis.

Ali GIE, Abd El-Hady HAM, and Abou Zeid MAM.
World Vet. J. 10(3): 286-296, 2020; pii:S232245682000037-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj37

ABSTRACT
Streptococcosis causes economic losses due to the high mortality in Nile tilapia (*Oreochromis niloticus*). The present study was carried out to detect the streptococci by PCR and chemical analysis in cultured tilapia fish. A total of 100 cultured tilapia fishes and 20 water samples were collected from 4 different fish farms at different locations in Kafr El-sheikh Governorate for bacteriological and chemical analysis. The results of water quality parameters examination revealed that the mean values of pH, dissolved oxygen, unionized ammonia, and nitrite were 8.2 ,0.73 ± 0.54 ± 7.44parts per million (ppm), 0.05 ± 0.008 ppm, and 0.00 ppm, respectively. All water quality parameters except for ammonia were within the permissible limit. The bacterial isolation results revealed % 38 positive samples for *Streptococcus* species isolated from tilapia fishes and 40 % were positive for *Streptococcus* species isolated from water samples. Of those 38 positive fish samples, 25 (65.78%) were *Enterococcus faecalis* (%39.47) 15 ,were *Streptococcus pyogenes* (%15.78) 6 ,were *Enterococcus faecium* (% 13.15) 5 ,were *Streptococcus agalactiae* ,and 3 (7.89 %) were *Streptococcus iniae* .On the other hand, from 8 positive farms water samples, 7 (87.5%) were *Enterococcus faecalis* 2 , (%25)were *Streptococcus pyogenes* (%25) 2 , were *Enterococcus faecium* (%37.5) 3 ,were *Streptococcus agalactiae* ,and 1 (12.5%) was *Streptococcus iniae* . Using mPCR to identify *Streptococcus* for some fish and water isolates, 6 *Enterococcus faecalis* with 310 base per (bp) were detected while 3 *Streptococcus agalactiae* with 153 bp and 2 *Enterococcus faecium* at 215 bp were detected. However, the mPCR from *Streptococcus* species directly from organs from fish and water samples revealed that 5 *Enterococcus faecalis* were detected at 310 bp while 2 *Streptococcus agalactiae* was identified at 153 bp. The biochemical results indicated that the infected fish with the streptococcal species had reduced total protein, albumin, and globulin in the blood serum while total cholesterol, urea, creatinine levels, and AST, ALT, GPX ,CAT, SOD activities significantly increased, compared to non- infected fish.
Keywords: Biochemical parameters, PCR, streptococci, Tilapia fish, Water examination.



Ali GIE, Abd El-Hady HAM, and Abou Zeid MAM (2020). **Rapid Detection of Streptococci in Cultured Tilapia Fish Using PCR and Chemical Analysis.** *World Vet. J.*, **10 (1): 286-296.** DOI: https://dx.doi.org/10.36380/scil.2020.wvj37

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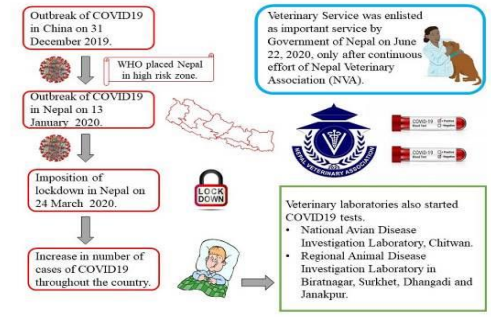
Research Paper

Contribution of Veterinary Sector to Control COVID-19 Pandemic in Nepal.

Gautam A, Khanal A, Subedi D, Bhandari S and Kaphle K.

World Vet. J. 10(3): 297-305, 2020; pii:S232245682000038-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj38

ABSTRACT



Gautam A, Khanal A, Subedi D, Bhandari S and Kaphle K (2020). **Contribution of Veterinary Sector in Handling of COVID-19 Pandemic in Nepal.** *World Vet. J.,* 10 (3): 297-305. DOI: https://dx.doi.org/10.36380/scil.2020.wvj38

Nepal has been identified in a high-risk zone by the World Health Organization (WHO) for the Corona Virus Disease of 2019 (COVID-19). Nepal's first official reported case was confirmed on 13 January 2020 and so far, 24,000 cases have tested positive with 91 deaths. The imposition of lockdown has begun since March 24 and it was extended until 22 July. Reverse transcriptase Polymerase Chain Reaction and rapid diagnostic tests are used by the Government of Nepal to identify COVID-19. Among the worst-hit sectors are tourism as well as travel and the farming industries, the later contributes to the employment of around 65% of the population and a significant share of the national economy. The loss of commercial sectors, such as dairy, feed, and poultry industries are estimated to be in billions of rupees. One shortcoming of resource poorly managed countries, such as Nepal is the lack of interdisciplinary coordination. Over half a decade long professional existence and crucial contributions in Nepal's overall development, veterinarians are just beginning to be recognized as essential service providers. Since veterinarians of Nepal have the experience of eradicating cattle plague dealing with Avian Influenza and Swine Flu among others, they study and seem to be mentally prepared to handle pandemics. Currently, five veterinary diseases diagnostic laboratories with RT-PCR facilities are serving as COVID-19 diagnosis labs. Veterinarians from Nepal are serving in innovating newer tools as well as performing, supervising, evaluating, reporting diagnosis, and actively in pursuit of establishing the One Health (OH) approach. The sincere and patriotic role played by veterinarians has pressurized the government of Nepal to recognize veterinary service as an essential commodity. In this regard, universities should take the lead and bring veterinarians in policy and planning as well as decision making. The Tribhuvan University is responsible to have the initiative and Agriculture and Forestry University to deliver the missed opportunities. The 17 sustainable development goals that are to be achieved by 2030 are not possible without a multi-sectoral approach and veterinarians are best suited in this regard. It also needs to be realized that veterinarians are better positioned to handle emerging and re-emerging zoonotic diseases and capable of combating the present situation of COVID19 pandemics. Reiterating the fact that OH approach should not only be in paper and there is no alternative but combined utilization of scarce resources for healthy animals, humans, and the environment with the veterinary profession at its core.

Keywords: COVID-19, Essential services, One Health, SARS-CoV-2, Veterinary.

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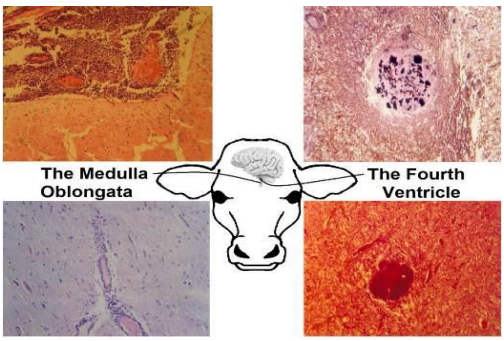
Research Paper

Histopathological Features of Listerial Rhombencephalitis in Dairy Calves in Kazakhstan.

Ibazhanova AS, Kenzhebekova ZhZh, Nurgazy BO, Khussainov DM, Namet AM, Alimov AA and Orynkhanov KA.

World Vet. J. 10(3): 306-311, 2020; pii:S232245682000039-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj39

ABSTRACT



Ibazhanova AS, Kenzhebekova ZhZh, Nurgazy BO, Khussainov DM, Namet AM, Alimov AA and Orynkhanov KA (2020). **Histopathological Features of Listerial Rhombencephalitis in Dairy Calves in Kazakhstan.** *World Vet. J.,* 10 (3): 306-311. DOI: https://dx.doi.org/10.36380/scil.2020.wvj39

Listeriosis is an infectious disease with zoonotic potential and can cause high fatality rates in animals and humans. Although the available evidence demonstrates the significance of post-mortem histological evaluation in the appropriate diagnosis, there is no sufficient evidence addressing histopathological alterations observed in calves clinically suspected of listeriosis. The current study aimed to carry out a post-mortem analysis of the dairy calves with clinical suspicion of listeriosis and determine a condition that contributed to death using histopathological evaluation. The tissue samples were obtained from 16 dairy calves with the age range of one day to one month, which died presumably due to listeriosis on several livestock farms in the Almaty region of Kazakhstan. The calves had symptoms of neurologic dysfunction prior to death. Necropsy and collection of brain histological specimens were performed, followed by procedures of fixation, dehydration, paraffinization, sectioning, staining with hematoxylin, and eosin along with Levaditi's method, and the microscopic examination. The histopathology findings were consistent with infection by *Listeria monocytogenes* and localized to the brainstem leading to the diagnosis of listerial rhombencephalitis.

Keywords: Brain, Calves, Histopathology, *Listeria monocytogenes,* Listeriosis, Rhombencephalitis.

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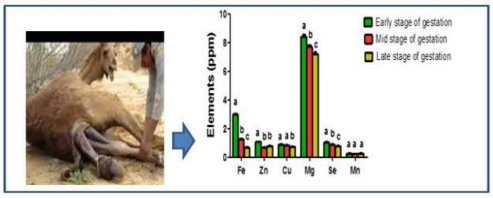
Research Paper

Trace Elements Profiles of Pregnant Camels (Camilus dromedaries), Fetus and Amniotic Fluid at Birth and their Association with Calf Birth Weight.

Essawi WM and Gouda HF.

World Vet. J. 10(3): 312-319, 2020; pii:S232245682000040-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

ABSTRACT



Essawi WM and Gouda HF (2020). **Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus and Amniotic Fluid at Birth and their Association with Calf Birth Weight.** *World Vet. J.*, 10 (3): 312-319. DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

Amniotic fluid is a dynamic complex mixture that carries components contributing to the regulation of fetal development. The present study aimed to measure the levels of trace elements, such as Fe, Zn, Cu, Mg, Se, and Mn in amniotic fluid, maternal serum, and venous umbilical cord serum at delivery. The study further investigated the relationships among levels of elements in amniotic fluid (AF), maternal serum (MS), and venous umbilical cord serum (VUCS) in order to assess the possibility of monitoring abnormal fetal growth. Blood samples were obtained from 30 pregnant female camels at delivery and the corresponding VUCS and AF were examined. The Fe, Zn, Cu, Mg, Se, and Mn were analyzed on the atomic absorption spectrophotometer. Concentrations of trace elements, Fe, Zn, Cu, Mg, Se, and Mn in VUCS were significantly higher, compared to MS or AF. The trace elements, Fe, Zn, Cu, Mg, Se, and Mn were present in significantly lower concentrations of AF than in MS or VUCS. Neonatal birth weight did not correlate with MS levels of the trace elements. However, neonatal weight correlated positively with venous cord serum Fe, Se, and Zn levels. There was a scarcity of correlation between maternal and fetus trace elements in the pregnant camels. In conclusion, AF could even be the result of simple filtration of maternal blood. Evaluation of selected trace element levels in MS did not appear to be useful within the assessment of fetus growth. The findings of this study indicated an active transport for Fe, Zn, Cu, Mg, Se, and Mn between pregnant camels and fetus.
Keywords: Calf birth weight, Dromedary camel, Placental barriers, Pregnancy, Trace element.

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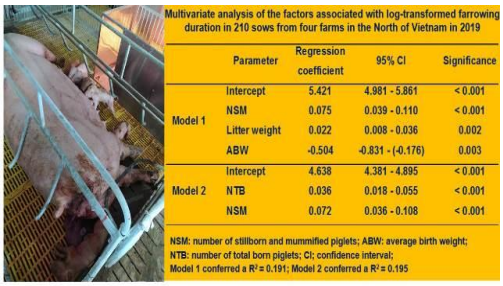
Research Paper

Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions.

Hoai Nam N and Sukon P.

World Vet. J. 10(3): 320-324, 2020; pii:S232245682000041-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj41

ABSTRACT



Multivariate analysis of the factors associated with log-transformed farrowing duration in 210 sows from four farms in the North of Vietnam in 2019

	Parameter	Regression coefficient	95% CI	Significance
Model 1	Intercept	5.421	4.981 - 5.861	< 0.001
	NSM	0.075	0.039 - 0.110	< 0.001
	Litter weight	0.022	0.008 - 0.036	0.002
	ABW	-0.504	-0.831 - (-0.176)	0.003
Model 2	Intercept	4.638	4.381 - 4.895	< 0.001
	NTB	0.036	0.018 - 0.055	< 0.001
	NSM	0.072	0.036 - 0.108	< 0.001

NSM: number of stillborn and mummified piglets; ABW: average birth weight; NTB: number of total born piglets; CI: confidence interval; Model 1 conferred a $R^2 = 0.191$; Model 2 conferred a $R^2 = 0.195$

Hoai Nam N and Sukon P (2020). **Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions.** *World Vet. J.*, 10 (3): 320-324. DOI: https://dx.doi.org/10.36380/scil.2020.wvj41

Modern pig farming worldwide has been facing substantial economic loss due to perinatal mortality which is mainly associated with the farrowing process. Therefore, the present study aimed to identify factors affecting the farrowing duration in natural farrowing sows in the intensive indoor conditions. In total, 210 farrowing sows in 4 commercial farms were included in the study. The association between potential risk factors and farrowing duration was analyzed by using general linear models. Two final models demonstrated that the number of total born piglets, number of stillborn and mummified piglets, litter weight, and average birth weight were significantly associated with farrowing duration. Among the four factors, average birth weight had a negative association with whereas the other three factors had positive associations with farrowing duration. Two models explained about 19.1-19.5% variation of the farrowing duration. The results also demonstrated that the number of total born piglets, stillborn, and mummified piglets were more important than litter weight and average birth weight in explaining the variation of farrowing duration.
Keywords: Birth weight; Farrowing duration, Sow, Stillbirth, Total born.

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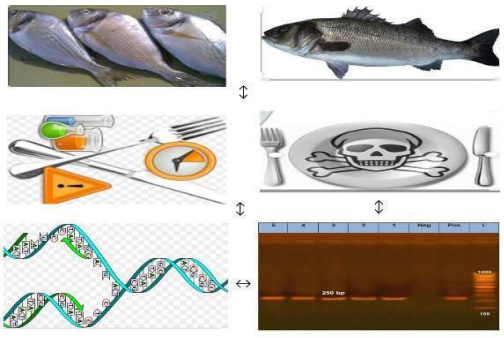
Research Paper

Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains.

Adel M. El-Gamal and Engy F. EL-Bahi.
World Vet. J. 10(3): 325-331, 2020; pii:S2322456820000042-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj42

ABSTRACT



Adel M, El-Gamal and Engy F. EL-Bahi (2020). **Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains.** *World Vet. J.*, 10 (3): 325-331. DOI: https://dx.doi.org/10.36380/scil.2020.wvj42

Vibrio species are the most common and serious pathogens in fish and shellfish marine aquaculture worldwide. The present study aimed to determine the prevalence of *Vibrio* spp. in seabass and seabream in fish markets, especially streptomycin-resistant strains that have great public health importance. A total of 30 seabass (*Dicentrarchus Labrax*) and 30 seabream (*Sparus aurata*) were purchased from fish markets at Kafr El Sheikh Governorate and subjected to bacteriological examination. The PCR assay was used for the detection of virulence genes (*tdh* and *trh*), aminoglycoside resistance gene (*aadA1),* and *toxR* gene. The results indicated that the total prevalence of *Vibrio* spp. was 26.66%, including *V. parahaemolyticus* (8.3%), *V. alginolyticus* (8.3%), *V. mimicus* (3.3%), *V. harveyi* (5%) and *V. vulnificus* (1.6%). The *toxR*, *trh,* and *aadA1* genes were found in all *V. parahaemolyticus* isolates while *tdh* gene was found in 80% of isolates. Antimicrobial sensitivity test of *V. parahaemolyticus* isolates showed sensitivity to ciprofloxacin, norfloxacin, cefotaxime, and chloramphenicol. *Vibrio parahaemolyticus* isolates were resistant to ampicillin, erythromycin, streptomycin, and gentamycin. The present results indicated that good hygienic measures should be taken to avoid infection with *Vibrio* species, especially *V. parahaemolyticus* that can pose a great risk to human health.

Keywords: Antibiotic resistance, Seabass, Seabream, Streptomycin, *Vibrio parahaemolyticus*.

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Research Paper

Effect of Dietary Dried Fennel and Oregano and Thyme Supplementation on Zootechnical Parameters of Growing Rabbit.

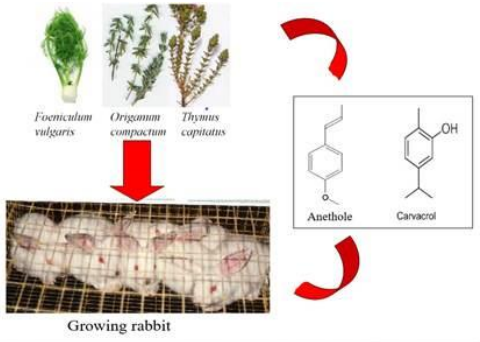
Benlemlih M, Barchan A, Aarab A, Bakkali M, Arakrak A and Laglaoui A.

World Vet. J. 10(3): 332-337, 2020; pii:S232245682000043-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj43

ABSTRACT

The objective of this study was to analyze and compare the effects of fennel, oregano, and thyme dietary supplements on the feeding of rabbits. In this regard, 96 weaned rabbits (30-day-old), white New Zealand, were divided into 4 groups and submitted to the following dietary treatments: Control diet, F diet (Control diet + 5% Foeniculum vulgaris), O diet (Control diet + 5% Origanum compactum), and T diet (Control diet + 5% Thymus capitatus). The essential oils of the above mentioned aromatic plants were extracted and were analyzed using a gas chromatograph coupled to a mass spectrometer. The treatment of fennel, oregano, and thyme had no beneficial effects on the growth performance of the rabbits but reduced the mortality rate. The phenylpropanoid and the phenolic monoterpenes were the major components of Foeniculum vulgaris, Origanum compactum, and Thymus capitatus essential oils. The aromatic plants and their active compounds can be used as additives in rabbit nutrition.

Keywords: Essential oil, Fennel, Growth performance, Oregano, Rabbit, Thyme.



Benlemlih M, Barchan A, Aarab A, Bakkali M, Arakrak A and Laglaoui A (2020). **Effect of Dietary Dried Fennel and Oregano and Thyme Supplementation on Zootechnical Parameters of Growing Rabbit.** *World Vet. J.*, 10 (3): 332-337. DOI: https://dx.doi.org/10.36380/scil.2020.wvj43

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Research Paper

Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies.

Azooz MF, SEl-Wakeel A, and Yousef HM.

World Vet. J. 10(3): 338-361, 2020; pii:S232245682000044-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

ABSTRACT

Bulk tank milk analysis was referred to as a useful and appropriate diagnostic tool to evaluate milk quality and mastitis pathogens in cattle dairy herds. Out of the total number of 150 pooled tank milk samples collected from 150 cattle dairy farms, 13 locally field *Staphylococcus aureus* isolates were detected and confirmed phenotypically by culturing, gram staining, biochemical, and molecular identification to be *Staphylococcus aureus* in the overall herd by the prevalence of 8.6%. Isolation and identification of *Salmonella* Typhimurium field isolates from bulk tank milk samples revealed that 20 locally field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemical and molecular identification to be *Salmonella* Typhimurium in the overall herd by the prevalence of 13.3%. The results of total

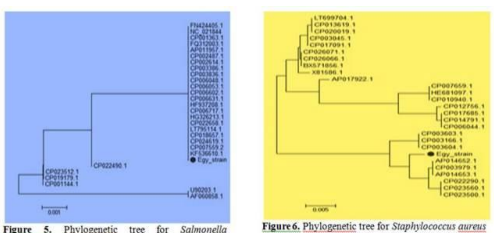

Figure 5. Phylogenetic tree for *Salmonella* Typhimurium (*sopB*) virulence gene partial nucleotide sequences that was generated using a neighbor joining in MEGA6. It shows a clear clustering of the Egyptian isolated strain and different *Salmonella* Typhimurium strains uploaded from gene bank.

Figure 6. Phylogenetic tree for *Staphylococcus aureus* (*hlg*) virulence gene partial nucleotide sequences that was generated using a neighbor joining in MEGA6. It shows a clear clustering of the Egyptian isolated strain and different *S. aureus* strains uploaded from gene bank.

Azooz MF, SEl-Wakeel A, and Yousef HM (2020). **Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies.** *World Vet. J.*, 10 (3): 338-361. DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

bacterial plate count (cfu/ml) revealed that the geometric mean of 150 dairy farms was 3.2×10^8 cfu/ml. The geometric mean of somatic cell count (SCC)/ml in Bulk tank milk samples of 150 cattle dairy farms were 556.7×10^3. The geometric mean value of *Staphylococcus aureus* count in this study was 3.7×10^3 cfu/ml. Serological identification of the 20 isolates revealed that they were *Salmonella* Typhimurium. The study provided various risk factors that had a clear and effective role in determining the level of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard plate count, and Somatic cell count in bulk tank milk. The PCR amplification with (*hlg*) gene-specific primers revealed a product with an approximate size of 937 bp. (*hlg*) gene found in 13 (54%) *Staphylococcus aureus* isolates. The PCR identification of *sopB* (*SigD*) virulence gene for *Salmonella* Typhimuriym revealed a product with an approximate size of 517 bp. *SopB* gene found in all *Salmonella* Typhimurium isolates (100%). Phylogenetic and partial gene sequence analysis of (*hlg*) *Staphylococcus aureus* gene of Egyptian isolated strain showed a great identity with the different *Staphylococcus aureus* strains uploaded from the gene bank. Phylogenetic analysis of *Salmonella* Typhimurium (*sopB*) virulence gene of Egyptian isolated strain indicated a great homology with the different *Salmonella* Typhimurium strains uploaded from the gene banks. The results of the present study emphasize the importance of more efficacious preventive programs for controlling the mastitis and bacteriological quality of bulk tank milk and monitoring mastitis economic losses.
Keywords: Mastitis, PCR, phylogenetic analysis, risk factors, *Salmonella* and *Staphylococcus aureus.*

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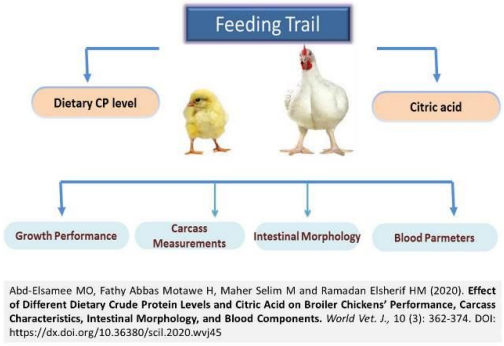
Research Paper

Effect of Different Dietary Crude Protein Levels and Citric Acid on Broiler Chickens' Performance, Carcass Characteristics, Intestinal Morphology, and Blood Components.

Abd-Elsamee MO, Fathy Abbas Motawe H, Maher Selim M and Ramadan Elsherif HM.

World Vet. J. 10(3): 362-374, 2020; pii:S232245682000045-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj45

ABSTRACT
The present study was conducted to investigate the effect of dietary protein levels and citric acid on the growth performance, carcass yield, abdominal fat, chemical composition of meat, intestinal morphology, and blood parameters of broiler chickens. A total of 160 Cobb 500 unsexed one-day-old broilers were assigned to 4 dietary treatments, 4 replicates of 10 chickens each. A factorial design arrangement 2×2 was used, including two protein levels, 100% (optimal level) and 90% (low level) of recommended dietary crude protein for cobb 500 broiler chickens, each protein level supplemented with or without 20-gram citric acid /kg. The results showed that chickens fed the diet containing 100% required Crude Protein (CP) supplemented with citric acid which could significantly improve body weight gain, feed conversion ratio, carcass yield, abdominal fat, fat content in meat, intestinal morphology, cecal microbial content, and blood parameters (Albumin, haemoglobin Ac1, fructosamine, and cholesterol). Chickens fed the low CP diet supplemented with citric acid could compensate for the growth performance equivalent to those fed the optimal CP diet. Both required protein level and citric acid were significantly improved blood albumin and reduced haemoglobin Ac1 and fructosamine, which could serve as indicators of the blood protein glycation. In conclusion, citric acid addition could alleviate the negative effect of feeding broiler chickens on low CP diets through its beneficial impact on intestinal morphology, cecal bacterial counts, blood cholesterol reduction, and glycated proteins.
Keywords: Broiler, Citric Acid, Glycation, Performance, Protein level.



Abd-Elsamee MO, Fathy Abbas Motawe H, Maher Selim M and Ramadan Elsherif HM (2020). **Effect of Different Dietary Crude Protein Levels and Citric Acid on Broiler Chickens' Performance, Carcass Characteristics, Intestinal Morphology, and Blood Components.** *World Vet. J.*, 10 (3): 362-374. DOI: https://dx.doi.org/10.36380/scil.2020.wvj45

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

Research Paper

Comparison of Probiotic *Lactobacillus acidophilus* and Oxytetracycline for the Treatment of Early Stage Interdigital Necrobacillosis in Dairy Cows.

Tulemissova ZhK, Torehanov MA, Myktybayeva RZh, Ibazhanova AS, Khussainov DM, Batanova ZhM and Usmangaliyeva SS.

World Vet. J. 10(3): 375-379, 2020; pii:S232245682000046-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj46

ABSTRACT
The objective of this multilocation field trial was to compare the effectiveness of the topical application of a powdered probiotic strain and intramuscular injection of oxytetracycline for the treatment of interdigital necrobacillosis in dairy cows. The current study was conducted from April 2018 to May 2020 on 230 dairy cows with early-stage interdigital necrobacillosis diagnosed by the research veterinarians on 6 farms situated in the Almaty region of Kazakhstan. The animals were recruited randomly to one of the following groups. A trial group in which the affected hoof of a cow was cleaned, coated with powder containing 10^6 colony-forming units of *Lactobacillus acidophilus* per gram, and then bandaged. Cows in the positive control group were subjected to a single

Tulemissova ZhK, Torehanov MA, Myktybayeva RZh, Ibazhanova AS, Khussainov DM, Batanova ZhM and Usmangaliyeva SS (2020). **Comparison of Probiotic *Lactobacillus acidophilus* and Oxytetracycline for the Treatment of Early Stage Interdigital Necrobacillosis in Dairy Cows.** *World Vet. J.*, 10 (3): 375-379. DOI: https://dx.doi.org/10.36380/scil.2020.wvj46

intramuscular injection of oxytetracycline at the dosage of 1.0 milligram per kilogram of bodyweight which is considered the routine treatment for interdigital necrobacillosis in the feedlots. Both procedures were executed every 72 hours during a period of two weeks with the subsequent two-week follow-up period. The treatment was discontinued when a cow was assessed as cured. Both groups were monitored daily for their limb condition and the degree of lameness during 28 days. Those cows having no evidence of lameness and lesions attributed to interdigital necrobacillosis with no disease recurrence recorded within the observation period, were considered cured. On day 15, the overall cure rates for *Lactobacillus acidophilus* 015k-1 strain and oxytetracycline were 80.87% and 83.48%, respectively. The overall odds ratio for the cure rate in the probiotic group versus oxytetracycline was 0.837. However, on day 28, all cows in both groups were found to be clinically free from the disease, and cure rates were 100%. No recurrence was recorded in any of the cases. It was concluded that the topical administration of the probiotic powder to dairy cows with early-stage interdigital necrobacillosis can result in cure rates nearly as high as those for intramuscular oxytetracycline within a period of 28 days. This is the first report on the treatment effect of *Lactobacillus acidophilus* locally applied to cattle with early-stage interdigital necrobacillosis.
Keywords: Dairy cows, Foot rot, Interdigital necrobacillosis, *Lactobacillus acidophilus*, Oxytetracycline, Probiotic, Topical administration.

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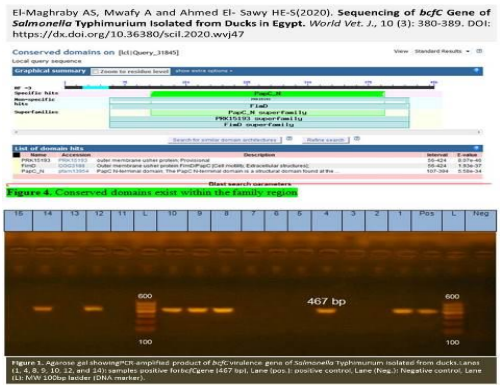
Research Paper

Sequencing of *bcfC* Gene of Salmonella Typhimurium Isolated from Ducks in Egypt.

El-Maghraby AS, Mwafy A and Ahmed El- Sawy HE-S.

World Vet. J. 10(3): 380-390, 2020; pii:S232245682000047-10; DOI: https://dx.doi.org/10.36380/scil.2020.wvj47

ABSTRACT



The main objective of this study was to applying *bcfC* gene sequence of *Salmonella* Typhimurium recently isolated from ducks to give insight into the source and origin, molecular epidemiology, disease pattern of *Salmonella* Typhimurium in Egyptian duck farms. Out of 75 fecal swab samples, 15 (20%) local field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemically and serologically to be *Salmonella* Typhimurium. The PCR amplification with *bcfC* gene-specific primers was conducted with genomic DNA, which revealed a product with the approximate size of 467 bp. The*BcfC* gene was found in 7 (46.6%) isolates of *Salmonella* Typhimurium. Phylogenetic and partial gene sequence analysis of *bcfC* gene of *Salmonella* Typhimurium showed clear clustering of Egyptian isolates of *Salmonella* Typhimurium and different *Salmonella* strains uploaded from GenBank. Sequence identities between the isolated Egyptian strain and different *Salmonella* Typhimurium strains from GenBank revealed 99.8-100% homology. Open reading frame (ORF) analysis of *Salmonella* typhimurium *bcfC* gene using NCBI tool and ORF analysis of *bcfC* gene protein translation using ExPasy (SIB Bioinformatics Resource Portal) indicated all open reading frames of a specified minimum size in a sequence of (453 bp). The 3 conserved domains region in the nucleotide sequence were PapC N-terminal domain (107-394bp), PRK15193 outer membrane usher protein (56-424bp), and FimD Outer membrane usher protein FimD/PapC (cell motility, extracellular structures, 56-424bp). The PapC N-terminal domain was a structural domain found at the N-terminus of *S.* typhimurium PapC protein and had a central role in the pili assembly chaperone usher system (CUP). Amino acids alignment report of the sequenced 415 amino acid of *Salmonella* Typhimurium *bcfC* gene showed great homology between the Egyptian *Salmonella* Typhimurium strain and the different *Salmonella* strains uploaded from GenBank. Nucleotide alignment report of the sequenced *Salmonella* Typhimurium *bcfC* gene at (417bp) demonstrated great homology between the Egyptian *Salmonella* Typhimurium strain and the different *Salmonella* strains uploaded from GenBank. In conclusion, the Egyptian *Salmonella* Typhimurium isolate was related to the common sequence types isolated from humans and bovine-based products across the world especially in the United Kingdom, USA, Ireland, and México. Most of the duck farms from which we isolated the Egyptian *Salmonella* Typhimurium isolates were located in the same geographical area of cattle farms in addition to the duck farms lacked the requirements of biosecurity, which could facilitate the circulatory transmission of salmonella strains between the human beings and other animal farms, including duck farms. Moreover, the PapC N-terminal domain was a central conserved domain encoded by *bcfC* gene of *S.* Typhimurium. A PapC N-terminal conserved domain can be used as a vaccine target for vaccine production against *S.* Typhimurium.
Keywords: *bcfC* gene, Conserved domain, Duck, GenBank, ORF, Phylogenetic tree, *Salmonella* Typhimurium, Sequencing.

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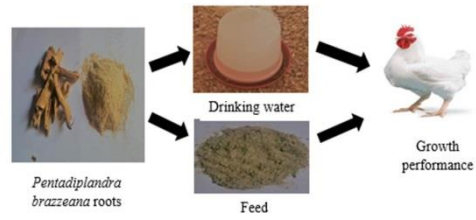
Research Paper

Effects of Dietary Inclusion of *Pentadiplandra brazzeana* Powder on Growth Performances, Gut Microbiota and Haemato-Biochemical Indices of Broiler Chickens.

Necdem TB, Kana JR, Ngouana TR, Ebile DA, Donfack M, Tchouan DG and Kengni NJ.

World Vet. J. 10(3): 391-397, 2020; pii:S232245682000048-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj48

ABSTRACT
This study was designed to assess the rearing performances of broiler chickens under two *Pentadiplandra brazzeana* powder feeding diets. A total of 256 day-old Cobb 500 broiler chicks were randomly assigned to 4 treatment groups with 4 replicates of 16 birds each in a completely randomized design. This trial involved administrating *Pentadiplandra brazzeana* powder to broiler chickens via drinking water (2 g/l) or dry feed (2 g/kg) and comparing the result to those obtained from chickens fed on antibiotic (1 g/kg) and chickens fed without additive. The results revealed that the incorporation of *Pentadiplandra brazzeana* powder in chickens' diet and drinking water could decrease feed intake, compared to the negative control diet. The inclusion of *Pentadiplandra brazzeana* powder in water and antibiotic in diet recorded the high live weight and weight gain, compared to the birds fed with the powder in feed and the negative control diet. The low feed conversion ratio was reported with the water supplemented with *P. brazzeana* powder. The carcass yield was significantly higher with the inclusion of the powder via drinking water, compared to the inclusion via feed and with the control diet. The feeding method did not affect the haemato-biochemical parameters, compared to the control diet. Chickens fed with a diet containing *Pentadiplandra brazzeana* powder in water recorded the highest ($p<0.05$) lactic acid bacteria count, compared to *E. coli* and *Salmonella*. In conclusion, dietary supplementing with *Pentadiplandra brazzeana* powder through drinking water at a rate of 2 g/l can be used as an alternative to antibiotics to improve the growth performances of broiler chickens.
Keywords: Broiler chicken, Growth performances, Gut microflora, Haemato-biochemical profile, *Pentadiplandra brazzeana*



Necdem TB, Kana JR, Ngouana TR, Ebile DA, Donfack M, Tchouan DG and Kengni NJ (2020). **Effects of Dietary Inclusion of *Pentadiplandra brazzeana* Powder on Growth Performances, Gut Microbiota and Haemato-Biochemical Indices of Broiler Chickens.** *World Vet. J.*, 10 (3): 391-397. DOI: https://dx.doi.org/10.36380/scil.2020.wvj48

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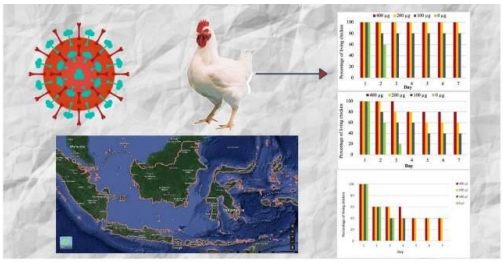
Research Paper

Cross Protectivity of Yolk Immunoglobulin Anti-Hemagglutinin Protein of High Pathogenic Avian Influenza A subtypes H5N1 Administered on Chicken Infected by High Pathogenic Avian Influenza A subtypes H5N1.

Suwarno, Ernawati R and Widjaja NS.

World Vet. J. 10(3): 398-404, 2020; pii:S232245682000049-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj49

ABSTRACT
Yolk Immunoglobulin (IgY) against Avian Influenza (AI) is commonly used as immunotherapy and immunodiagnostic techniques. Application of IgY mixed in drinking water is known effective to inhibit AI replication. The effectivity of IgY anti-Hemagglutinin Protein (anti-HA) of High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) was tested against infection of High Pathogenic Avian Influenza clade 2.3.2 (A/Duck/Sidoarjo/2012). The inhibiting activity was observed through Immunohistochemistry. Sixty chickens were infected with 105 EID50/ml of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012). Yolk Immunoglobulin with different amounts (0 µg, 100 µg, 200 µg and 400 µg) were administered at three different times which were 24 hours before infection, at the time of infection, and 24 hours after infection. The observation was conducted for 7 days. During post infection observation, death chickens were managed for immunohistochemistry assay to observe the present of virion and IgY sialic acid 2,3-alfa galactosa (SA α 2,3 gal) blocking activity in septa alveoli. By the end of observation all chickens were euthanized for immunohistochemistry assay. The result showed that anti-HA IgY obtained from HPAI clade 2.1 could protecting infection of HPAI clade 2.3.2. According to immunohistochemistry assay, the administration of IgY can neutralize the infecting virus marked by the number of virions observed in septa alveoli of the lungs. Regarding the assay, the dose of 200 µg and 400 µg of IgY applied 24 hours before the infection, can reduce clinical signs and mortality of infected chicken (80-100%). The best dose of the IgY to protect them from infection of clade 2.3.2 (A/Duck/Sidoarjo/2012) was 400 µg administered 24 hours before infection. It could be concluded that administration of IgY anti-Haemaglutinin Protein (anti-HA) of High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) could protect chickens against the infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012), even though they belong different clades. The protection rate was 80-100%. Further research should be done to discover the cross-protectivity of IgY as preventive method against HPAI outbreak.
Keywords: Avian influenza virus, IgY anti-HA, Immunotherapy, Productivity.



Suwarno, Ernawati R and Widjaja NS (2020). **Cross Protectivity of Yolk Immunoglobulin Anti-Hemagglutinin Protein of High Pathogenic Avian Influenza A subtypes H5N1 Administered on Chicken Infected by High Pathogenic Avian Influenza A subtypes H5N1.** *World Vet. J.*, 10 (3): 398-404. DOI: https://dx.doi.org/10.36380/scil.2020.wvj49

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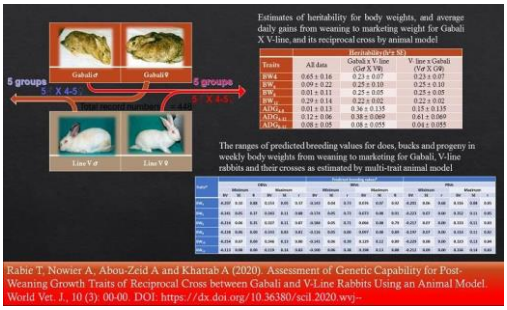
Research Paper

Assessment of Genetic Capability for Post- Weaning Growth Traits of Reciprocal Cross between Gabali and V-Line Rabbits Using an Animal Model.

Rabie T, Nowier A, Abou-Zeid A and Khattab A.

World Vet. J. 10(3): 405-413, 2020; pii:S232245682000050-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj50

ABSTRACT
This study aimed to assess the conceivable impact of hereditary factors on the crossbreeding rabbit groups set up by proportional going between Sinai Gabali and V-Line. Reciprocal cross on the post-weaning performances was performed by estimating the genetic capability for their crosses. The study samples included two pure rabbit breeds (a male Saini Gabali (G) and a female V-Line (V)), and reciprocal crosses to compromise 10 groups. Records of 448 kits delivered by 45 does and 16 bucks were utilized to estimate Heritability (h^2), genetic and phenotypic correlations, and breeding values of litter weight traits. First generation was created from the consequences of four parities (½G ½V, and ½ V ½G; sire breed was demonstrated first). Weaning was implemented on the 28th day of the kits' age. Post-weaning litter traits were measured Body weight (BW) at 4, 5, 6, 8, 10, and 12 weeks of their age; and average daily gain was measured during 4-8 weeks (ADG_{4-8}, ADG_{4-12}, ADG_{8-10}, and ADG_{8-12}). Data were examined by animal model, which was performed utilizing derivate free limited maximum likelihood. The results revealed that h^2 was moderate for both breeds, and its reciprocal cross ranged from 0.2 to 0.25, and BW at weaning was 0.22 ± 0.07. Meanwhile, there was a positive genetic correlation between BW and ADG at different age ranges (ranged 0.02 to 0.77, 0.04 to 0.76, respectively). Assessments of environmental correlation between BW at different age ranges were negative, except of those between BW_8 and BW_{12} which were positive, but not significant. Additionally, the progeny had higher predicting breeding values for BW at 4, 8,10, and 12 weeks for both breeds, but that was obtained from G×V exceeding those from their reciprocal cross. In conclusion, direct additive variance was considerably effective, and consequently body weight at weaning and post-weaning growth traits could be improved by utilizing bucks of Sinai Gabali with doesof V-line based on the performance of their progenies, and selection of sires and dams.
Keywords: Genetic correlation, Heritability, Post-weaning, Sinai gabali, V-line, Weaning weight



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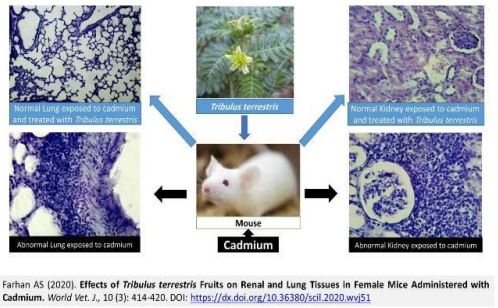
Research Paper

Effects of Tribulus terrestris Fruits on Renal and Lung Tissues in Female Mice Administered with Cadmium.

Farhan AS.

World Vet. J. 10(3): 414-420, 2020; pii:S232245682000051-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj51

ABSTRACT
The current study was designed to evaluate the protective effects of *Tribulus Terrestris* on kidney and lung tissues against cadmium toxicity in female mice. In this regard, 20 female albino mice were randomly assigned into three groups; the first group served as the control group, the second group was given the toxic substance (cadmium 6 mg/kg) only, and the third group was given cadmium (6 mg/kg) plus the alcoholic extract of the *Tribulus terrestris* fruit (200 mg/kg). The substances were administered orally by stomach tube daily for 10 days. On the last day of the study, the animals were euthanized, and their kidney and lung were sampled for histological study. The kidney tissue in mice exposed to cadmium showed cellular inflammation, necrosis, hyperplasia, and large urinary space in Bowman's capsule in comparison to the normal appearance of tissues in the mice in the other two groups. The large aggregations of lymphocytes around the bronchus and edema in the lungs exposed to cadmium were observed. The lungs of some mice exposed to cadmium and treated with *Tribulus terrestris* fruit indicated normal tissue appearance, while others showed large aggregations of lymphocytes between alveolar sacs and thick interalveolar septa. The *Tribulus terrestris* protected the kidneys against the toxicity of the cadmium while this plant had fewer protective effects against cadmium in the lung tissue. These results demonstrated that *Tribulus terrestris* ameliorated cadmium toxicity.
Keywords: Cadmium, Kidney, Lung, Mice, *Tribulus terrestris*



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Research Paper

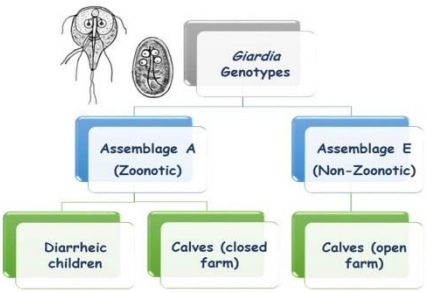
The Use of *Giardia* immunogenic Protein Fraction to Distinguish Assemblages in Humans and Animals.

Ramadan RM, Khalifa MM, Kamel NO, Abdel-Wahab AM and El-Bahy MM.

World Vet. J. 10(3): 421-428, 2020; pii:S232245682000052-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj52

ABSTRACT
Buffalo calves have a high adverse effect on public health, specifically zoonotic Giardiasis in Egypt. The current study was carried out to investigate the use of enzyme-linked immunoelectrotransfer blot technique (EITB) as a preliminary step for the diagnosis of giardiasis before genotyping. For this purpose, fecal and blood samples were collected from diarrheic

calves and workers in closed and non-closed beef buffalo farm. Zoonotic *Giardia* assemblage A was isolated from both diarrheic children and their close contact calves while the calves were infected by animal genotype assemblage E under low contact conditions. This was identified after the amplification and sequencing of a 292 bp fragment of 16S-rRNA ribosomal unit from 20 children and 28 calves *Giardia* isolates using nested PCR. Fractionation of different isolated *Giardia* assemblage and identification of specific fraction versus anti-*Giardia*-IgG antibodies in infected humans or animals using EITB revealed that α-1 giardin antigen with molecular weight ranged 29-34KDa. This specific immunogenic assemblage was fraction-related where it reacted specifically versus antibodies in sera of humans or animals infected by this assemblage and did not cross-react with giardin of other assemblage or with sera of non-infected cases. The results of the current study highlighted the use of EITB as a preliminary investigation before genotyping in the identification of zoonotic giardiasis.



Ramadan RM, Khalifa MM, Kamel NO, Abdel-Wahab AM and El-Bahy MM (2020). **The Use of *Giardia* immunogenic Protein Fraction to Distinguish Assemblages in Humans and Animals.** *World Vet. J.*, 10 (3): 421-428. DOI: https://dx.doi.org/10.36380/scil.2020.wvj52

Keywords: Buffalo calves, Genotype, *Giardia*, Human, Immunoblot, Nested PCR

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Research Paper

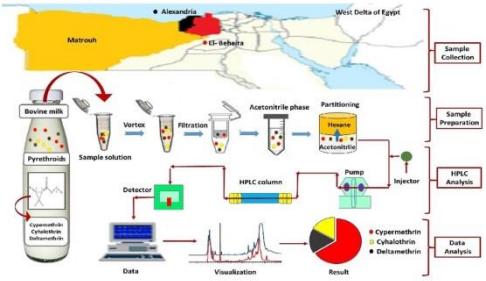
Spatial Monitoring of Pyrethroid Residues by RP-HPLC in Raw Bovine Milk in West Delta Region of Egypt.

Amer AA and Abou-Alella SA

World Vet. J. 10(3): 429-436, 2020; pii:S232245682000053-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj53

ABSTRACT

The massive implementation of pesticides in agronomy, public health series, manufacturing, and production can increase pesticides in the environment leading to tremendous effects on human health. Hence, the current study aimed to evaluate the concentration and frequency of pyrethroid residues in raw bovine milk sourced from local producers at West Delta of Egypt carefully chosen from three cities on High-Performance Liquid Chromatography system. A total of 200 of raw bovine milk samples (Alexandria 75, El- Behera 75, and Matrouh 50) were analyzed. The achieved results indicated that Cypermethrin, Cyhalothrin, and Deltamethrin residues were respectively screened at frequencies of 18.66%, 17.33%, and 14.66% in Alexandria, 21.33%, 17.33%, and 16% in El- Behera, and 44%, 28%, and 24 % in Matrouh. Cypermethrin was detected in a mean concentration level of 6.63, 5.98, and 10.74 in Alexandria, El- Behera, and Matrouh, respectively. However, Cyhalothrin and Deltamethrin had lower mean values of 4.66 and 2.45 in Alexandria, 5.14 and 3.05 in El- Behera, and finally 2.84 and 2.69 in Matrouh. There was no α- Cypermethrin in all the investigated samples while Cypermethrin was present with the highest mean concentration and frequency levels in Matrouh, compared to other cities. Concerning the compatibility of examined samples with maximum residue levels (MRLs) set by the European Commission and Codex regulation, indicated only 2.66%, 1.33%, and 0.00 % for Cyhalothrin, and 1.33%, 4%, and 2% for Deltamethrin examined samples in Alexandria, El- Behera, and Matrouh cities exceeded MRLs, respectively. As for Cypermethrin, its concentrations did not exceed MRLs. These results indicated a significant amount of these chemical pollutants in raw bovine milk offered by local producers in the West Delta of Egypt, which can threaten human life. Accordingly, continual assessment of pyrethroid residues in milk is of utmost importance to guarantee Egyptian consumers' health.



Amer AA and Abou-Alella SA (2020). **Spatial Monitoring of Pyrethroid Residues by RP-HPLC in Raw Bovine Milk in West Delta Region of Egypt.** *World Vet. J.*, 10 (3): 429-436. DOI: https://dx.doi.org/10.36380/scil.2020.wvj53

Keywords: Bovine milk, Cyhalothrin, Cypermethrin, Deltamethrin, MRLs, Pesticides, Pyrethroids, RP-HPLC

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

Research Paper

The Role of Afferent C-Fibers in Muscle Contraction of Trachea and Bronchi in Rat.

Kirilina VM, Smirnova OE, Blazhevich LE and Chepurina IV.

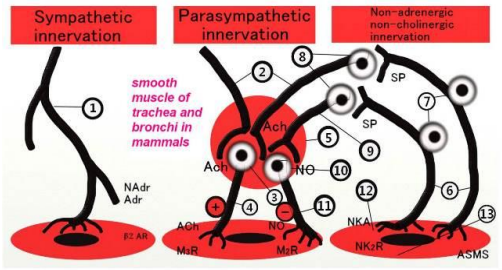
World Vet. J. 10(3): 437-445, 2020; pii:S232245682000054-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj54

ABSTRACT

The present experimental study on some basal neurophysiological systems aimed to evaluate the effect of the non-adrenergic non-cholinergic system on muscle contraction of the trachea and bronchi. Moreover, the study was targeted toward the investigation of the effect of the local intramural ganglion. The obtained results indicated that C-fibers, which represent the excitatory non-adrenergic non-cholinergic system, caused smooth muscle contraction by the realization of reflex through local intramural ganglia. Furthermore, it was observed that C-fibers affected the muscle by releasing tachykinins for constricting effect. The constricting influence of fibers was greater in the case of involving local reflex through the ganglion, and less significant in the case of activating of the humeral mechanism related to tachykinins. This finding became apparent as a result of the comparison of contractile muscle responses in Krebs-Henseleit's solution with

atropine, and with activating C-fiber capsaicin applications. It was also observed that in the rat trachea and bronchus the elimination of NO-ergic mechanisms led to an increase in the contraction, and the dilatation effect of nitric oxide was associated with preganglionic and postganglionic nerve structures of the intramural ganglia. In conclusion, it is identified that afferent C-fibers increase the contractions of the smooth muscle of the trachea and bronchi of the rat mainly with the involvement of ganglion neurons. The humoral mechanism of C-fibers performs a minor and additional role in muscle contraction by the release of tachykinins. The obtained data can contribute to the study of the interaction of the autonomous nervous system and non-adrenergic non-cholinergic system.

Keywords: Bronchi, Intramural ganglion, Non-adrenergic non-cholinergic system, Tachykinins



Kirilina VM, Smirnova OE, Blazhevich LE and Chepurina IV (2020). **The Role of Afferent C-Fibers in Muscle Contraction of Trachea and Bronchi in Rat.** *World Vet. J.*, 10 (3): 437-445. DOI: https://dx.doi.org/10.36380/scil.2020.wvj54

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Research Paper

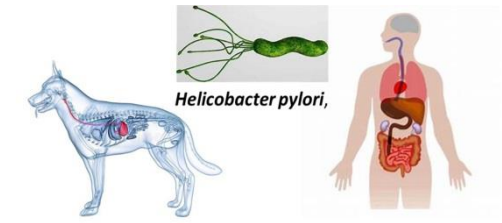
Dog as Potential Source of *Helicobacter pylori* in Egypt: Public Health Significance.

Elhelw R, Elhariri M, Ragab E, Kadry M and Hamza D.
World Vet. J. 10(3): 446-450, 2020; pii:S232245682000055-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj55

ABSTRACT

Helicobacter species are a group of Gram-negative, microaerophilic bacteria, which are known to colonize the gastrointestinal and biliary tracts of humans and various animal species. The objective of the present study was to determine the prevalence of *Helicobacter pylori* in owned dogs and their role in the transmission of *H. pylori* to the dog owners. For this purpose, 60 gastric biopsy samples from dog owners and 80 stool samples from owned dogs were collected and examined for the presence of *H. pylori 16s rRNA* gene by nested PCR. The PCR positive samples from human and dog isolates were further subjected to partial *Helicobacter* genus-specific *16s rRNA* gene sequencing. Phylogenetic analysis based on partial sequence of this gene was performed to determine the relationship between human and dog isolates. *H. pylori* was detected in 62.5% and 91.6% of dog and human samples, respectively. The nucleotide sequence of *Helicobacter* genus-specific *16s rRNA* gene of human and dog isolates were similar. In conclusion, this study indicated a high prevalence of *H. pylori* in both dogs and dog owners in Egypt. Zoonotic transmission of *H. pylori* between dogs and humans is probable and represents a public health concern.

Keywords: Dogs, *Helicobacter pylori,* Humans, Phylogenetic analysis, 16s rRNA sequencing



Elhelw R, Elhariri M, Ragab E, Kadry M and Hamza D (2020). **Dog as Potential Source of *Helicobacter pylori* in Egypt: Public Health Significance.** *World Vet. J.*, 10 (3): 446-450. DOI: https://dx.doi.org/10.36380/scil.2020.wvj55

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Research Paper

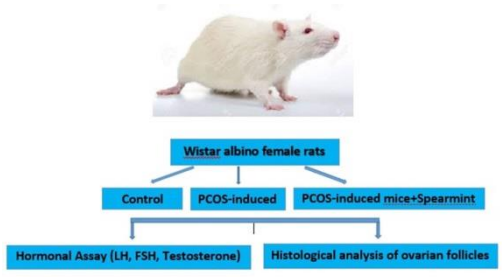
Capacity of *Mentha spicata* (spearmint) Extract in Alleviating Hormonal and Folliculogenesis Disturbances in Polycystic Ovarian Syndrome Rat Model.

Alaee S, Jafar Bagheri M, Sadeghi Ataabadi M and Koohpeyma F.
World Vet. J. 10(3): 451-456, 2020; pii:S232245682000056-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj56

ABSTRACT

Polycystic ovary syndrome, a common cause of infertility among women in the reproductive age, is associated with high levels of androgens. Recognizing the anti-androgenic effects of spearmint, the present study aimed to evaluate the effects of its hydroalcoholic extract on the levels of luteinizing hormone, follicle-stimulating hormone, and testosterone and ovarian folliculogenesis in normal and letrozole-induced polycystic ovary syndrome rats. Female mature rats were divided into six groups (n=8 per group), as follows: Normal rats (I or Control), normal rats which received 250 mg/kg spearmint extract (II) or 500 mg/kg spearmint extract (III), and PCOS-induced rats (IV), PCOS-induced rats which received 250 mg/kg spearmint extract (V), or 500 mg/kg spearmint extract (VI). At the end of the experiment the animals were euthanized, and then mentioned parameters were evaluated. Administration of spearmint extract to PCOS rats resulted in a decrease of body weight and testosterone level, higher corpus luteum, and lower ovarian cysts and atretic follicles, compared to PCOS rats which received no spearmint. Accordingly, the spearmint can attenuate polycystic ovarian syndrome-related problems, such as a high testosterone level and ovarian cysts.

Keywords: Folliculogenesis, *Mentha spicata*, Ovary, PCOS, Rat



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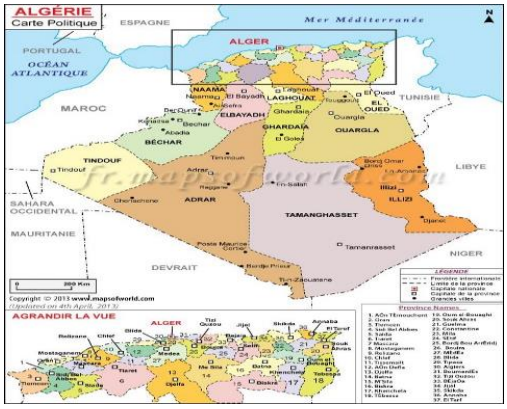
Research Paper

Comparison of Serological Tests in Cattle and Ovine Brucellosis; an Abattoir Study in Algeria.

Khames M, Zúñiga-Ripa A, Pérez Gómez S, Oumouna M and Moriyón I.

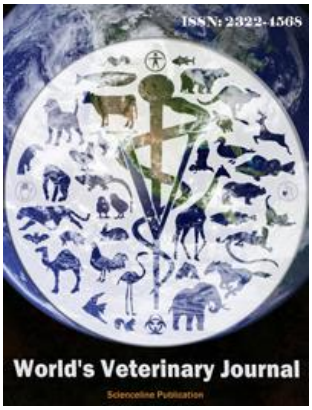
World Vet. J. 10(3): 457-464, 2020; pii:S232245682000057-10 ; DOI: https://dx.doi.org/10.36380/scil.2020.wvj57

ABSTRACT



ThDespite the paucity of data, brucellosis is considered as a major problem in Algeria. The aim of present study was to assess the presence of bovine and ovine brucellosis in the areas close to the capital city (Algiers) where its vaccination is not implemented. A total of 402 cattle and 203 ovine sera were collected from two slaughterhouses, and examined by the Rose Bengal Test (RBT). Positive samples were then tested by Complement Fixation Test (CFT) and Hypertonic Double Gel Diffusion (DDG) with a smooth lipopolysaccharide, and the extract of native hapten was also tested by Indirect Enzyme Linked Immuno Sorbent Assay (iELISAs) with smooth lipopolysaccharide and polyclonal or protein G conjugates. Twenty-four bovine sera (5.97%) were RBT positive. Of these, 23 were positive in CFT, DDG, and 16 samples were also positive in iELISA when the assay was adjusted to 100% specificity. Only two ovine sera were RBT positive; one was CFT and DDG positive, and the other one had a CFT-titer of 1/4, and was DDG negative. This preliminary study confirmed that bovine brucellosis is a major problem in Algeria, and indicated that some field studies are needed to determine the prevalence of Brucellosis in Algeria urgently. Similarly, other studies are necessary in areas with dominance of ovine breeding system. Further studies in the areas with a dominance of ovine breeding system are necessary. The results of this work showed that simple tests like RBT and DDG are not outperformed by CFT or iELISA for assessing the apparent prevalence of brucellosis in the absence of vaccination. Finally, isolation and typing of the involved Brucella species are also necessary in order to have a complete epidemiological picture of brucellosis in Algeria.

Keywords: Abattoirs, Algeria, Brucellosis, Cattle, Serology, Sheep, Prevalence



World's Veterinary Journal

ISSN: 2322-4568

Frequency: Quarterly

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

Current Issue: 2020, Vol: 10, 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

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- 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)
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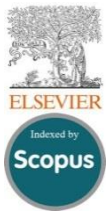

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 260-266, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj34

Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran

Ali Fattahi Bafghi[1,2], Mohammad Reza Yavari[2,3], Farzaneh Mirzaei[1*], Abolghasem Siyadatpanah[4], Watcharapong Mitsuwan[5], Maria de Lourdes Pereira[6], Veeranoot Nissapatorn[5], Roghayeh Norouzi[7] and Seyed Abdollah Hosseini[8]**

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ORGINAL ARTICLE
pii: S232245682000034-10
Received: 02 Jul 2020
Accepted: 10 Aug 2020

ABSTRACT

Consumption of raw vegetables is an important route of parasites transmission. It is an important source for foodborne outbreaks in both developed and developing countries, and outbreaks of parasitic diseases in humans. The objective of the present study was to detect the presence of *Cryptosporidium* oocysts in raw fresh vegetables in Yazd city, Iran, from 2017 to 2018. A total of 275 fresh vegetable samples were collected and tested using a sucrose flotation medium of 1.21 specific gravity and a Modified Ziehl-Nielsen staining procedure. Of the 275 vegetables examined, 85 (31.5%) samples were positive for *Cryptosporidium* oocysts. Lettuce had the highest rate (n= 16, 47.1%) of contamination with *Cryptosporidium* oocysts while basil and parsley showed the lowest rates of contamination (n= 6, 20%). There was a significant association between the occurrence of *Cryptosporidium* oocysts and the investigated vegetable types. According to the locations of the vegetable field, Amir Abad and Bahaman Hospital area had the highest (n: 16, 59.3%) and lowest (n= 5, 18.5%) rates of *Cryptosporidium* oocysts contamination, respectively. The plant part showed that the root vegetables had the highest contamination rates (n= 41, 45.6%), followed by leafy vegetables (n= 44, 24.4%). The analysis further indicated a significant association between the occurrence of *Cryptosporidium* oocysts and the route of vegetable consumption. Based on these results, the edible vegetables in Yazd city are one of the potential sources of *Cryptosporidium* infections in humans. Moreover, the vegetable fields within the city of Yazd are contaminated with *Cryptosporidium* oocysts which can pose public health problems.

Keywords: *Cryptosporidium*, Oocysts, Raw vegetables, Yazd city, Iran

INTRODUCTION

Cryptosporidium is a protozoan coccidian intestinal parasite that causes a diarrheal disease called cryptosporidiosis, a major public health problem in both developed and developing countries, and is an opportunistic infection among patients with AIDS and responsible for outbreaks of gastrointestinal disease (Desai et al., 2012). Many species of *Cryptosporidium* infect both humans and animals. This parasite has an outer layer that protects it from harsh external conditions as some disinfectants such as chlorine (Rossle and Latife, 2013). *Cryptosporidium* is one of the most frequent microbial causes of diarrhea, accompanied by pain and abdominal colic and a notable loss of weight. Since the 1980s, cattle have considered the most important source of zoonotic cryptosporidiosis (McDaniel et al., 2014). Although it is typically an opportunistic and short-term acute infection, the parasite can cause severe and unresolved condition in immune-compromised individuals such as the patients with AIDS, whose its presence remains in the lower intestine, and can remain for up to one month (Rossle and Latif, 2013; Mohaghegh et al., 2017).

Foodborne outbreaks of cryptosporidiosis have been identified from several food items, mainly fruits, vegetables, and seafood (Fayer et al., 2003; Sherbini et al., 2016). These products are usually consumed raw which raises public concerns. The evidence suggests that the consumption of raw products is a risk factor, at least in outbreak situations. Contamination of vegetables can occur if they are irrigated by contaminated water with human and/or animal feces, due to poor hygienic conditions in the environment in which *Cryptosporidium* is the most likely to be the parasite (Snelling et al., 2007; McDaniel et al., 2014). Although vegetables contain fiber, vitamins, and minerals, and are an essential

To cite this paper: Fattahi Bafghi A, Yavari MR, Mirzaei F, Siyadatpanah A, Mitsuwan W, Pereira ML, Nissapatorn V, Norouzi R, and Hosseini SA (2020). Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran. *World Vet. J.*, 10 (3): 260-266. DOI: https://dx.doi.org/10.36380/scil.2020.wvj34

source for maintaining health, the consumption of raw vegetables play an important role in the transmission of parasites, and have been shown to be an important source of foodborne outbreaks in developed and developing countries (Ortega et al., 1997). Therefore, the present study was carried out to investigate the presence of *Cryptosporidium* oocysts in fresh/raw vegetables in Yazd city, Iran.

MATERIALS AND METHODS

Study area

This study was carried out in Yazd city, Iran. It has a total land space of 300 km^2. Yazd is the driest main city in Iran with an average annual rainfall of 55 to 60 millimeters. Shortages of water supply are commonly seen in many areas like Yazd which leads to the scarcity of clean water consumption, and can increase the contamination by waterborne pathogens through water and food supplies including vegetables (DeNicola et al., 2015).

Sample collection and *Cryptosporidium* oocysts detection

A total number of 275 samples of raw vegetables were collected from particular vegetable fields around Yazd city, and subsequently examined for the presence of *Cryptosporidium* oocysts (Table 1). In this study, the whole plant was purchased and transported to the laboratory of Shahid Sadoughi University of Medical Science, Yazd city, Iran. Then, these vegetables were washed with normal saline, and stored until use. In the first stage of sample collection, the survey was primarily done in the selected vegetable fields on the source of vegetables, the source of water used for washing vegetables and the condition of vegetables (fresh and edible) on the farms. Cluster sampling was done in two stages, the city was divided into five sections: north, south, east, west, and central. Simple randomization was carried out in each section. Finally, five vegetable fields were selected. Samples were collected in the clean polythene bags during the spring of 2017 to winter of 2018 and transported to the laboratory at the Department of Medical Parasitology and Mycology at the School of Medicine University of Medical Sciences of Shahid Sadoughi, Yazd, Iran for further examination. In brief, vegetables were prepared and modified according to the protocol provided in study of Abougrain et al. (2010). Two hundred gram of a collected vegetable sample was weighed, washed with 250 mL of saline solution, and left for 10 hours for the sedimentation process. The supernatant was discarded, and the residue was transferred into a centrifuge tube and spun at 1500 rpm for 5 minutes. The supernatant was decanted, and the residue was gently stirred in a sucrose flotation medium with a specific gravity of 1.21. Each test tube was then covered to the edge with the flotation medium to form a meniscus. A coverslip was placed in the test tubes for 3 minutes and was removed and placed on a clean glass slide. Observations for the presence of *Cryptosporidium* oocysts were conducted under a light microscope (Olympus BX41TF, Okayama, Japan). Positive slides for oocysts were allowed to air dry and stained using the standard modified Ziehl-Nielsen technique (Abougrain et al., 2010). Air-dried slides were fixed in methanol for 2-3 minutes. Slides were flooded with cold carbol fuchsin for 5 to10 minutes, and then with 1% hydrochloric acid ethanol until the color stopped flowing and rinsed with tap water. It was then counterstained with 0.25% methylene blue for 30 seconds, rinsed again in tap water and air-dried.

Morphological identification of *Cryptosporidium* oocysts

The prepared slides were examined using a light microscope (Olympus BX41TF, Okayama, Japan) with 40 and 100 magnifications. *Cryptosporidium* oocysts appeared on a pale green background as bright rose-pink spherules (Figure 1). The positives slides identified were used for photomicrographs of *Cryptosporidium* oocysts.

Statistical analysis

All data were analyzed using SPSS 20 (IBM SPSS Statistics 20.0, NY, USA). Pearson chi-square or Fisher's Exact Test were used to analyze the association between the presence of *Cryptosporidium* oocysts and factors such as type, nature, consumption, and source of vegetables. The odds ratio (OR) values greater than the unit denoted association and less than unit denoted that the factor may have a protective effect. The occurrence of *Cryptosporidium* oocysts was estimated by dividing the number of positive samples by the total number of samples. A p value ≤ 0.05 was considered to be statistically significant.

Table 1. List of vegetables used in the study

Common name	Botanical name	Used Part
Mint	*Mentha spicata*	Leaf
Tarragon	*Artemisia dracunculus*	Leaf
Basil	*Ocimum bacilicum*	Leaf
Lettuce	*Lactuca sativa*	Leaf and stem
Spring onion	*Allium cepa*	Leaf and stem
Coriander	*Coriandrum sativum*	Leaf
Leek	*Allium ampeloprasum*	Leaf
Radish	*Raphanus sativus*	Root
Parsley	*Petroselinum crispum*	Leaf

To cite this paper: Fattahi Bafghi A, Yavari MR, Mirzaei F, Siyadatpanah A, Mitsuwan W, Pereira ML, Nissapatorn V, Norouzi R, and Hosseini SA (2020). Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran. *World Vet. J.*, 10 (3): 260-266. DOI: https://dx.doi.org/10.36380/scil.2020.wvj34

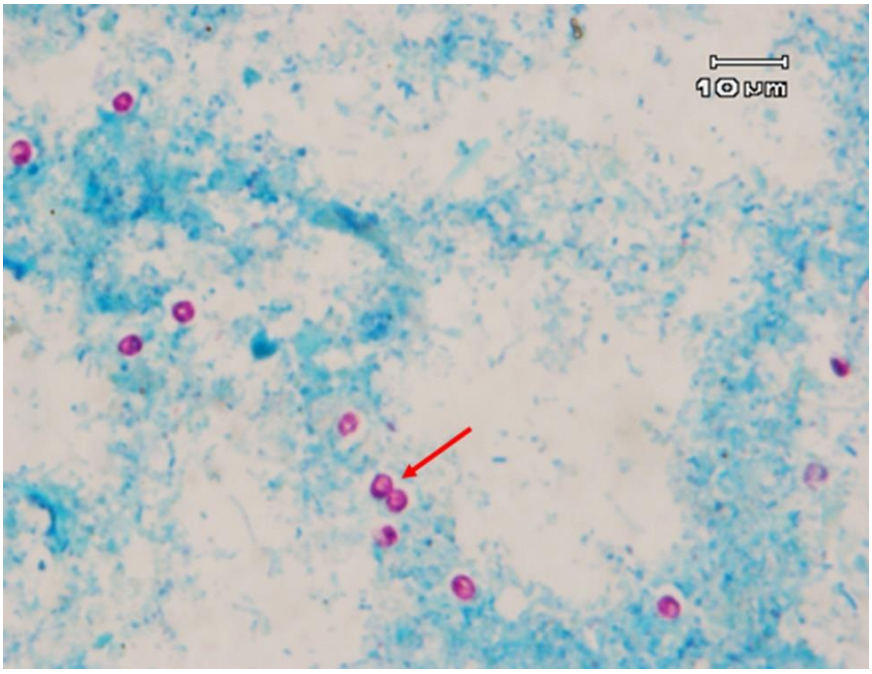

Figure 1. *Cryptosporidium* oocysts stained using the standard modified Ziehl-Nielsen technique. Oocysts appeared as bright rose-pink spherules (arrow).

RESULTS

Contamination of *Cryptosporidium* oocysts in the different vegetables

The oocysts appeared as bright rose-pink spherules with the size of 6-10 µm (Figure 1). Of the 275 samples of vegetables examined, 85 (31.5%) vegetables were positive and 190 (68.5%) were negative for the presence of *Cryptosporidium* oocysts (Figure 2). A total of nine varieties of vegetables were examined, and all were positive for *Cryptosporidium* oocysts. Of these, lettuce had the highest contamination rate (n: 16, 47.1%), followed by radish (n: 14, 46.7%), spring onion (n: 11, 35.5%), leeks (n: 9, 30%), mint and coriander (n: 8, 26.7%), tarragon (n: 7, 23.3%) basil and parsley (n: 6, 20%). There was a statistical significance between the occurrence of *Cryptosporidium* oocysts and types of vegetables examined ($p<0.05$) (Table 2).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to the marketplace

The occurrence of *Cryptosporidium* oocysts in the different vegetable fields showed that Amir Abad area had the highest contamination rate (n: 16, 59.3%), followed by Ghiam square (n: 14, 51.9%), Imam Shahr street (n: 12, 44.4%), Imam Khomeini Avenue (n: 9, 33.3%), Silage (n: 7, 25.9%), Ayatollah Kashani Avenue, Shahid Sadoughi boulevard, Homafar square (n: 6, 2.2%), and Bahaman Hospital area (n: 5, 18.5%) (Figure 3). The results further demonstrated that fresh vegetables from the area of Amir Abad had significantly the highest rate of contamination with *Cryptosporidium* oocysts (OR: 8.36; 95% confidence interval (CI) in OR: 2.25 < OR < 31; p: 0.001), followed by Ghiam square, and Imam Shahr. The lowest contamination was found in the Bahaman hospital area (OR: 1.3; 95% CI on OR: 0.3 < OR< 5.5; p: 0.7), although no statistical significant was found between the association (Table 3).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to the plant parts

The root vegetables had the highest contamination rate (n: 41, 45.6%), followed by leafy vegetables (n: 44, 24.4%). There was a significant association (OR: 2.58; 95% CI in OR: 1.5 < OR < 4.4; p: 0.001) between the occurrence of *Cryptosporidium* oocysts and the way in vegetables were consumed (Fisher's Exact Test; p: 0.000). There was a statistical significant (p=0.000) between the occurrence of *Cryptosporidium* oocysts and the nature of examined vegetables (Figure 4).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to types of vegetables consumption

The present study showed that 108 (39.27%) out of 275 consumed raw vegetables and 167 (60.73%) of those cooked before consuming were positive for *Cryptosporidium* oocysts (Figure 5).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to types of water uses

The present study showed that 40% of retailers had a direct wholesale from vegetables fields (more in the center), while 60% were from middlemen who got vegetables from farms and sold to retailers (more uptown) before being sold to consumers. In addition, the water used in the vegetable farm was from different sources: 40% came from the farmland, 30% from the nearby stream water, and 30% from the tap water. Water is always sprinkled on growing vegetables to maintain freshness, and to attract more consumers. The water, therefore, seemed to expose unhygienic conditions of reuse and containers (data were not shown).

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Table 2. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the types of vegetables

Vegetable	Odd ratio	CI*	P value
Mint	1.455	(.435 4.860)	0.543
Tarragon	1.217	(.355 4.170)	0.754
Lettuce	4.571	(1.452 14.389)	0.009
Spring onion	2.316	(.724 7.407)	0.157
Coriander	1.455	(.435 4.860)	0.543
Leek	1.714	(.523 5.621)	0.374
Radish	3.500	(1.112 11.017)	0.032
Parsley	1.000	(.282 3.544)	1.000

*Confidence Interval. There was a statistical significance between the occurrence of *Cryptosporidium* oocysts and types of vegetables examined (X2: 15.487a, DF: 8; p: 0.049).

Table 3. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the location of vegetables fields

Vegetable field location	Odd ratio	CI*	P value
Amir Abad	8.36	(2.25 31)	0.001
Ayatollah Kashani	1.64	(0.4 6.6)	0.48
Silage	2.01	(0.5 7.8)	0.31
Shahid Sadoughi Blv	1.64	(0.4 6.6)	0.4
Bahaman hospital area	1.3	(0.3 5.5)	0.7
Ghiam square	6.1	(1.6 22.7)	0.006
Imam Shahr	4.6	(1.2 16.9)	0.02
Imam Khomeini Avenue	2.8	(0.7 10.8)	0.1
Homafar Square	1.64	(0.4 6.6)	0.4

*Confidence Interval

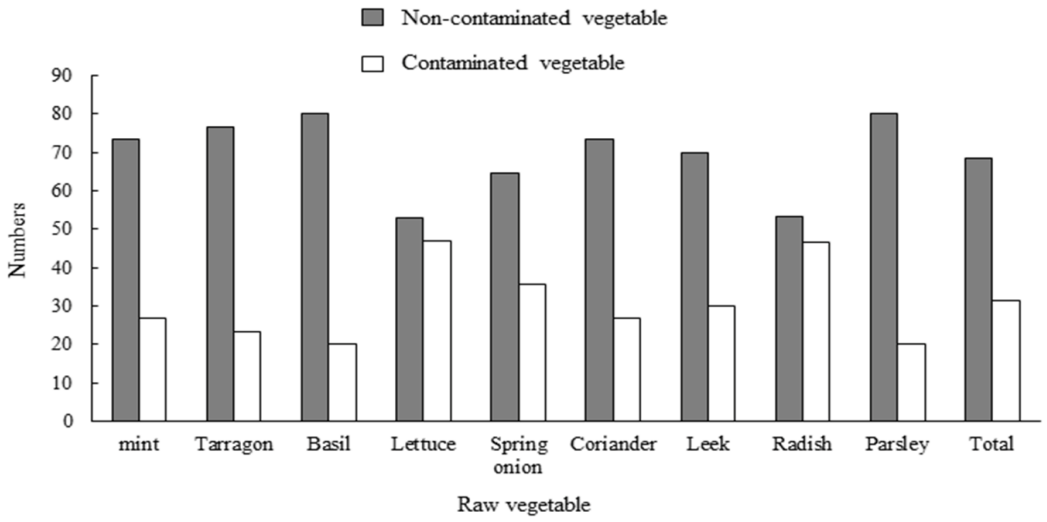

Figure 2. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the types of vegetables

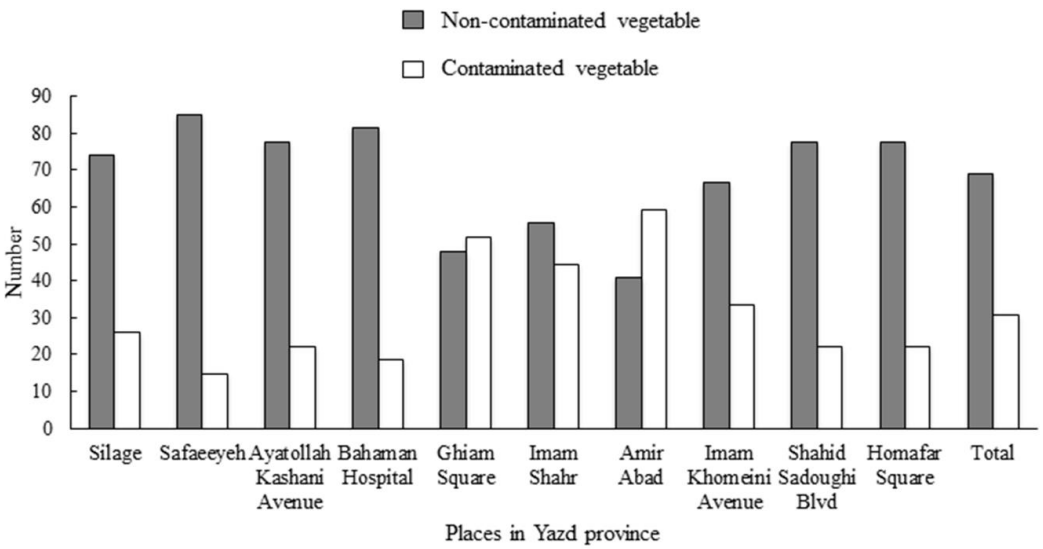

Figure 3. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the marketplace

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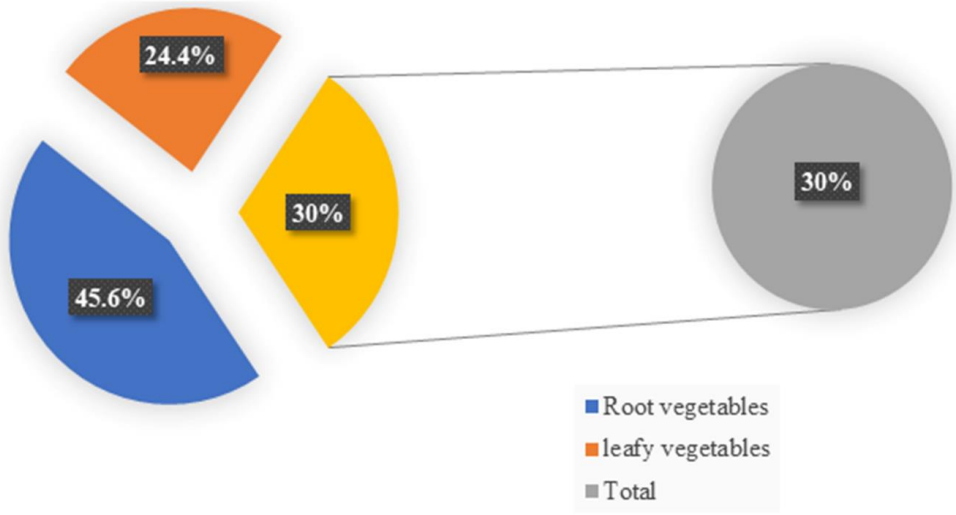

Figure 4. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the plant parts.

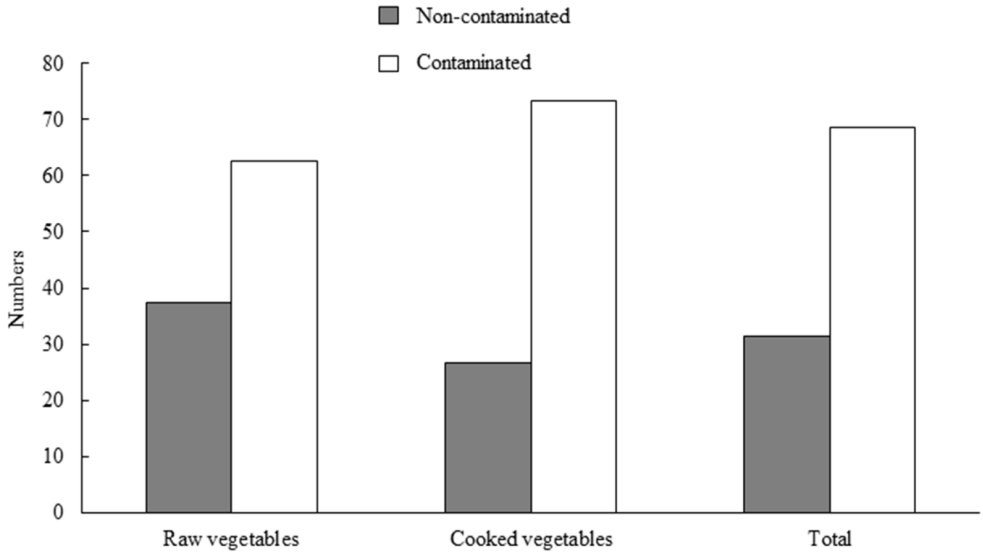

Figure 5. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the types of vegetable consumption

DISCUSSION

Yazd is the driest and the warmest major city in Iran, located in the north of the Persian Gulf coast. A general practice of "Save to Safe Water" is commonly seen among people living in the city of Yazd due mainly to a shortage of water supply. This economic condition of water use, therefore, plays a critical role in water consumption, and can increase the feasibility of contamination in food, including the consumption of vegetables in general. In the current study, the occurrence of *Cryptosporidium* oocysts was high in vegetable farms due to the dry and hot climate in the city of Yazd. This also indicates the lack of hygiene in personal behavioral practices of food and water consumption such as the use of unclean water for washing vegetables. In the fruit and vegetable fields, wholesalers regularly wash vegetables which sprinkle vegetables using the raw water from natural sources to prevent the fading of vegetables (Abedi et al., 2014).

Cryptosporidium is a cosmopolitan protozoan parasite, and water is a significant vehicle for the transmission of this intestinal pathogen. The potential of this parasite for waterborne transmission is further enhanced by the perpetual infectivity of the oocysts, small size (3.5 to 6.0 mM), and low precipitation rate (0.5 mM/s) (Fletcher et al., 2012; Ryan et al., 2014). The surface water is more likely to be contaminated with human and/or animal feces in the environment. It can also be contaminated by feces entering the agricultural run-off of adjacent farm animals or from human sewage (Ryan et al., 2014; Widerström et al., 2014). The proximity of the vegetables to the soil may also played a key role in the contamination of the plants with *Cryptosporidium* oocysts, since the vegetables were kept in an unhygienic condition. Moreover, the reinforcement of horticultural crops, such as vegetables with cattle and sheep dung (dried fecal matters)

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(Rossle and Latif, 2013) with viable oocysts of *Cryptosporidium* represented a significant risk for contamination of vegetables (Snelling et al., 2007; Kinyua et al., 2016).

A high occurrence of *Cryptosporidium* oocysts in lettuce is possibly due to its flat leafy nature which may increase it, trapping a great number of oocysts from the contaminated sources. Contamination of lettuce with food toxins and foodborne pathogens can occur in fields resulting from the entry of microorganisms from non-potable water irrigation or fertilizers that still contains viable pathogens (Chau et al., 2014; Bintsis, 2018). Some foodborne pathogens associated with lettuce have been observed, and the ingestion of raw vegetables, such as salad, played a key role in the transmission of parasitic infections (Snelling et al., 2007). In the Amir Abad area, the vegetable field is well supported, more centrally located, and therefore this area can provide more vegetables for the wholesale and retail outlets to customers. However, the greater possibility of obtaining contaminated vegetables from Amir Abad fields was significantly observed. Furthermore, it has been found that this area increases activities that can trigger an easier introduction of infectious agents, including *Cryptosporidium*, into vegetables sold on the market. Consequently, there was a higher rate of recovery of *Cryptosporidium* oocysts in the vegetable field of Amir Abad compared to other places in the city of Yazd.

The findings of this study also suggest that a higher rate of contamination was found in the root part compared to the leafy vegetables. Results of the present study, however, contrary to a previous study, showed a higher rate of contamination in leafy vegetables (Maikai et al., 2013). The reason could be attributed to the nature of the contacted soil-root vegetables which appeared to retain more trapped pathogens than leafy vegetables. Besides, it is obvious that these vegetables were consumed raw or cooked (Snelling et al., 2007). Interestingly, the findings also showed contamination rates in vegetables that are generally eaten raw or cooked. Of these, radish, basil, parsley, and lettuce are normally eaten raw, while spring onion, mint, leek, tarragon and coriander are also eaten raw as well as cooked. Although there was a higher rate of contamination in vegetables normally cooked before consuming, all vegetables should be washed sufficiently with clean/chlorinated water before consuming, whether they are eaten raw or cooked to decrease the chances of contamination with *Cryptosporidium* oocysts.

CONCLUSION

Based on this preliminary study, vegetables sold in Yazd city, were contaminated with *Cryptosporidium* oocysts. The study also indicated a higher occurrence of *Cryptosporidium* oocysts on the root part of vegetables than in the leafy ones. It was found that more densely populated and more supported facilities in the Amir Abad area were more widely contaminated with *Cryptosporidium* oocysts than the remaining vegetable fields in this city. As vegetables are an important part of a healthy diet, and can be consumed either raw or cooked, the findings of the current study raised great public health concerns. Moreover, it is mandatory for the higher authority to strictly and consistently monitor the level of contamination in all edible vegetables and water consumption with *Cryptosporidium* in a larger scale. More comprehensive studies on molecular and phylogenetic analyses are also strongly recommended to confirm the pathogenic strains of *Cryptosporidium* as a zoonotic transmission in this part of Iran.

DECLARATIONS

Acknowledgments

This study was supported by the Shahid Sadoughi University of Medical Sciences (GN: 1394.522-2276) for the cooperation and financial management. The authors would also like to thank our colleagues: Mrs. Marziyeh Beigom Modares Sanavi, Sayyedeh Zahra Hosseini and Zahra Sadeghi for their assistance in data collection. Maria de Lourdes Pereira thanks to project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 & UIDP/50011/2020.

Competing interests

The authors have not declared any conflict of interest.

Author`s contribution

Ali Fattahi Bafghi, Mohammad Reza Yavari, and Farzaneh Mirzaei designed the study. Farzaneh Mirzaei supervised the laboratory examinations. Abolghasem Siyadatpanah performed the experiments. Manuscript preparation and the statistical analysis were conducted by Watcharapong Mitsuwan, Maria de Lourdes Pereira, Veeranoot Nissapatorn, Roghayeh Norouzi, Seyed Abdollah Hosseini. All authors read and approved the final manuscript.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 267-275, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj35

Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against *Escherichia coli* in Minced Meat

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ORIGINAL ARTICLE
pii: S232245682000035-10
Received: 28 Jun 2020
Accepted: 07 Aug 2020

ABSTRACT

Antibacterial nanoparticles are a new approach to control the safety of meat and meat products. This work aimed to investigate the antibacterial effect of zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles, alone or together, against *Escherichia coli*. Antibacterial activity of these nanomaterials was evaluated using the disc diffusion method. In this regard, minced meat samples were inoculated with *E. coli* and treated with different concentrations of two nanomaterials (approximately 20 nm), including 6 mM and 12 mM ZnO, 6 mM and 12 mM TiO2, and a combination of 6 mM ZnO and 6 mM TiO2, then stored at 4°C for 17 days. The results indicated that ZnO (12 mM) had a significant reduction effect on *E. coli* count in minced meat, followed by the combination of ZnO and TiO2, and 12 mM TiO2 alone. The antibacterial activity of ZnO, TiO2, and combination of ZnO and TiO2 was also examined using a transmission electron microscope and it was found that 12Mm ZnO had a higher destructive effect on bacterial cell than the mixture of ZnO + TiO2, and 12Mm TiO2 alone. The disc diffusion method showed that ZnO (12 mM) was the most effective concentration used against *E. coli*. It is concluded that 12 mM ZnO nanoparticles have the best antibacterial effect against *E. coli* in minced meat stored at 4 °C for 17 days.

Keywords: *E. coli*, Minced meat, Nanoparticles, TEM, Titanium dioxide, Zinc oxide

INTRODUCTION

Increasing meat consumption throughout the world presents excessive challenges to meat safety and hygiene (Sofos and Geornaras, 2010). Meat product deterioration during distribution and exposure through the markets has negative effects on the meat industry from an economic point of view (Domínguez et al., 2018). Ground meat not only is highly susceptible to spoilage, but also is frequently involved in the spread of pathogens (Ahmed and Ismail, 2010) especially pathogenic strains of *E. coli* (Marcous et al., 2017), therefore, it is necessary to search for substances that are effective against bacteria especially *E. coli* and improve the microbiological condition of ground meat. Source tracking of *E. coli* is the main step to control foodborne infections (Liu et al., 2019). Great efforts are being made in the food industry for improving hygiene and increasing the shelf life of meat products through preventing the growth and multiplication of food-borne pathogens (Baltić et al., 2013).

Nanotechnology can provide the method that can be applied throughout different aspects of the food chain processing to improve food safety and quality control and increase food shelf life (Baltić et al., 2013). The most important nanomaterials that commonly used for antibacterial activity in the food industry are oxides of zinc (Zn) and titanium (Ti) (Duncan, 2011). Titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles are known to be one type of inorganic multifunctional substances that are able to inhibit the growth of microbes and they have been listed as Generally Recognized As Safe (GRAS) by the U.S. FDA (Zambrano-Zaragoza et al., 2018). These nanoparticles act as biocides and do not have any toxic effect and have approved by FDA for application in food processing fields (Toker et al., 2013). TiO2 is commonly used as a food additive and authorized for use in the European Union as E171 (Directive, 1994).

It is supposed that the toxicity of nanoparticles depends on their morphology, size, and amount consumed. Although possible risk to human health after eating food having TiO2 nanoparticles has been poorly explored, scientific databases inform that TiO2 nanoparticles can induce inflammation due to oxidative stress and also can have a genotoxic effect leading to chromosomal instability (Baranowska et al., 2020).

The antibacterial effect of TiO_2 and ZnO nanoparticles against *E. coli* O157:H7 in fresh calf minced meat have been investigated (Marcous et al., 2017). The antibacterial activity of TiO_2 nanoparticle against *E. coli* was investigated by Othman et al. (2014), who proved the importance of TiO2 nanoparticles for ensuring the safety of food.

To cite this paper: Abd El-Aziz M.A., Ibrahim H.M., EL-Roos NA, Anis B and Elsabagh R (2020). Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against *Escherichia coli* in Minced Meat. *World Vet. J.,* 10 (3): 267-275. DOI: https://dx.doi.org/10.36380/scil.2020.wvj35

One of the most important ways for applications of nanomaterials in food and meat is to place nanoparticles directly into food as food additives (Coles and Frewer, 2013) to preserve colors and prevent spoilage (Xie et al., 2011). Most previous studies link nanoparticles to external coating or packaging of meat but these methods are not appropriate for use in the minced meat industry. In addition, EFSA (2008) and Avella et al. (2005) supposed that migration of nanoparticle from packaging materials is either nil or very low, therefore, this study aimed to examine the antibacterial effect of ZnO and TiO2 nanoparticles, alone or together, directly mixed with minced meat. Also, the present study evaluated the *in vitro* antimicrobial effect of these nanomaterials against *E. coli.*

MATERIALS AND METHODS

The experiment was conducted in the Animal Health Research Institute, Egypt.

Minced beef

Fresh minced beef used in this study was purchased and immediately transported to the laboratory in an icebox and stored at 4 °C until use. Thin sheets of minced beef were treated with ultraviolet light (wavelength 385 nm) for 30 min, 15 min to each side to eliminate background microflora (Morsy et al., 2018).

Bacterial strain

Escherichia coli (ATCC® 25922™) ~ 8 log CFU/ ml was used in this study and obtained from Media Unit, Food Hygiene Department, Animal Health Research Institute, Dokki, Giza, Egypt.

Synthesis and preparation of zinc oxide nanoparticles

Zinc oxide nanoparticles were prepared by dissolving 11 g zinc acetate hydrate with 99.9% purity ($Zn(Ac)_2$•2H2O, Sigma-Aldrich) in 500 ml ethanol. Then, 2.9 g sodium hydroxide was added into the solution through ultra-sonication, and a transparent solution was obtained. The conical flask containing the transparent solution was put into a water tank with a constant temperature of 60 °C. After that, 10 ml of distilled water was added to the solution into the conical flask. The solution was stirred for 30 min at 60 °C. The prepared ZnO nanoparticles were collected by centrifuging and drying at 60 °C (Wang et al., 2007).

Synthesis and preparation of titanium dioxide nanoparticles

Titanium tetrachloride ($TiCl_4$) (Fluka 98%) was used as a starting material. TiO2 nanoparticles were prepared by dropwise addition of 4 ml of $TiCl_4$ into 400 ml of water/ethanol solution (3:1) at 0 °C with vigorous stirring. Subsequently, a dilute solution of NH_4OH was used to adjust the pH at 9. The solution was refluxed for 4 h with continues stirring. Then the solution was cooled down to room temperature naturally. The TiO2 nanoparticles were obtained by centrifuging at 4000 rpm. The formed TiO2 was washed using acetone several times and then dried at 100 °C for 5 h. The powder was annealed at 400 °C in air for 2 h by raising the temperature at a rate of 10 °C/min (Yin et al., 2001).

Assessment of *in vitro* antibacterial activity of nanomaterials using disc diffusion method

To assess the inhibitory range of nanoparticles of ZnO (2, 3, 5, 6, and 12mM) and TiO2 (3, 4, 5, 6, and 12mM) against *E. coli*, they were suspended in double-distilled water and constantly stirred until a uniform colloidal suspension was formed to yield solutions of different concentrations. An appropriate volume of test bacteria was inoculated on Mueller-Hinton agar medium. Sterile paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of suitable media plates freshly inoculated with bacterial cells, then 10µL from each dilution was dispensed onto the surface of each disc. Plates were then incubated for 24 h at 37 °C (Bauer et al., 1966).

Assessment of antibacterial activity of nanomaterials in minced meat

In a sterile bag, minced meat was inoculated with *E. coli* (~ 8 log CFU/ml) to achieve final concentration ~ 6 log CFU/g of minced meat. Then, they were mixed thoroughly by gently squeezing the bags by hand till even distribution of microbe occurred, and left for 30 min for complete attachment between inoculated *E. coli* and minced meat. The initial load of *E. coli* was determined before the addition of nanomaterials. Phosphate buffer saline (PBS) was used for the treatment of control samples. Minced meat sample was divided into six groups (200 g each); Group 1 (PBS + *E. coli*), Group 2 (6mM ZnO + *E. coli*), Group 3 (6mM TiO2 + *E. coli*), Group 4 (12mM ZnO + *E. coli)*, Group 5 (12mM TiO2 + *E. coli*), and Group 6 (6mM ZnO + 6mM TiO2 + *E. coli*). Nanomaterials were mixed with the minced beef samples for a further 30 seconds to ensure even mixing. All samples were transferred individually into a standard sterile polyethylene bag (self-closed). Packed samples were labeled and kept at 4 ± 1 °C till spoilage of minced meat. Counting of *E. coli* and sensory evaluation were performed on days 0, 3, 6, 9, 12, 15, and 17. The experiment was repeated in triplicate for each group and mean values were calculated.

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E. coli enumeration

Accurately, 100 µl from each previously prepared serial dilution was spread over duplicated plates of EMB agar (OXOID, CM0 069) using a sterile bent glass spreader. The inoculated and control plates were incubated at 37 °C for 24 h (FDA, 2001). The suspected colonies of *E. coli* were greenish metallic colonies with a dark purple center. These colonies were enumerated and expressed as log CFU/g of sample.

Electron microscopy observations

Transmission Electron Microscopy (TEM) techniques used to evaluate biocidal action of nanomaterials on *E. coli* using TEM Negative Staining method**.** JEOL JEM1400 transmission electron microscope was used (Yashroy, 1990). TEM was conducted in Cairo University Research Park, Egypt.

Sensory evaluation

Sensory evaluation was performed under the controlled condition of temperature (28 °c), humidity (65%), and light by five well-trained female panelists of 30 to 35 years of age, who were selected according to ISO (2012). The panelists were able to perform descriptive sensory analysis for treated samples and control one and give reliable comparative judgments. The criteria used as the basis of the organoleptic descriptive assessment and the samples were rated on a continuous hedonic scale (ISO, 2003). The panel received a list of descriptors (odor, color, and texture) to score on numerical and continuous scales from 0 (the lowest score for each attribute, very bad) to 10 (the highest score for each attribute, very good) according to Cullere et al. (2018). Every one of panelists took disposable dish containing three samples (two identical and another different) in triangle form randomly coded with four numbers and worksheet to give the score for each point. A mean score of lower than 5 indicated unacceptable quality. Totally, 210 samples (50 g) were examined on 7 sessions, 30 samples per session on five rounds per session at 1st day, 3rd day, 6th day, 9th day, 12th day, 15th day and 17th day.

Statistical analysis

The experiment was designed in completely randomized design in a 6×7 factorial design; 6 treatments (6 mM ZnO, 12 mM ZnO, 6 mM TiO2, 12 mM TiO2, 6 mM of ZnO + TiO2 and control one) during 7 sampling days (1st day, 3rd, 6th, 9th, 12th, 15th and 17th) at refrigerated storage (4±1 °C). The experimental model was made according to Butler et al. (2009). All data were subjected to analysis of variance (ANOVA) using SPSS program for Windows (Version 22) (SPSS Inc. Chicago, IL, USA). F-values at the $p \leq 0.05$ were indicated significantly different. Duncan's multiple range test was used for measuring the specific differences between pairs of means (Duncan, 1955). Values presented as the means ± standard error

RESULTS AND DISCUSSION

In vitro antibacterial activity of nanomaterials using disc diffusion method

As shown in table 1, zones of inhibition were differed according to the concentration of nanoparticles used. Results showed that 12 mM ZnO had the widest inhibition zone, followed by 12mM TiO2, 6 mM ZnO, and 6 mM TiO2. While 2mM ZnO and 3 mM TiO2 do not show any antibacterial effect. According to a study, 3 mM and 6 mM concentrations of ZnO nanoparticles resulted in less bacterial growth compared to the control, while the growth of *E. coli* O157:H7 was completely inhibited by 12 mM ZnO nanoparticles (Liu et al., 2009). The antibacterial action of ZnO nanoparticle was studied by Emami-Karvani and Chehrazi (2011) against Gram-negative bacteria (*E. coli*) were used as test microorganisms. It was found that the antibacterial activity of Zn O nanoparticles increased with decreasing particle size ~20 nanometers and increasing powder concentration 12 mM. These small concentrations were able to prevent the growth of *E. coli* on its media so we used it in our challenge study.

Table 1. Antibacterial activity assessment of nanoparticles against *E. coli* using disc diffusion method

Nanoparticle (concentration)	Zone of inhibition(mm)
ZnO (2mM)	ND*
ZnO (3mM)	6 ± 0.12
ZnO (5mM)	7.5 ± 0.11
ZnO (6mM)	10 ± 0.10
ZnO (12mM)	15 ± 0.14
TiO_2 (3mM)	ND*
TiO_2 (4mM)	6 ± 0.02
TiO_2 (5mM)	7 ± 0.025
TiO_2 (6mM)	8 ±0.22
TiO_2 (12mM)	13 ±0.11

ND*: Not detected

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Electron microscopy observations on nanoparticle against *E. coli*

Figure 1 shows normal *E. coli* with an intact cell membrane (control one). As shown in figure 2, ZnO had the largest effect on bacterial cell as it affects the cell wall of bacteria so it becomes irregular, perforated, swelling, and over enlargement. Figure 3 shows the accumulation of nanoparticles (ZnO and TiO2) around the bacterial surface and agglomeration of cytoplasmic material. These results were in agreement with the findings of Ashe (2011), who reported ZnO nanoparticles are bactericidal and disrupt membrane thus cause membrane dysfunction, resulting in leakage the content outside and cell death. There are components found in Gram-negative bacteria, and not in Gram-positives, which can oppose nanoparticles to attachment onto cell walls; the possible mechanism is the extra layer of outer membranes and the pathogen-associated molecular patterns which include lipopolysaccharide and particular fragments of peptidoglycan. Bacterial cell wall properties can play a crucial role in the diffusion of nanoparticles inside the bacterial cell (Espitia et al., 2012). Figure 4 shows the effect of TiO2 on *E.coli* was less affected by TiO2 compared to ZnO, which showed over swelling and thinning of cell wall. The antibacterial effect of TiO_2 on some bacteria such as *E. coli* has been studied (Mihaly et al., 2015).

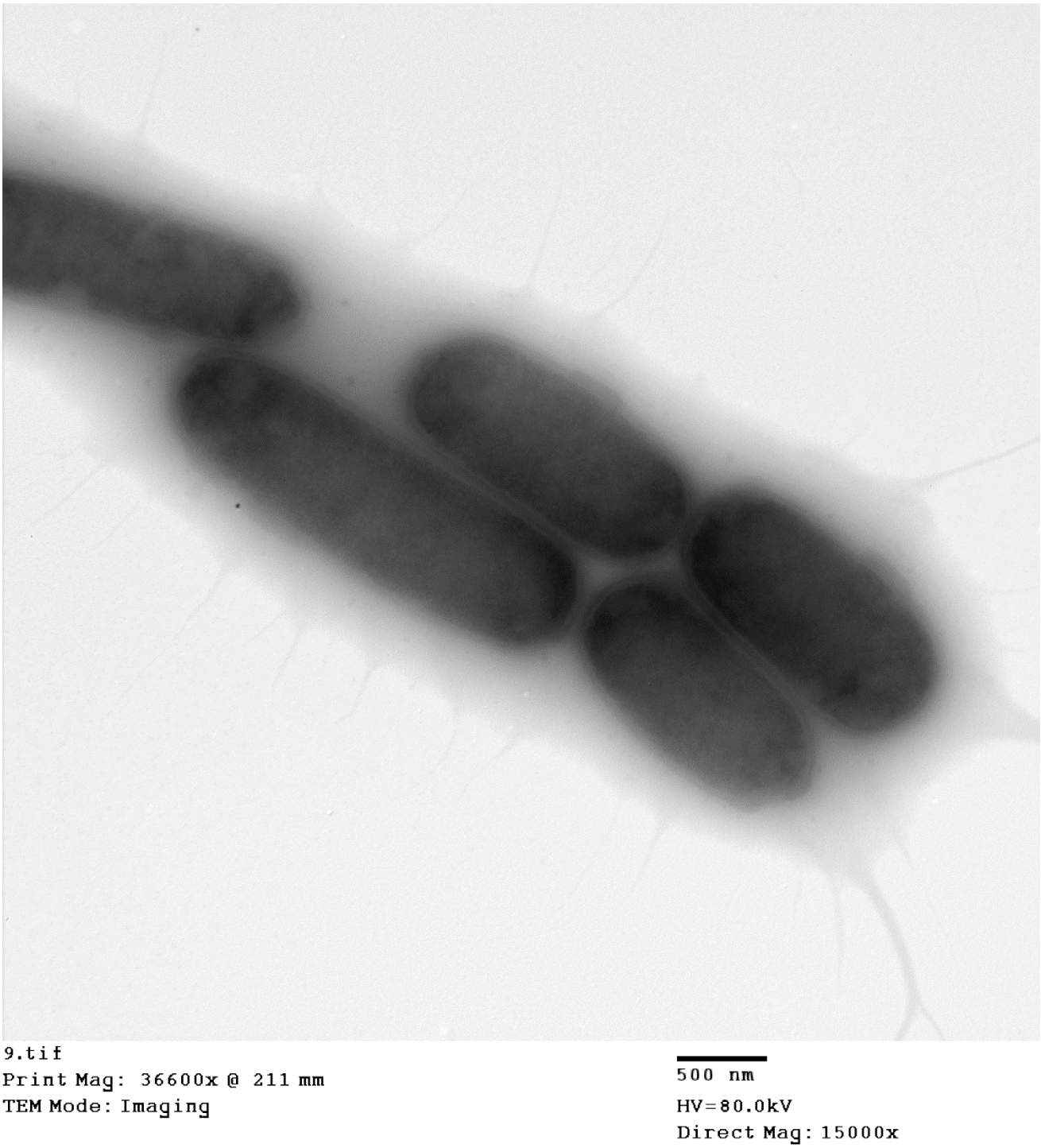

Figure 1. Normal morphology of *E. coli* using transmission electron microscope

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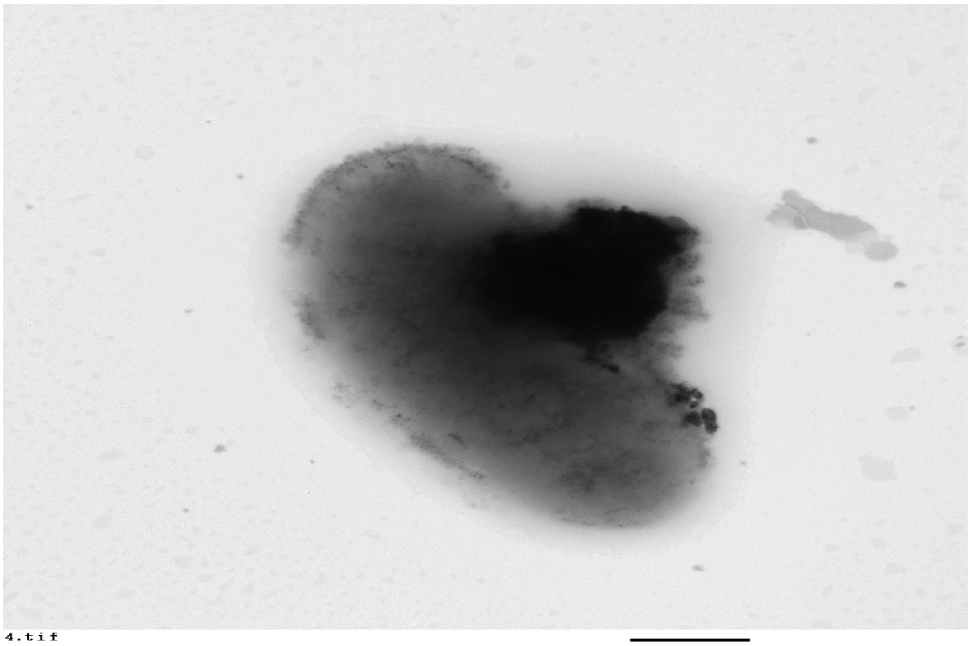

Figure 2. Antibacterial activity of ZnO nanoparticles against *E. coli* evaluated by transmission electron microscopy. ZnO nanoparticles adhered to *E. coli* cause pores in cell wall, elongation of cell, and ruptured cell.

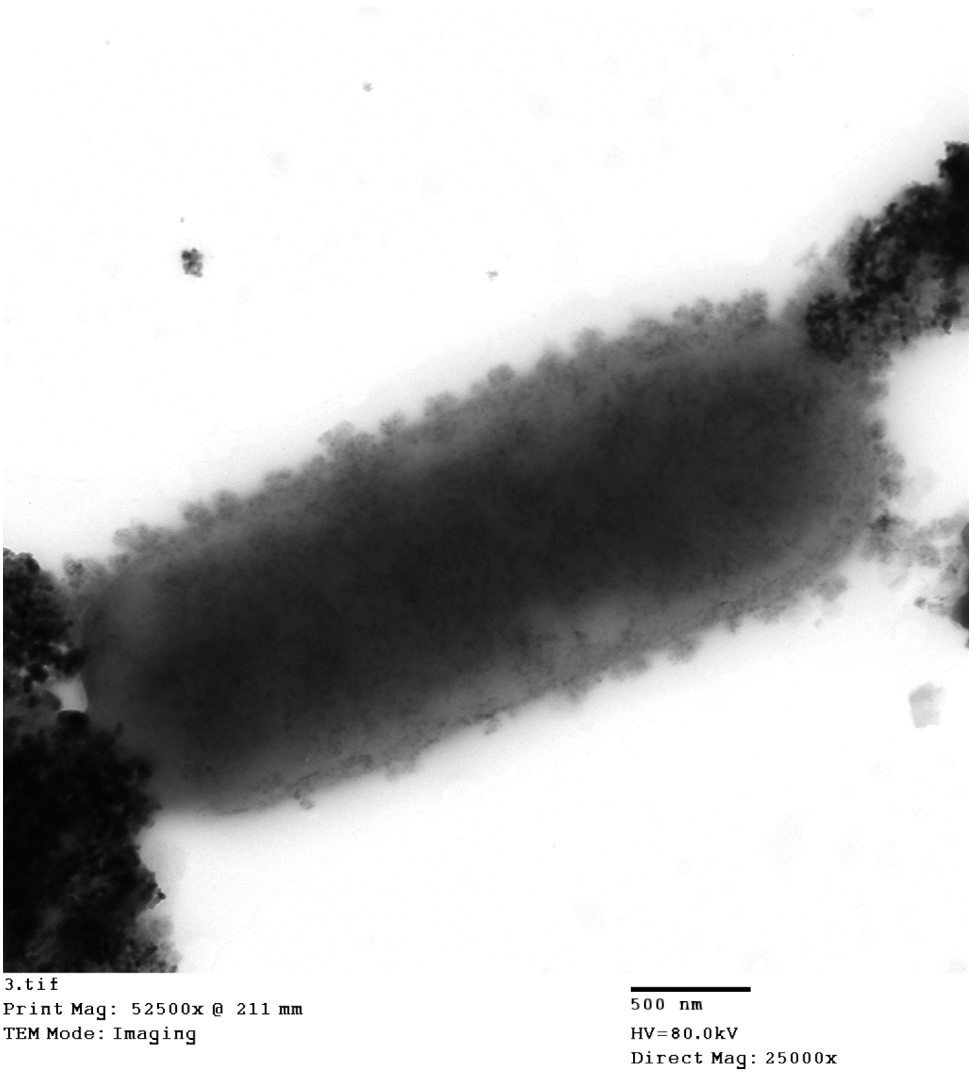

Figure 3. Antibacterial activity of nanoparticles mixture (ZnO + TiO2) against *E. coli* evaluated by transmission electron microscopy. Nanoparticles accumulated around the bacterial cell cause elongation of cell and disruption of cytoplasm.

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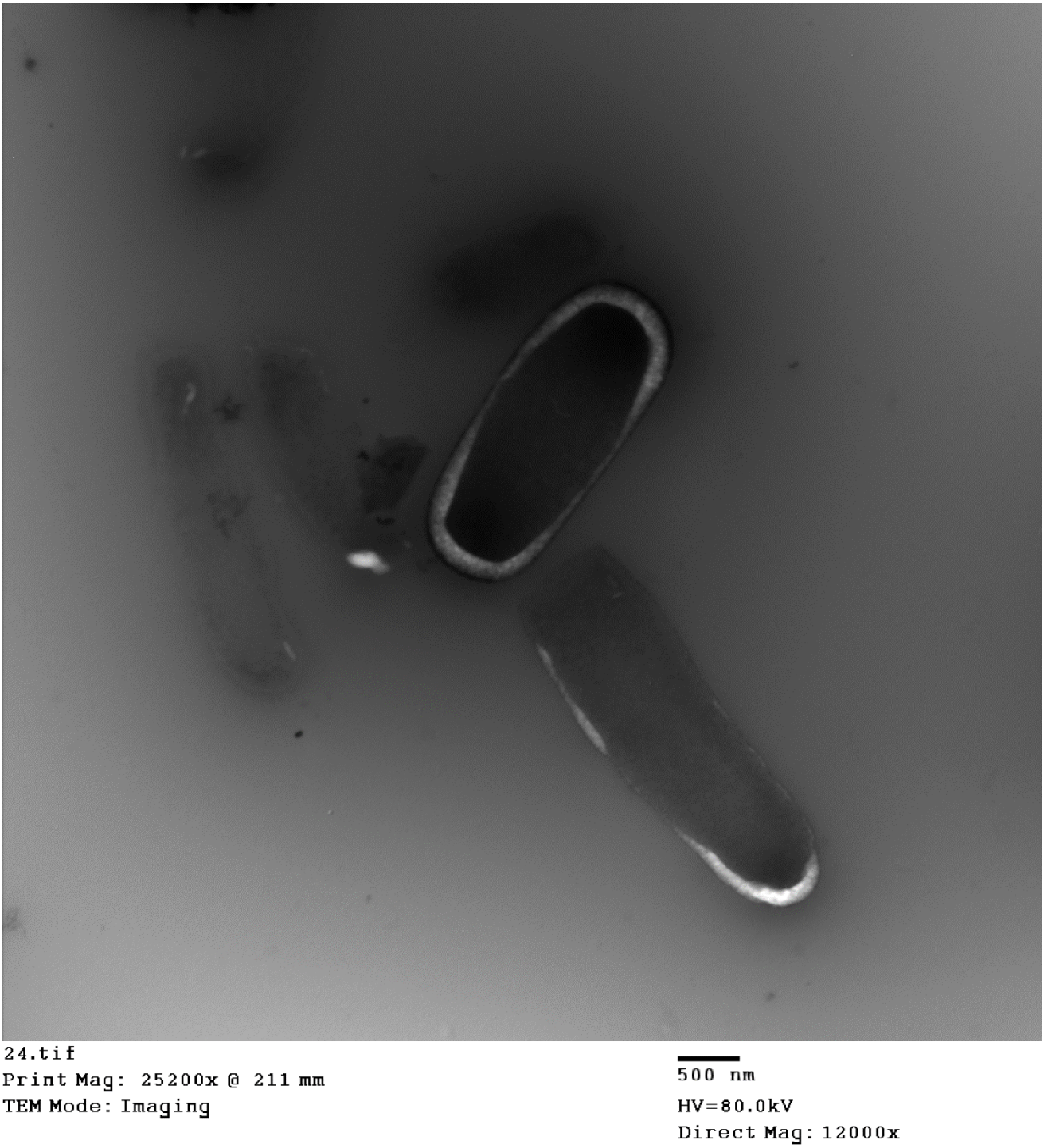

Figure 4. Antibacterial activity of TiO2 nanoparticles against *E. coli* evaluated by transmission electron microscopy. TiO2 caused elongation of cell, thinning of cell wall and pores in plasma membrane

***E. coli* count in minced meat stored at 4 °C**

The effect of nanoparticles on the count of inoculated *E. coli* in minced meat during storage at 4 °C is presented in table 2. *E. coli* counts increased in the control sample during the storage period of minced meat by ~ 2-4 log (CFU/g), which was significantly different from all treated samples. Count of *E. coli* in nanoparticles treated samples decreased throughout storage, indicating antibacterial activity of nanoparticles. ZnO (12 mM) exhibited great antibacterial effect against *E. coli* and it decreased the count by ~ 5 log (CFU/g) which was significantly different from the effects of obtained by the mixture of ZnO +TiO2 (6 mM) and TiO2 (12 mM) which decreased the count by ~ 4 log (CFU/g). Also, ZnO (6 mM) and TiO2 (6 mM) decreased the count by ~ 2log (CFU/g). These results are nearly similar to the findings of Marcous et al. (2017) who examined the antibacterial action of ZnO and TiO2, alone and together, against *E. coli* in calf minced meat and reported that ZnO was the most effective antimicrobial nanoparticles. ZnO nanoparticles are a novel material controlling foodborne pathogens, thus can be applied for food safety (Ali et al., 2020). Also, Morsy et al. (2018) studied the synergistic antimicrobial effect of ZnO nanoparticle and other compounds as nisin, lysozyme and EDTA nanoparticles on different foodborne pathogens including *E. coli* O157:H7 and proved that ZnO has great antimicrobial

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effect. As ZnO nanoparticles have great antibacterial activity, it has received significant interest worldwide particularly by the implementation of nanotechnology. Reduction of the particle size of ZnO nanoparticles leads to an increase in the particle surface reaction thus it exhibits great antibacterial activity (Sirelkhatim et al., 2015). Many studies investigated the effect of ZnO and TiO2 nanoparticles on *E. coli* at concentrations near concentrations used in this study but a very small diameter used in this study enhanced the effect of these nanoparticles. The concentrations of nanoparticles used in this study were less than the permissible limits approved by FDA (2015). ZnO and TiO2 nanoparticles are cheap antibacterial substances that have a wide range of antibacterial activity against microbes present in meat, therefore, they help to ensure the quality of meat, increase the shelf life for minced meat, and maintain the health of human.

Table 2. Antibacterial activity of different concentrations of ZnO and TiO2 nanoparticles against *E. coli* counts on minced beef inoculated with *E. coli* (~ 6 log CFU/g of minced meat) during storage at 4 °C for 17 days

Groups	1st day	3rd day	6th day	9th day	12th day	15th day	17th day
Control group	6.41 ± 0.26 ^{a}	6.28 ± 0.22 ^{a}	7.14 ± 0.1 ^{a}	8.64 ± 0.32 ^{a}	8.95 ± 0.6 ^{a}	8.99 ± 0.73 ^{a}	9.22 ± 0.14 ^{a}
12 mM ZnO	6.03 ± 0.6 ^{a}	5.38 ± 0.2 ^{c}	5.35 ± 0.1 ^{d}	4.78 ± 0.21 ^{c,d}	3.60 ± 0.2 ^{d}	1.65 ± 0.14 ^{c}	1.31 ± 0.9 ^{e}
6 mM ZnO + 6 mM TiO2	6.16 ± 0.7 ^{a}	5.55 ± 0.3 ^{c}	5.21 ± 0.1 ^{d}	4.33 ± 0.19 ^{d}	4.47 ± 0.3 ^{c}	2.11 ± 0.3 ^{c}	2.02 ± 0.10 ^{d}
12 mM TiO2	6.38 ± 0.8 ^{a}	5.63 ± 0.1 ^{b,c}	5.32 ± 0.2 ^{e}	4.90 ± 0.3 ^{b,c}	4.60 ± 0.23 ^{c}	3.02 ± 0.7 ^{d}	2.92 ± 0.10 ^{c}
6 Mm ZnO	6.40 ± 0.6 ^{a}	5.56 ± 0.09 ^{c}	5.43 ± 0.1 ^{c}	5.40 ± 0.6 ^{b}	4.71 ± 0.1 ^{b}	4.61 ± 0.68 ^{b}	4.30 ± 0.19 ^{b}
6 mM TiO2	6.40 ± 0.9 ^{a}	5.93 ± 0.1 ^{b}	5.51 ± 0.1 ^{b}	5.14 ± 0.5 ^{b}	4.94 ± 0.3 ^{b}	4.85 ± 0.73 ^{b}	4.27 ± 0.24 ^{b}

The values are expressed as Mean ± standard error of three experiments. Means within a column and rows followed by different letters are significantly different ($p \leq 0.05$).

Sensory evaluation

The chemical stability of meat during storage affects the sensory parameters. In general, sensory parameters are considered the most important factors in using any antibacterial nanoparticles. The effect of nanoparticles on overall acceptability (odor, color, and texture) of minced meat during refrigerated storage at 4 °C is presented in table 3. Sensory properties were satisfactory for all the samples on the initial day of the storage (1st day), however, they decreased during the storage period ($p \leq 0.05$). The results showed that all sensory attributes of control samples were acceptable by the 3rd day of the storage period and spoiled at 6th day; while treated samples were acceptable by the 15th day of storage for texture, color, and overall acceptability attributes, by the 12th day of storage for odor attribute. There was a significant difference ($p \leq 0.05$) for overall acceptability attribute between the treated and control samples on the days 3rd, 6th, 9th, 12th, 15th, and 17th of the storage time. The concentrations of 12 mM ZnO, 12mM TiO2, and the mixture of ZnO + TiO2 (6mm) enhanced shelf lifetime of minced meat and delayed its spoilage until 17th day, while minced meat treated with concentrations of 6mM ZnO and 6mM TiO2 spoiled on 15th day. Similarly, it is reported that the use of TiO2 delay spoilage of meat to 15 days (Alizadeh-Sani et al., 2020). Sensory evaluation of meat products allows researchers to evaluate how consumers perceive associated palatability. The reactions during lipid oxidation can lead to the formation of off-odors and off-flavors, texture, and color changes due to the myoglobin oxidation cause discoloration, which influences consumer's choice and acceptance. Aldehydes are important toxic compounds resulted during lipid oxidation cause organoleptic changes (Banerjee et al., 2017).

Table 3. Effects of different concentrations of ZnO and TiO2 nanoparticles on overall acceptability of minced meat during storage at 4 °C for 17 days.

Groups	1st day	3rd day	6th day	9th day	12th day	15th day	17th day
Control group	8.85 ± 0.08 ^{a}	5.66 ± 0.33 ^{b}	3.66 ± 0.06 ^{b}	3.10 ± 0.09 ^{c}	2.20 ± 0.06 ^{e}	1.50 ± 0.08 ^{e}	1.10 ± 0.10 ^{e}
12 mM ZnO	8.95 ± 0.03 ^{a}	8.88 ± 0.06 ^{a}	7.66 ± 0.33 ^{a}	7.16 ± 0.16 ^{a}	6.50 ± 0.29 ^{a}	5.66 ± 0.33 ^{a}	3.11 ± 0.06 ^{a}
6 mM ZnO + 6 mM TiO2	8.95 ± 0.03 ^{a}	8.71 ± 0.11 ^{a}	7.50 ± 0.29 ^{a}	7.13 ± 0.13 ^{a}	6.16 ± 0.16 ^{a}	5.73 ± 0.39 ^{a}	2.50 ± 0.06 ^{b}
12 mM TiO2	8.95 ± 0.03 ^{a}	8.56 ± 0.29 ^{a}	7.27 ± 0.30 ^{a}	7.13 ± 0.09 ^{b}	6.20 ± 0.15 ^{b}	5.46 ± 0.26 ^{a,b}	2.10 ± 0.06 ^{d}
6 Mm ZnO	9.25 ± 0.28 ^{a}	8.61 ± 0.06 ^{a}	7.10 ± 0.16 ^{a}	6.00 ± 0.48 ^{b}	5.26 ± 0.12 ^{c}	3.00 ± 0.06 ^{b}	2.00 ± 0.12 ^{c}
6 mM TiO2	8.95 ± 0.03 ^{a}	8.62 ± 0.12 ^{a}	7.10 ± 0.13 ^{a}	6.10 ± 0.06 ^{b}	5.23 ± 0.14 ^{c}	2.50 ± 0.06 ^{b}	2.00 ± 0.14 ^{c}

The values are expressed as Mean ± standard error of three experiments. Means within a column and rows followed by different letters are significantly different ($p \leq 0.05$).

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CONCLUSION

It is concluded that nanoparticles have an antimicrobial effect against *E. coli* and their effect is concentration-dependent. It is demonstrated that 12 mM ZnO nanoparticles had a greater antimicrobial effect against *E. coli* than the mixture of ZnO + TiO2 (6 mM), followed by 12 mM TiO2. It is found that these nanoparticles can prevent bacterial growth and enhance the shelf life of minced meat.

DECLARATIONS

Acknowledgments

The authors express their sincere gratitude to Animal Health Research Institute, Egypt for all the support provided, Cairo University Research Park (CURP), Egypt for using TEM and Spectroscopy Department, Physics Division, National Research Centre, Egypt for providing the nanoparticles.

Authors' contributions

Hemmat M. Ibrahim and Rasha Elsabagh designed the plan of study, revised the research article. Mohebat A. Abd El- Aziz, Rasha Elsabagh and Nahla Abo EL-Roos analyzed the data, performed laboratory experiments, and drafted the manuscript. Nahla Abo EL-Roos provided the experimental tools, revised the research article, Badawi Anis helped in the synthesis of nanoparticles and helped in the analysis of TEM pictures.

Competing interests

The authors declare no conflicts of interest.

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To cite this paper: Abd El-Aziz M.A., Ibrahim H.M., EL-Roos NA, Anis B and Elsabagh R (2020). Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against *Escherichia coli* in Minced Meat. *World Vet. J.,* 10 (3): 267-275. DOI: https://dx.doi.org/10.36380/scil.2020.wvj35

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To cite this paper: Abd El-Aziz M.A., Ibrahim H.M., EL-Roos NA, Anis B and Elsabagh R (2020). Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against *Escherichia coli* in Minced Meat. *World Vet. J.,* 10 (3): 267-275. DOI: https://dx.doi.org/10.36380/scil.2020.wvj35

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 276-285, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj36

Incidence and Prevalence of Hard Ticks in Ruminants of Al-Ahsa Oasis Region, Kingdom of Saudi Arabia

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ORIGINAL ARTICLE
pii: S232245682000036-10
Received: 01 Jul 2020
Accepted: 04 Aug 2020

ABSTRACT

In the present study, a number of camels, cattle, sheep, and goat herds have been examined and followed up to record the incidence of tick infestation in Al-Ahsa Oasis in the Eastern Region of the Kingdom of Saudi Arabia. From 24 herds distributed in eleven localities, a total of 4068 animals (123 camels, 60 cattle, 1780 sheep, and 2105 goats) were individually examined every second week during the period from January to December 2010. In total, 5320 ticks were collected from 1125 infested animals (27.65%). The overall prevalence rate of identified Ixodide ticks was: *Hyalomma anatolicum excavatum* (18.33%), *Hyalomma dromedarii* (17.63%), *Hyalomma anatolicum anatolicum* (14.29%), *Rhipicephalus turanicus* (14.04%), *Hyalomma impeltatum* (11.28%), *Rhipicephalus praetextatus* (8.56%), *Hyalomma marginatum turanicum* (6.20%), *Haemaphysalis sulcata* (3.57%), *Rhipicephalus kohlsi* (2.33%), *Hyalomma marginatum rufipes* (2.09%), *Hyalomma schulzei* (1.03%), *Amblyomma variegatum* (0.47%), and *Amblyomma gemma* (0.18%). Tick infestation in terms of mean intensity, abundance, and prevalence rates was highest in camels (41 tick/infested camel, 30 ticks/camel, and 73.17%, respectively). This was followed by cattle (15 tick/infested cow, 5 ticks/cow, and 33.33%, respectively), goats (1.35 tick/infested goat, 0.33 tick/goat, and 23.52%, respectively), and sheep (1.27 tick/infested sheep, 0.3 tick/sheep, and 29.21%, respectively). The incidence rate of ticks significantly increased during the warmest summer months of the year (highest recorded temperature ranged between 47℃ and 50℃), mainly due to the management practices of farmers. During this period animals were housed and water was supplied *ad libitum* during hot months, thereby increasing animal density and humidity in the shaded farms. According to the obtained results of the current study, tick control can be started in Al-Ahsa area in early May for all animals with the focus on camels as they are the harbor of the tick biomass, or on goats as they had initially increased in incidence rates.

Keywords: Kingdom of Saudi Arabia, Prevalence, Ruminants, Ticks

INTRODUCTION

Hard ticks of the family Ixodidae include more than 700 species (Guglielmone et al., 2014), most of which are considered highly specialized and obligate blood-sucking vectors that transmit pathogens to human and animals (Jongejan and Uilenberg, 2004; Karim et al., 2017). Among such pathogens are protozoan species of veterinary importance, for example *Theileria* spp., *Babesia* spp. and *Anaplasma* spp., which affect red and white blood cells, leading to severe damages to the infected hosts (Friedhoff, 1997; Alessandra and Santo, 2012). Furthermore, viral tick-borne infections can be zoonotic with grave consequences, and worst of all is the emerging Crimean-Congo Hemorrhagic Fever (CCHF) (Tonbak et al., 2006). CCHF virus can be transmitted via hard ticks of *Hyalomma* (Dohm et al., 2004) and *Rhipicephalus* species from animals to humans (Aktas et al., 2012; Bartikova et al., 2017). Both of these ticks were reported from Saudi Arabia (El-Azazy et al., 1997; El-Azazy et al., 2001; Al-Khalifa et al., 2007).

Al-Ahsa oasis is located in the Eastern region of the Kingdom of Saudi Arabia. It has been inhabited by humans for more than 7000 years, mainly as an agricultural heaven in the middle of the desert. It has the world largest palm gardens along with a plethora of agricultural corps and domesticated animals. Camels are dominant in this area, but local breeds of cattle, sheep and goats are also indigenous. In this region, the culture of farming in many places has somewhere lost momentum of developing and adhering to the modern methods of production and health control (Abdallah and Faye, 2013). This situation has created a rare model to study the tick fauna of domesticated animals in this area, and thereby presenting a snapshot of the situation that has been there in many areas in the world but then one century ago.

The present study aimed to explore the hard tick species richness in this oasis and the dynamics of the incidence of ticks in animals. Most of the domesticated animals in the study area did not receive proper acaricide treatment, and planning tick control strategies here aims to reduce risk of outbreak of tick-borne infections. Such outbreak, if occurs,

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might have deep health consequences in a dynamic city that houses hundreds of animal farms within a total population of around one million (https://www.alhasa.gov.sa/SitePages/Home.aspx). Previously, limited survey has reported the presence of some tick species in a small animal sample (Hoogstraal et al., 1981). Therefore, the present study will follow up a number of camels, cattle, sheep and goat herds for a whole year to record the tick species and to establish background information on dynamics of tick infestations for later designing of mitigation plans.

MATERIALS AND METHODS

Ethical approval

This research has been conducted according to the national guidelines for research ethics on living organisms (Kingdom of Saudi Arabia, Act No. 321).

Areas of study

Al-Ahsa Oasis is about 160 Km south of Dammam, the capital of the Eastern Region of Saudi Arabia (Figure 1). Geographically, the sampled areas are located between 49°10′E to 49°50′E and 25°25′ N to 25°40′ N, and about 130-160 m above sea level. The weather in Al-Ahsa oasis is typical oasis, in which two major lakes are located within an arid desert area, and many springs and wells are found in scattered agricultural farms. Meteorological data on relative humidity and temperature in Al-Ahsa oasis are provided by the Saudi General Authority of Meteorology and Environmental Protection (www.pme.gov.sa; temperature data are summarized in figure 2). Rainfall in that area is scares. In fact, in the year 2010 it rained for a whole week in February, otherwise intermittent showers of rain were experienced a few days during September and November.

Sampled herds

Location of the farms and number of animals sampled are summarized in table 1 and figure 1. Within Al-Ahsa oasis, 24 herds in farms were included in this study according to the availability of the animals and willingness to cooperate with the researchers. Farming in this area undergoes two different practices depending on the season. The first method was permanent housing, in which animals were kept indoors and fed *ad libitum* during hot months of the year (from early May to end of September). In the second method of farming practice in this area, herds were "semi-nomad" and were grazing is practiced in the open fields around the owners' tents or at a maximum distance of 10km away to portable houses during moderate and cold months of the year (from October to April). Camel, sheep and goat herds undergo these two practices yearly, but cattle herds are always kept indoors throughout the year. Herd size in sheep farms had a range of 60 to 500 animals per farm, all of which were Naimi breed. Goat farms had a range of 30 to 600 animals per farm of Shami or Aardhiat breeds. Cattle farms had 3 to 25 animals of a local breed. Camel farms had 30 to 80 animals that all were Arabian breed. Derived by the market demands, animals in sheep and goat farms were constantly replaced with new individuals, unlike animals in camel and cattle farms. Accordingly, an incidence and prevalence rate presented in this study represents farm-level indices.

Sampling methodology

Farms were regularly visited every second week during the period starting from January 2010 until the end of December 2010. Approximately 10% of all animals in any farm were subject to examination. Upon animal examination, all present ticks were removed from the animals using tweezers and rubber gloves by following instructions of Hoogstraal et al. (1981) and Abdally (2008). The locations of the collected ticks were marked, and they were stored in 70% ethyl alcohol. In some cases where alcohol was not available fresh tick samples were kept in plain containers and transported to the examination lab, where they were kept in a freezer (-20℃) for a short period (up to 30 minutes) to stabilize the tick before morphological examination. From each examined animal the ticks were removed and counted. Tick identification was done according to the keys of Hoogstraal et al. (1981) and Walker et al. (2003). Due to logistic constrains, only adult female ticks were diagnosed to the species level and included in this study. As well, it was not possible to collect information on age and sex of the examined animals in all occasions, therefore such data was not included in the statistical analysis.

Statistical analysis

Collected data were transformed to templates in Microsoft Excel software to produce summary and descriptive statistics. The incidence rate of tick infestation was employed here as the percentage of infested animals at a given time interval within the year. The overall prevalence rate of tick infestation was employed here as the percentage of animals infested with ticks during the whole period of the study. The mean intensity rate employed here was calculated as the total number of collected ticks divided by the number of infested animals, while the mean abundance rate was calculated as the total number of collected ticks divided by the total number of examined animals. Seasons

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were defined as winter: December-February, spring: March-May, summer: June-August, and autumn: September-November. To explore the seasonal differences in the incidence of ticks, Chi-suqare test was employed, where observed integers were the cumulative number of found ticks of all species in all animal types, and the expected percentages were set as 25% for each season. Depending on sample size, Fisher's exact text or Student's T-test were employed to explore differences within the study populations. Using any statistics, the results were considered significant at p values < 0.05.

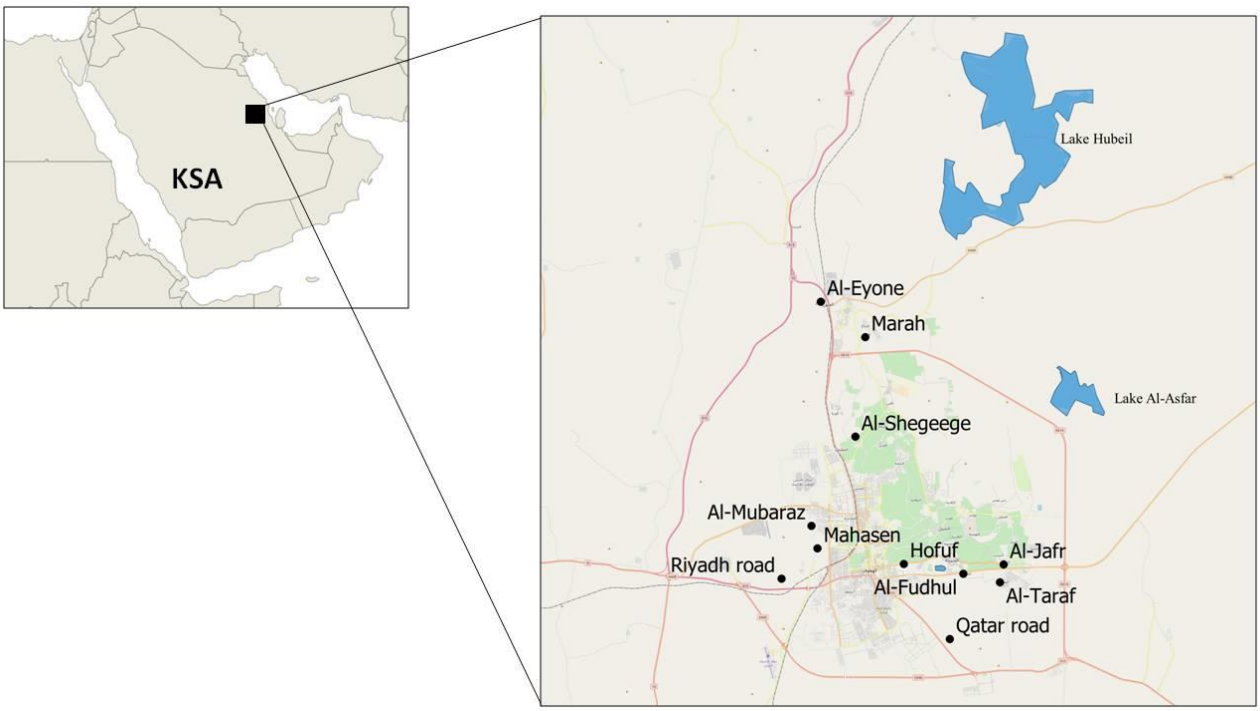

Figure 1. Map of the Kingdom of Saudi Arabia (KSA), showing the 11 areas in Al-Ahsa oasis (black circle) where animal sampling took place from January to December 2010.

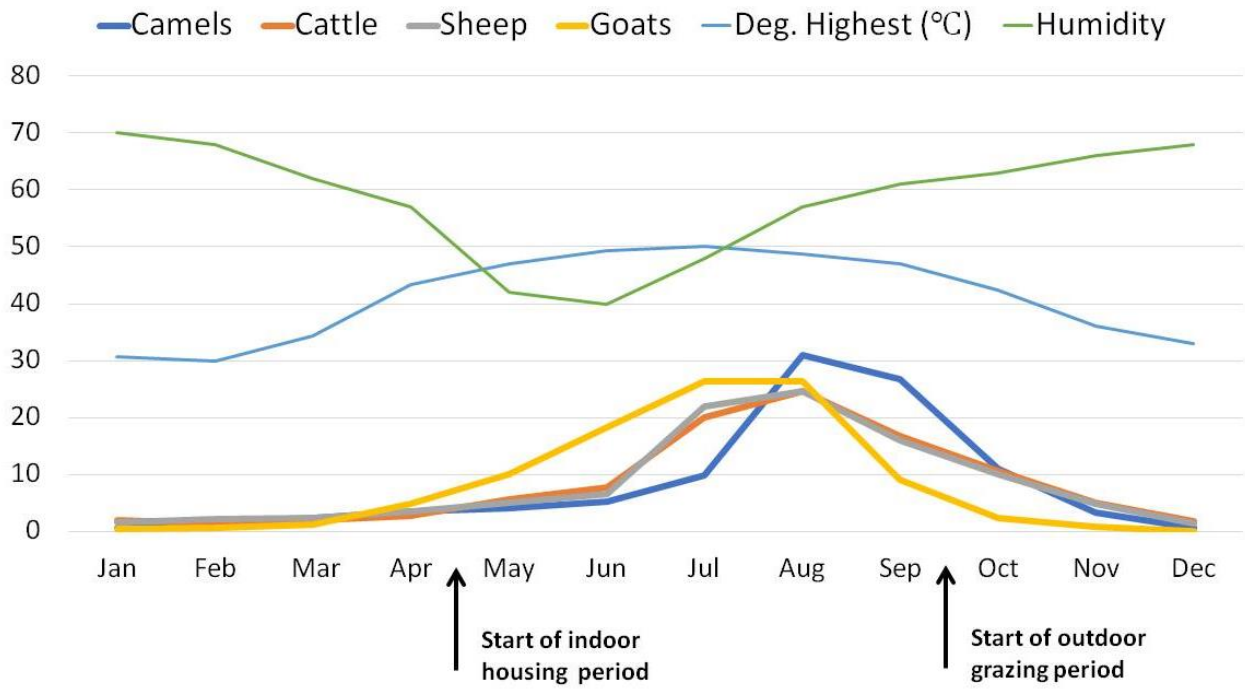

Figure 2. Seasonal variations in incidence of recovered hard ticks from camels, cattle, sheep and goats examined in 2010 in Al-Ahsa oasis, Kingdom of Saudi Arabia.

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Table 1. Number of animals examined and incidence rates of hard ticks found in domesticated ruminant herds in 11 localities in Al-Ahsa oasis, Kingdom of Saudi Arabia, during the period from January 2010 to December 2010

Examined Animals / Locality	Camel		Sheep		Goats		Cattle		Total
	Examined	Infested (%)	Examined	Infested (%)	Examined	Infested (%)	Examined	Infested (%)	
Al-Hufof	5	5 (100%)	80	20 (25%)	40	13 (30.23%)	6	1 (16.67%)	134
Al-Mubaraz	5	5 (100%)	80	30 (37.5%)	40	20 (50%)	4	1 (25%)	129
Mahasen	-	-	70	30 (42.86%)	95	35 (36.84%)	-	-	165
Ryiadh Rd *	32	22 (68.75%)	490	120 (24.49%)	320	120 (37.5%)	-	-	842
Qatar Rd.*	22	10 (45.45%)	430	130 (30.23%)	530	130 (24.53%)	25	8 (32%)	1007
Al-Oyone	13	8 (61.54%)	90	40 (44.44%)	180	30 (16.67%)	5	3 (60%)	288
AL-Marah	13	10 (76.92%)	75	25 (33.33%)	225	25 (11.11%)	4	2 (50%)	317
Al-Shegeege	7	7 (100%)	58	18 (31.03%)	150	30 (20%)	4	1 (25%)	219
Al-Fudhule	3	3 (100%)	93	23 (24.73%)	100	20 (20%)	5	2 (40%)	201
Al-Jafr	18	15 (83.33%)	104	24 (23.08%)	131	31 (23.66%)	4	1 (25%)	257
Al-Taraf	5	5 (100%)	210	60 (28.57%)	291	41 (14.09%)	3	1 (33.33%)	509
Total	123	90 (73.17%)	1780	520 (29.21%)	2105	495 (23.52%)	60	20 (33.33%)	4068

Rd.*: road; herd farms located in vicinity of the main road.

RESULTS

A total of 4068 animals were examined (Table 1), among which 1125 animals were infested with hard ticks, giving an overall prevalence rate of 27.65%. The highest prevalence of ticks was found in camels (χ^2 = 65.5031, the p-value is < 0.0p1), in which 90 of the examined 123 camels (73.17%) were found infested with at least one tick during this one-year cohort study. Camels had also the highest mean intensity rate of tick infestations; a total of 3690 ticks were recovered from 90 camels (69.36% of all collected ticks in this study), giving a mean intensity of 41 tick/infested camel, and mean abundance rate of 30 ticks/camel. Cattle were second in mean intensity and abundance rates; 15 tick/infested cow and 5 ticks/ cow, respectively, and had an overall prevalence rate of 33.33%. In goats the mean intensity rate was 1.35 tick/infested goat, and the mean abundance rate was 0.33 tick /goat, while the overall prevalence rate was 23.52%. Similarly, sheep had intensity and abundance rates of 1.27 tick/infested sheep and 0.3 tick/sheep, respectively. However, the overall prevalence rate of ticks in sheep was 29.21%.

In this study, a total of 5320 ticks were collected (Table 2), among which 13 species belonging to four genera were identified as follows in a descending order of prevalence rates: *Hyalomma anatolicum excavatum* (18.33%), *Hyalomma dromedarii* (17.63%), *Hyalomma anatolicum anatolicum* (14.29%), *Rhipicephalus turanicus* (14.04%), *Hyalomma impeltatum* (11.28%), *Rhipicephalus praetextatus* (8.56%), *Hyalomma marginatum turanicum* (6.20%), *Haemaphysalis sulcata* (3.57%), *Rhipicephalus kohlsi* (2.33%), *Hyalomma margin rufipes* (2.09%), *Hyalomma schulzei* (1.03%), *Amblyomma variegatum* (0.47%), and *Amblyomma gemma* (0.18%). Ticks of the genus *Hyalomma* were generally the most diverse and commonly found in all species of examined animals, followed by *Rhipicephalus* then *Haemaphysalis*, while ticks of the genus *Amblyomma* were the least found and were restricted to cattle.

Ticks that had the highest incidence rates were mostly collected from camels, albeit being found in other types of examined animals (Table 2). The least incident tick species were those found in one or two animal species. One tick species was found only in camels (*Hy. schulzei*), two tick species were only found in cattle (*A. variegatum* and *A. gemma*) and two tick species were only found in sheep and goats (*R. kohlsi* and *Haem. sulcata*).

The four types of examined domesticated animals had similar tick species richness; goats were found infested with eight tick species, camels, sheep, and cattle were infested with nine tick species (Table 2 and figure 3). Nonetheless, during the study period, at least one animal per farm was found infested with a hard tick regardless of the animal type, herd type and herd size; hence none of the included farms were tick-free. Furthermore, all animal herds were found infested with at least one tick species. All camels in herds that had three to seven animals (N=5) had prevalence rates of 100%. In larger size camel herds, the prevalence rates ranged from 45.45% to 83.33%. Prevalence of ticks in cattle herd ranged from 16.76% to 60%. All cattle farms except one had less than six animals in the herd. Prevalence level of ticks in sheep herd ranged from 24.49% to 44.44%. Prevalence level of ticks in goat herd ranged from 11.11% to 50%.

Ticks were most abundant in the warmest summer months of the year (Table 3; χ^2 = 2288.289, the *p*-value is < 0.01), where ticks of all species were found in June, July, and August with an overall incidence rates of 10.83%, 16.20%, and 24.59%, respectively. In these months, the highest recorded degrees ranged from 47℃ to 50℃, while the lowest temperatures ranged from 22.9℃ to 26.6℃, with no precipitation at all at any time during these months, with

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maximum humidity of 70% in January and slowly dropping to 40% in June. On the other hand, the incidence and abundance of ticks dropped during the colder winter season (Table 3 and figure 2), were only seven out of the total 13 tick species were found in January, February and December months, giving an overall abundance rates of 2.07%, 2.88% and 2.01%, respectively.

Animal species differed in their trend of harboring ticks during the year (Figure 2). The first animals that exhibited increase in incidence rates of hard ticks were goats, which showed gradual increase in incidence rate during May and peaked during August, then dropped sharply until October. The incidence rates of ticks showed almost identical trend in cattle and sheep, in which the rate started to rise sharply in June, peaked from July until August, and then dropped slowly towards December. Camels were the last animals to show increase in the incidence rates, which started to build up sharply in July, peaked in August, and then slowly dropped from September through October.

Tick species that had prevalence rates of more than 3% were observed throughout the year (Table 4), namely: *Hy. a. excavatum, Hy. dromedarii, Hy. a. anatolicum, R. turanicus, Hy.m. turanicum, R. praetextatus* and *Haem. sulcata*. An exception of the previous observation is *Hy. impeltatum*, which had a prevalence rate of 11.28%, but was not observed in any examined animal in December and January. The rest of the tick species could not be seen in the examined animals for at least two months during that year (*R. kohlsi, Hy. impelt,* and *Hy. m. rufi*). Some of the tick species were not found for five months in row (*Hy. schulzei*), six months (*A. variegatum*) and up to eight months in row during that year (*A. gemma*).

In camels, *Hy. dromedarii* was the most abundant tick species and accounted for 24.39% of all tick infestations (Figure 3), followed by *Hy. a. excavatum* (21.68%). Infestation with *Hy. a. anatolicum* in cattle accounted for 33.3% of all tick infestations, followed by *R. turanicus* (15.7%). However, infestation with *R. turanicus* accounted for 34.92% of all tick infestations in sheep and 45.71% of all tick infestations in goats. The second most important tick species infesting sheep was *Hy. a. excavatum* (22.22%), while in goats it was *Haem. sulcata* (17.14%).

All the observed ticks placed themselves in different parts of host body (Figure 4). None of the examined animals had ticks on places readily exposed to sun light, for example on the backs, on top of the head or on the back of the neck. The majority of the found ticks were collected from the necks (χ^2 = 41.074, the *p*-value is <0.01) of camels, goats and cattle (41%, 29%, and 26%, respectively). Astonishingly enough, the neck was the least chosen place on sheep for tick to take its blood from (10% of all found ticks). Generally, 35.90% (1910) of all collected ticks were found in the necks, followed by chest (23.12% of all collected ticks), legs (19.17%), on udders (10.53%), on ears (5.83%), and least were under the tail.

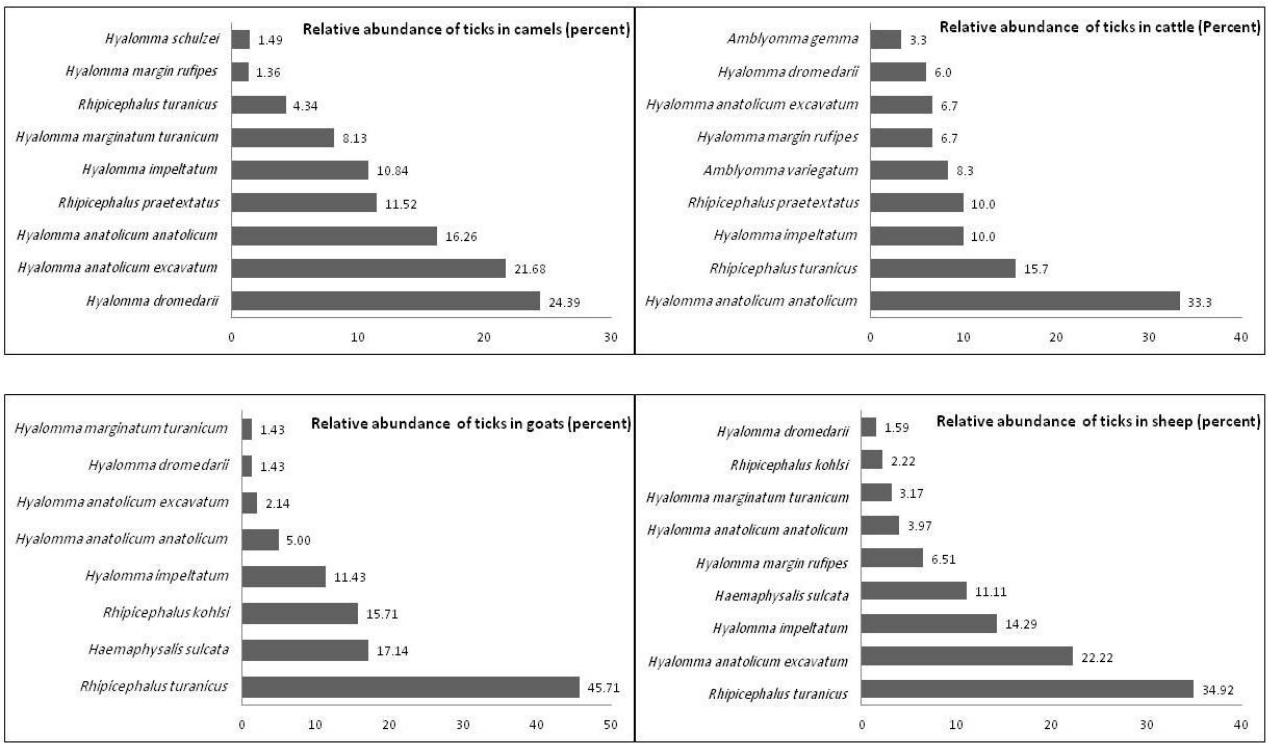

Figure 3. Percentage of tick species found in sampled animals in Al-Ahsa oasis in 2010.

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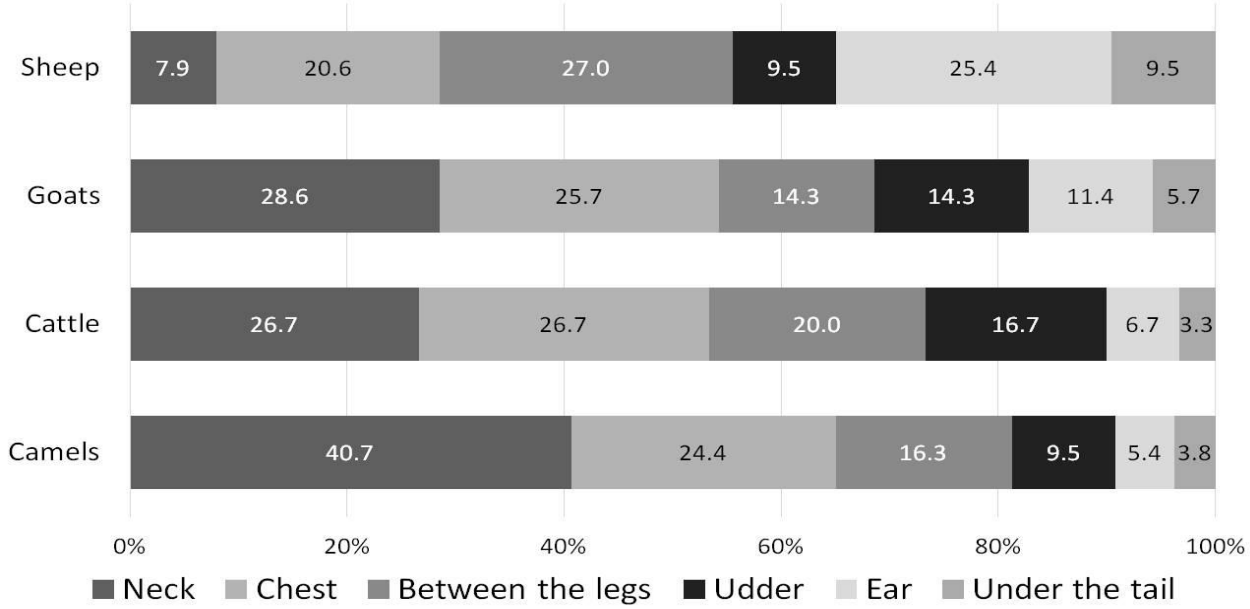

Figure 4. Predilection sites of ticks naturally infesting domesticated ruminants in Al-Ahsa oasis, Kingdom of Saudi Arabia.

Table 2. Numbers and prevalence of recovered hard tick species from domesticated ruminants during January - December 2010 in Al-Ahsa Oasis, KSA.

Tick species	Hosts				Total	Prevalence %
	Camels	Sheep	Goats	Cattle		
Amblyomma variegatum	-	-	-	25	25	0.47
Amblyomma gemma	-	-	-	10	10	0.18
Rhipicephalus kohlsi	-	14	110	-	124	2.33
Haemaphysalis sulcata	-	70	120	-	190	3.57
Hyalomma anatolicum anatolicum	600	25	35	100	760	14.29
Hyalomma anatolicum excavatum	800	140	15	20	975	18.33
Hyalomma dromedarii	900	10	10	18	938	17.63
Hyalomma impeltatum	400	90	80	30	600	11.28
Hyalomma marginatum turanicum	300	20	10	-	330	6.20
Hyalomma margin rufipes	50	41	-	20	111	2.09
Hyalomma schulzei	55	-	-	-	55	1.03
Rhipicephalus praetextatus	425	-	-	30	455	8.56
Rhipicephalus turanicus	160	220	320	47	747	14.04
Total number of collected ticks	3690	630	700	300	5320	-
%	69.36	11.84	13.16	5.64	-	-

Table 3. Seasonal variation in incidence of recovered hard tick species from domesticated ruminants during January - December 2010 in Al-Ahsa Oasis, KSA.

Tick species	Counts per season				Total	χ^2	*p*-value
	Winter	Spring	Summer	Autumn		2288.29	< 0.00001
Amblyomma variegatum	-	3	17	5	25		
Amblyomma gemma	-	-	8	2	10		
Rhipicephalus kohlsi	5	29	59	31	124		
Haemaphysalis sulcata	15	41	94	40	190		
Hyalomma anatolicum anatolicum	75	196	321	168	760		
Hyalomma anatolicum excavatum	85	225	464	201	975		
Hyalomma dromedarii	62	97	590	189	938		
Hyalomma impeltatum	10	130	350	110	600		
Hyalomma marginatum turanicum	20	46	175	89	330		
Hyalomma margin rufipes	1	19	64	27	111		
Hyalomma schulzei	-	6	31	18	55		
Rhipicephalus praetextatus	17	121	229	88	455		
Rhipicephalus turanicus	80	109	344	214	747		
Total number of ticks and incidence rate	370 (7%)	1022 (19.2%)	2746 (51.6%)	1182 (22.2%)	5320 (100%)		

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Table 4. Monthly variation in number of recovered tick species from camels, cattle, sheep and goats during January - December 2010 in Al-Ahsa Oasis, KSA

Tick species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Amblyomma variegatum	0	0	0	1	2	3	4	10	5	0	0	0	25
Amblyomma gemma	0	0	0	0	0	1	1	6	2	0	0	0	10
Rhipicephalus kohlsi	0	5	6	10	13	15	19	25	18	10	3	0	124
Haemaphysalis sulcata	5	8	8	12	21	25	37	32	25	13	2	2	190
Hyalomma anatolicum anatolicum	20	35	40	71	85	105	116	100	70	60	38	20	760
Hyalomma anatolicum excavatum	30	30	50	80	95	104	140	220	101	52	48	25	975
Hyalomma dromedarii	19	23	26	30	41	100	200	290	100	51	38	20	938
Hyalomma impeltatum	0	10	20	50	60	70	100	180	40	36	34	0	600
Hyalomma marginatum turanicum	5	5	10	11	25	30	65	80	40	30	19	10	330
Hyalomma margin rufipes	0	1	3	6	10	10	15	39	18	5	4	0	111
Hyalomma schulzei	0	0	0	3	3	5	10	16	10	8	0	0	55
Rhipicephalus praetextatus	6	6	18	51	52	64	55	110	53	24	11	5	455
Rhipicephalus turanicus	25	30	31	38	40	44	100	200	100	60	54	25	747
Total number of ticks	110	153	212	363	447	576	862	1308	582	349	251	107	5320

DISCUSSION

Observations of the current study clearly demonstrate local adaptation of ticks to survive and nourish albeit the harsh desert environment. High species richness of ticks in the examined animals can ensure constant supply of blood meals, especially from domesticated livestock in the oasis, which have shown to be highly susceptibility to various tick species. Livestock that lived longer in this oasis, such as the local breeds of camels and cattle, were those harbored most of the tick biomass in the studied areas, which suggests probable reduced immunity against tick infestations and/or increased susceptibility to tick infestations (Brossard and Wikel, 2004). Furthermore, the type of camel farming systems sampled in this study were those solely or partially devoted to camel farming. Such farmers in the KSA have traditional way of managing camel health issues (Abdallah and Bernard Faye, 2013). This explains the reason for not treating their animals with anthelmintics, albeit obvious presence of ticks.

The conceived experience among local camel owners is that camels are generally in a better condition and health when they are roaming out in the desert. Although grazing in open fields might have increased the chance of harboring ticks from infested wildlife, grazing animals might on the contrary experience reduced tick abundance due to host-diluting effect (Lydecker et al., 2019). However, population dynamics of ticks can be further complicated by the continuous influx of new animals into the herds, uncontrolled administration of acaricides or anthelmintics, the abundance of susceptible wildlife (Alanazi et al., 2019), and the introduction of ticks by migrating birds (Klitgaard et al., 2019). Indeed, Al-Ahsa oasis is a heaven for thousands of migratory birds, which supports their routs by hosting naturally protected places for bird nesting, good plant cover and plenty of clean surface water (Bird Life International, 2019).

This study reports incidence of *Rh. praetextatus* in Saudi Arabia. Here, this tick had a relatively high prevalence rate of 8.56%, mainly found in camels but also in cattle. The origin of this tick could not be traced in this study, but ticks of other species could be introduced to new territories by migrating birds (Klitgaard et al., 2019; Tonelli and Dearborn, 2019) or via imported animals. *Rh. praetextatus* can transmit Nairobi sheep disease, while in cattle and humans it may cause toxicosis and paralysis (Gothe, 1999; Mans et al., 2004).

Recent reports originated from a nearby governorate showed high prevalence rates of hard tick in camels (85.2%; Alanazi et al., 2019). However, the later study reported a much higher prevalence rate of ticks in sheep, goats and cattle (79.1%, 74.2%, and 69.2%, respectively) than that in this study. Several factors may have resulted in the observed high incident and abundance of ticks in camels. These factors may include the lack of proper control measures against ticks. Having a semi-nomad farming system may further contribute to the spread of ticks to other farms and being in contact with wildlife reservoirs of ticks (Alanazi et al., 2019). Furthermore, keeping animals in farms for extended periods of time might increase the chances of exposure to ticks, depending on the abundance of ticks in the surrounding environment and in other farm animals as well as in visiting wildlife. In addition, other factors could have contributed to the high abundance of ticks in camel and cattle, which may include the reluctance of using Doramectin in its prescribed dosage to treat camel infested with ecto-parasites due to its potential to induce toxicity in local camels (Abdaly, 2008; Lumaret et al., 2012). Improper and/or uncontrolled usage of acaricides have

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been personally noticed during the present study and might possibly have facilitated building resistance against the locally administered acaricides by indigenous ticks, but that is yet to be tested and proved.

In the present study, it was noticed that adult female ticks of seven species were active throughout the year, while six other species were completely absence from the examined animals for periods extend from two and up to eight months in row during colder months of that year. It is not expected in this study that given the condition of Al-Ahsa oasis ticks might diapause in winter, as temperatures do not reach low critical points that stimulate this pattern of lifestyle here. Alternatively, the limited sample size of five of the least abundant ticks might also influence its prevalence rates (below 2% in general), but this is not the case for *Hy. impeltatum*, which had an overall prevalence rate of 11.28%. The latter tick species is a specialist for livestock living in deserts and hot areas (Walker et al., 2003), but their younger life stages, larval and nymph stages, are usually feeding on birds and/or rodents and probably other domesticated or wild animal species during the colder months of the year (Walker et al., 2003; Alanazi, et al., 2019), which might represent the reservoir for this tick during its absence from domesticated animals.

Locally, the ticks found in this study were previously reported in domesticated livestock in Saudi Arabia (Banaja and Roshdy, 1978; Diab et al., 2006; Abdally, 2008), with *Hy. dromedarii* being the most prevalent tick in camels followed by *R. tauranicus* in sheep and goats (Alanazi et al., 2019). However, Alanazi et al. (2019) found *R. tauranicus* and *Hy. schulzei* only in spring season, whereas in the current study *R. tauranicus* was abundant throughout the year, and *Hy. schulzei* was found in spring but mainly in summer. Disagreement in prevalence rates may be merely attributed to the sample size and the local adaptation of ticks that have tuned its seasonal dynamics to maximize the chances of its survival (Alanazi et al., 2019).

Ticks in other areas of Saudi Arabia (Alanazi et al., 2019) and in similar climatic areas in nearby countries (Robson et al., 1969) have shown similar trend of increased prevalence rates in summer season, both in domesticated animals and wildlife. In tropical areas, adult ticks were more abundant in domesticated animals during the summer season (Walker et al., 2003; Barbieri et al., 2019; Hornok et al., 2020), but conversely in eastern and southern Africa, dramatic increase in mortality rate of ticks was found during the dry season (Randolph, 1994). The summer here in Al-Ahsa is relatively hot and dry, and adult ticks have though no chance to survive in nature under these conditions. Consequently, better chances for tick survival exist if ticks would continually feed on animals that are kept indoors during the dry, hot season. Such housing conditions provide the ticks with suitable moisture and endurable temperature levels, together with higher level of animal aggregation, which is ideal for ticks to persist in the dessert (Robson et al., 1969; Alanazi et al., 2019). On the other hand, larvae and nymph stages were presumably feeding on smaller mammals, such as rodents, canines and/or birds, especially during the relatively colder months of the year (Alanazi et al., 2019).

The current finding suggests that ticks in Al-Ahsa area will first appear in goats starting from May, then in June the tick populations will start to infest cattle and sheep simultaneously, and finally the ticks will build up in numbers in camels. This observed trend coupled with the fact that the sampled animals at Al-Ahsa area got infected with different tick species at different intensity and abundance rates clearly indicates different preference of ticks to certain animal species more than others (Guglielmone et al., 2014). Nonetheless, goats in this area can be considered as a bio-forecaster that predicts the beginning of tick infestation in other domesticated animals, and camels as the major contributors of tick biomass to the environment.

The recovered ticks in present study preferably chose the neck, the chest, then between the legs for feeding. An exception to the above trend was found in sheep, where the neck was the least preferable place for ticks to reside, and the chest and between the legs were the predilection site of most of the recovered ticks. Several factors may contribute to the preference of ticks to specific sites on the body of its hosts, including easiness in accessing blood and choosing protected areas (Chandler and Read, 1994), or influence of chemotaxis induced by locally emitted odors (Kariuki et al., 2018).

It is believed that this is the completive and systematic study to explore the seasonal variation of ticks in Al-Ahsa oasis in Saudi Arabia. Hence, providing important information, that can aid planning control programs against ticks in this region and similar regions. The currently found tick species are known vectors of infections for both man and animals. Al-Ahsa oasis is wealthy in animal resources which can be negatively influenced by diseases that can be transmitted by the abundant ticks in the area. Tick borne pathogens of medical and veterinary importance had been reported in ticks from neighboring areas in Saudi Arabia (Hussein et al., 1991; Harrison et al., 2015).

CONCLUSIONS

The current results showed that the prevalence rates of ticks started to raise synchronizing the raise in ambient temperatures, but was first evident in goats, then sheep and cattle and lastly in camels. However, the biomass burden of tick infestations has highest in camels, then sheep and goats, and lastly in cattle. The predominant tick species in camels were *Hy. dromedarii* (24.39%) and *Hy. anatolicum excavatum* (21.68%), in cattle *Hy. anatolicum anatolicum*

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(33.3%), and in sheep and goats *Rh. turanicus* (34.92% and 45.71%, respectively). The overall prevalence rates of ticks started to drop in all animals when the temperature started to drop in August until October. This may provide a seasonal bottleneck that should be targeted for control measures. A proposed tick control plan can be initiated in Al-Ahsa area in early May for all animals or can be focused on camels as the animals that harbored most of the tick biomass in the area, or on goats as those that first experience increases in incidence rates.

DECLARATIONS

Competing interests

The authors declare no competing interests.

Acknowledgements

The authors of this article would like to thank all farmers for accepting participation on this study and making their animals available for sampling. The authors acknowledged Mr. Hussein Al-Isa for his support in tick collection. Special thanks also go to Dr. Mohammad Nafi Solaiman Al-Sabi and Dr. Al-Awad Al-Ombashi for reading and commenting on drafts of this manuscript. Funding of this study was on the expense of the authors and did not receive any funds from any agencies. Nonetheless, the authors acknowledged the support and cooperation of the administration and staff of the Department of Microbiology and the College of Veterinary Medicine at King Faisal University in Al-Ahsa.

Author's contribution

M. H. Abdally has contributed in conceptualization of the project ideas, funding of the project from his own acquisitions, project administration along the duration of the study, secured resources, contributed in sampling methodology and lab diagnosis, secured the investigation process, facilitating and contributing to the writing of original draft, and overall supervision on the research work. Theeb M. Al-Marri and Hussain M. Abdally have equally secured the availability and using of the resources, secured and used relevant software for data curation, performed formal analysis and validation of the data. Omar A. Al-Jabr has contributed in visualization of the results, discussion, and secured writing, reviewing and editing of the final version of the manuscript.

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To cite this paper: Abdally MH, Al-Marri TM, Abdally HM and Al-Jabr OA (2020). Incidence and Prevalence of Hard Ticks in Ruminants of Al-Ahsa Oasis Region, Kingdom of Saudi Arabia. *World Vet. J.*, 10 (3): 276-285. DOI: https://dx.doi.org/10.36380/scil.2020.wvj36

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 286-296, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj37

Rapid Detection of Streptococci in Cultured Tilapia Fish Using PCR and Chemical Analysis

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ORIGINAL ARTICLE
pii: S232245682000037-10
Received: 11 Jul 2020
Accepted: 20 Aug 2020

ABSTRACT

Streptococcosis causes economic losses due to the high mortality in Nile tilapia (*Oreochromis niloticus*). The present study was carried out to detect the streptococci by PCR and chemical analysis in cultured tilapia fish. A total of 100 cultured tilapia fishes and 20 water samples were collected from 4 different fish farms at different locations in Kafr El-sheikh Governorate for bacteriological and chemical analysis. The results of water quality parameters examination revealed that the mean values of pH, dissolved oxygen, unionized ammonia, and nitrite were 8.2 ± 0.73, 7.44 ± 0.54 parts per million (ppm), 0.05 ± 0.008 ppm, and 0.00 ppm, respectively. All water quality parameters except for ammonia were within the permissible limit. The bacterial isolation results revealed 38 % positive samples for *Streptococcus* species isolated from tilapia fishes and 40 % were positive for *Streptococcus* species isolated from water samples. Of those 38 positive fish samples, 25 (65.78%) were *Enterococcus faecalis*, 15 (39.47%) were *Streptococcus pyogenes*, 6 (15.78%) were *Enterococcus faecium*, 5 (13.15 %) were *Streptococcus agalactiae*, and 3 (7.89 %) were *Streptococcus iniae*. On the other hand, from 8 positive farms water samples, 7 (87.5%) were *Enterococcus faecalis*, 2 (25%) were *Streptococcus pyogenes* , 2 (25%) were *Enterococcus faecium*, 3 (37.5%) were *Streptococcus agalactiae*, and 1 (12.5%) was *Streptococcus iniae* . Using mPCR to identify *Streptococcus* for some fish and water isolates, 6 *Enterococcus faecalis* with 310 base per (bp) were detected while 3 *Streptococcus agalactiae* with 153 bp and 2 *Enterococcus faecium* at 215 bp were detected. However, the mPCR from *Streptococcus* species directly from organs from fish and water samples revealed that 5 *Enterococcus faecalis* were detected at 310 bp while 2 *Streptococcus agalactiae* was identified at 153 bp. The biochemical results indicated that the infected fish with the streptococcal species had reduced total protein, albumin, and globulin in the blood serum while total cholesterol, urea, creatinine levels, and AST, ALT, GPX, CAT, SOD activities significantly increased, compared to non- infected fish.

Keywords: Biochemical parameters, PCR, Streptococci, Tilapia fish, Water examination.

INTRODUCTION

The second most common fish species in tropical and subtropical freshwater aquaculture is *Oreochromis niloticus* (FAO, 2018). Tilapia is a common aquacultured fish and an important seafood source for human consumption. Up to date, little known is about their usual physiology and reaction to disease infections. Subsequently, ceaseless development of tilapia wellbeing evaluation strategies is fundamental. In any event, the hematology methods commonly utilized for demonstrative clinical infections in the veterinary world are still restricted in aquatic animal pharmaceuticals (Chen et al., 2003).

Bacterial infections in cultivated tilapia were considered the foremost critical causes for financial misfortunes. *Vibrio anguillarum, Aeromonas species, Flavobacterium columnare, Pseudomonas fluorescens, Streptococcus* species, *Edwardsiella tarda*,, and *Enterococcus* species Were found commonly in aquaculture establishment (Plumb, 1997). Various studies help detect fish bacteria, laboratory infection or resistance to disease (Azad et al., 2001; Al-Harbi and Uddin, 2004; Cai, et al, 2004) still few links haematological parameters with experimental bacterial infection. Hematological parameters are important diagnostic tools that indicate the health state of fish (Blaxhall, 1972; Rehulka, 2002; Martins et al., 2004).

Streptococcus species, for the most part, are astute aquaculture pathogens, the pathogenicity of which is influenced by environmental stresses such as low oxygen levels and a high nitrite concentration (Bunch and Bejerano, 1997), water hardness, overfilling with removed scales (Wedmeyer, 1997) and water temperatures over 20 ˚C (Ohnishi and Jo, 1981). All vital organs of the infected fish with streptococci are heavily infected and the mortality is enormous (50-60 %) (Hubbert, 1989)**.**

Streptococcosis is a bacterial disease in fish that causes economic losses in the freshwater and marine fish production, which is economically important in many countries, including the tilapia industry. *Streptococcus* species that cause disease in fish include *S. agalactiae*, *S. dysgalactiae*, and *S. equi*, *S. equisimilis*, *S. faecium*, *S. pyogenes*, *S. zooepidemicus*, and *S.iniae.* Previous case reports showed that *S. agalactiae* and *S. iniae* are the main causative agents of

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streptococcosis in Tilapia. Moreover, the World Animal Health Organization (OIE) has declared that *S.iniae* is a zoonosis (Amal, 2011). The acute form of streptococcal infection in *O. niloticus* has resulted in more than 50% mortalities for about 3 days to one week, while the chronic form could last for several weeks, with the low rate daily mortality being approximately one or two percent (Osman et al., 2017).

The aim of the present project was to isolate *streptococcus* species from tilapia fish and water samples by the traditional method by isolation and identification, then by using multiplex PCR for conformation of some *streptococcus* isolates and a rapid method to detect *streptococcus* species directly from organs of fish and water samples. Also, this experiment was conducted to diagnose streptococcal infection using water quality parameters estimation and biochemical parameters, since diagnostic techniques to detect pathogenic bacteria responsible for fish streptococcosis are usually based on the cultured technique, which requires several days to reach a definitive diagnosis leading to increased disease outbreak risk.

MATERIALS AND METHODS

Ethical approval

The experimental design was performed in accordance with the Guidelines for Animal Experimentation of the Ethics Review Committee of the Animal Health Research Institute, Giza, Egypt (Approval No 83429).

Samples collection

A total of 100 Nile tilapia fishes were collected from live or freshly dead fishes with at least one or more clinical signs of eye lesions and opacity, septicemia, skin lesions, detached scales, skin congestion, ulcers, hemorrhage, and congestion of fins. Twenty water samples were collected from four different fish farms at different locations in Kafr El sheikh Governorate and transported to the Animal Health Research Institute Kafr El sheikh branch, Egypt. The fishes were transported in a sterile polythene bag, which was supplied with aerated tap water with chlorine-free water from fish farms and subjected to biochemical and bacteriological examinations.

Water quality sampling and measurement

Water quality at a depth of 1 meter in each farm was measured at five clear sampling points. A similar technique was used to sample the fish. The water quality parameters for pH and dissolved oxygen were measured using a handheld meter (ORION 5 STAR). The concentration of water nitrite and unionized ammonia was measured using powder pillow procedures and using a spectrophotometer to measure the concentration (HACH Company, Loveland, CO, USA). According to (APHA, 1998)

Bacteriological examinations

Bacterial isolation

Tissue samples from the brain, kidney, liver, spleen, and eyes of the fish were homogenized in a sample, which was used for the fish sampling according to Aboyadak et al. (2016) representative. Tissue samples were divided into two parts, one processed directly for multiplex PCR by sending it to the Dokki Laboratory, and the other was primarily cultivated on Tryptic Soy Broth (TSB) from each sample at 37˚C for 48 hours. A loopful from each tryptic soy broth tube was streaked on Edwards medium (modified) with additional 5-7% bovine blood, TSA, and blood agar plates, then the streaked plates were incubated at 37˚C for 48 hours. Pure bacterial isolates were identified according to their cultural, morphological, and biochemical characteristics (Holt et al., 1994).

Identification of *Streptococcus* species

All purified isolates were identified by studying colony growth characteristics ,morphological analysis (Cruickshank et al, 1975), biochemical analysis as catalase activity test, oxidase test, detection of hemolysis, growth at 6.5% NaCl, growth at 10^{o}C and 45^{o}C, detection of arginine decarboxylase (ADH), hippurate hydrolysis test, bile esculin test, fermentation of sugers (MacFaddin, 2000) and multiplex polymerase chain reaction (m- PCR) for detection of some pathogenic bacteria directly from the samples and from isolates The extraction of DNA from tested samples was done utilizing the QIAamp DNA small kit (Qiagen, Germany, GmbH) with alterations compared to the manufacturer's suggestions. Briefly, 200 µl of the sample suspension tested was incubated for 10 minutes with 20 µl proteinase, potassium and 200 µl of lysis buffer at 56^{o}C. After incubation 200 µl of 100% ethanol added to the lysate l. The sample was washed and centrifuged according to manufacturer's protocol. The nucleic acid was eluted with 100 µl elution buffer, which was given within the kit. PCR specific primers from Metabion (Germany, table 1), were used in a 50- µl multiplex PCR response containing 25 µl of Emerald Amp Max PCR Master blend (Takara, Japan), 1 µl of each primer of 20 picomols (pmol) concentrations, 11 µl water, and 8 µl DNA template. The reaction was performed in an applied 2720 thermal cycler biosystem. PCR products were isolated on 1.5% agarose gel (Applichem, Germany, GmbH) in

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1xTBE buffer at room temperature using 5 Volts/cm slopes by electrophoresis. 40 µl of the products were loaded into each gel slot for gel analysis. The part sizes were calculated using Gelpilot 100 base per Ladder (Qiagen, Germany, GmbH). The gel documentation system (Alpha Innotech, Biometra) captured the gel and the computer program analyzed the information

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target Agent	Target gene	Primers sequences	Amplified segment (base per)	Primary denaturation	Amplification (35 cycles)			Final extension	References
					Secondary denaturation	Annealing*	Extension		
E. faecalis	16S rRNA	GTT TAT GCC GCA TGG CAT AAG AG / CCG TCA GGG GAC GTT CAG	310	94˚C 5 minutes	94˚C 30 seconds	50˚C 40 seconds	72˚C 45 seconds	72˚C 10 minutes	Zoletti et al., 2006
S. agalactiae	cfb	TTTCACCAGCTGTATTAGAA GTA / GTTCCCTGAACATTATCTTT GAT	153						Ke et al., 2000
E. faecium	sodA	GAAAAAACAATAGAAGAAT TAT / TGCTTTTTTGAATTCTTCTTT A	215						Jackson et al., 2004

*The annealing temperature of the primers was modified, and validated to 50˚C to be suitable for multiplex PCR.

Biochemical examinations

The fish body surfaces were cleaned and dried with absorbent paper. Blood samples obtained from caudal vessels of 2ml/fish from each farm using disposable 3 milliliters syringes, were transferred to serum separation tubes without anticoagulants, such as defined for total serum protein, albumin, globulin, total cholesterol, urea, creatinine levels, and AST, ALT, ALP, GPX, CAT, SOD activities, which were estimated spectrophotometrically (LABOMED Co., Lab. American Inc., USA) according to the manufacturer's instructions.

RESULTS

Table 2. Water quality parameters in examined tilapia fish farms (n = 20) at different locations in Kafr El sheikh Governorate, Egypt

Parameters	Values	Permissible limit*
pH	8.2 ± 0. 73	6.5 – 9
Dissolved oxygen (ppm)	7.44 ± 0.54	5.61 - 9.4
Unionized ammonia (ppm)	0.05 ± 0.008	0.00 - 0.02
Nitrite (ppm)	0.00	0.00 - 0.2

Values are means ± standard error. *Permissible limit according to Egyptian law No. 48 (1982).

Clinical and post mortem examinations

The samples of infected fishes revealed the presence of eye lesions (bilateral exophthalmia and opacity), skin lesions and congestion of the internal organs (Figures 1 and 2).

Figure 1. Bilateral exophthalmia and skin lesion in tilapia fish

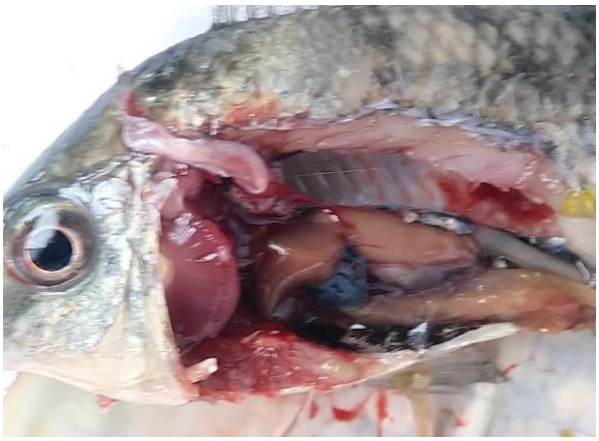

Figure 2. Congestion of the internal organs in tilapia fish

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Table 3. Percentage of positive *Streptococcus* species isolated from tilapia fish (n =100) and water samples (n = 20) was done at the Animal Health Institute - Kafer El sheikh Regional Laboratory, in Egypt and in summer 2018.

Samples types	Samples number	Positive samples number	Percentage (%)*
Tilapia fish samples	100	38	38
Water samples	20	8	40

*Percentage (%) calculated according to total number of samples.

Table 4. Identification of *Streptococcus* species isolated from positive tilapia fish samples was done at the Animal Health Institute - Kafer El sheikh Regional Laboratory, in Egypt and in summer 2018.

Identified organism	Positive samples (n = 38)	Percentage (%)*
Enterococcus faecalis	25	65.78
Streptococcus pyogenes	15	39.47
Enterococcus faecium	6	15.78
Streptococcus agalactiae	5	13.15
Streptococcus iniae	3	7.89

*Percentage (%) calculated according to the number of positive fish samples (38).

Table 5. Identification of *Streptococcus* species isolated from positive farms water samples from four different farms in Kafer El sheikh Governorate, in Egypt and in summer 2018

Identified organism	Positive samples (n = 38)	Percentage (%)*
Enterococcus faecalis	7	87.5
Streptococcus agalactiae	3	37.5
Streptococcus pyogenes	2	25
Enterococcus faecium	2	25
Streptococcus iniae	1	12.5

*Percentage (%) calculated according to the number of positive water samples (8).

Detection of some *Streptococcus* species by using multiplex Polymerase Chain Reaction

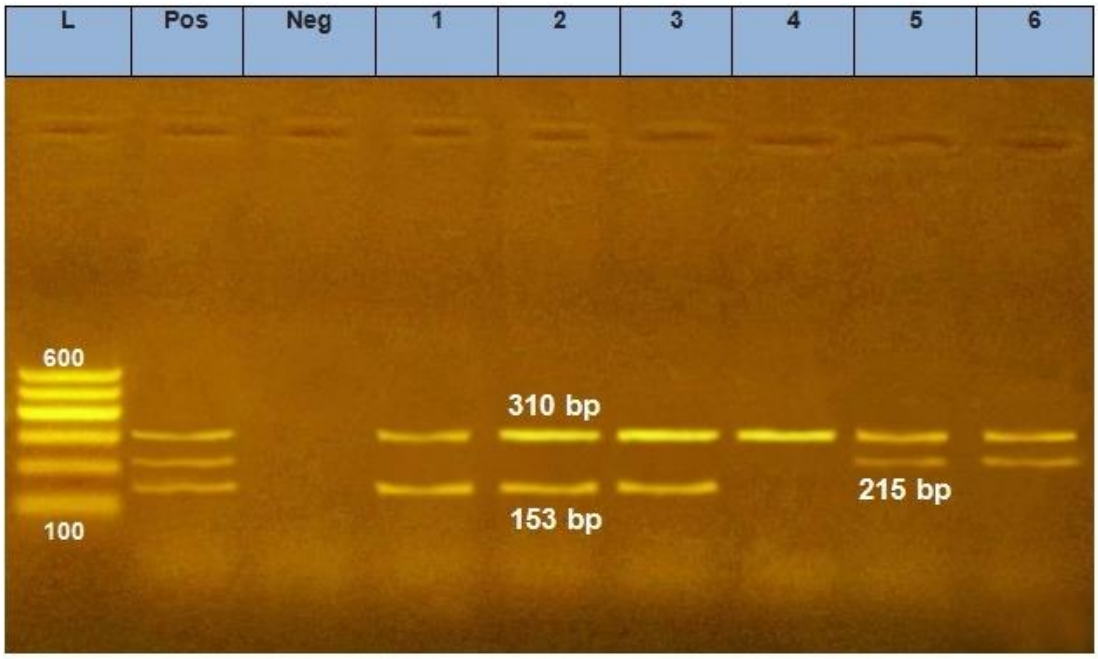

Figure 3. Agarose gel electrophoresis of multiplex PCR amplification products of *Streptococcus* isolated from tilapia fish samples and water samples. Lane L: 100 base per (bp) ladder as a molecular size DNA marker. Lane Pos: Control positive *streptococcus* species genes. Lane Neg: Control negative. Lanes1, 2, and 3: Positive for *Enterococcus faecalis* at 310 bp, and *Streptococcus agalactiae* at 153 bp. Lane 4: Positive for *Enterococcus faecalis* at 310 bp. Lanes 5 and 6: Positive for *Enterococcus faecalis* at 310 bp and *Enterococcus faecium* at 215 bp.

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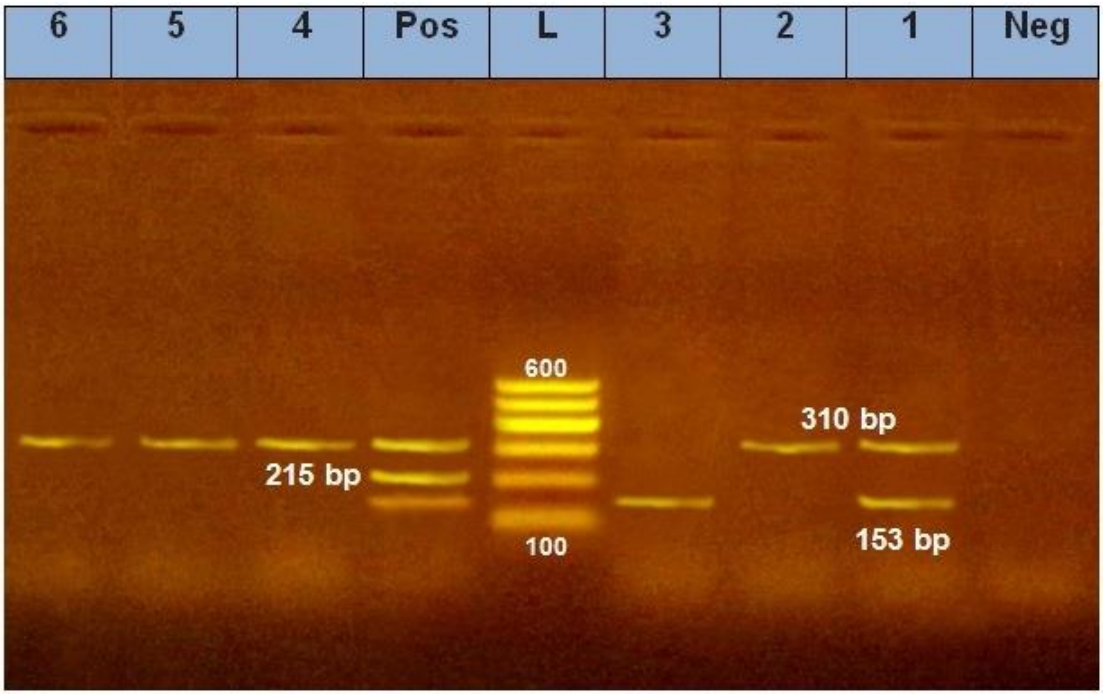

Figure 4. Agarose gel electrophoresis of multiplex PCR of *Streptococcus* species directly from organs of tilapia fish and water samples. Lane L: 100bp ladder as a molecular size DNA marker. Lane Pos: Control positive *Streptococcus* species genes. Lane Neg: Control negative. Lane1: Positive for *Enterococcus faecalis* at 310 bp and *Streptococcus agalactiae* at 153 bp. Lanes 2, 4, 5 and 6: Positive for *Enterococcus faecalis* at 310 bp. Lane 3: Positive for *Streptococcus agalactiae* at 153 bp.

Table 6. Variation of some blood serum parameters of Nile tilapia on exposure to *Streptococcus* infection in Kafer El sheikh Governorate, Egypt

Parameters	Non infected	Infected
Total protein (g/dl)	6.01 ± 0.08	5.81 ± 0.05
Albumin (g/dl)	5.44 ± 0.03	5.21 ± 0.09
Globulin (g/dl)	0.76 ± 0.11^{a}	0.40 ± 0.08^{b}
Urea (mg/dl)	4.99 ± 0.05^{b}	5.12 ± 0.02^{a}
Creatinine (mg/dl)	1.89 ± 0.08	1.99 ± 0.03
AST(u/ml)	31.3 ± 0.46^{b}	47.3 ± 0.38^{a}
ALT (u/ml)	54.3 ± 0.27^{b}	82.3 ± 1.2^{a}
ALP (u/ml)	76.3 ± 0.27	74.8 ± 0.61
Glucose (mg/dl)	159.4 ± 0.54^{b}	235.7 ± 0.27^{a}
Cholesterol (mg/dl)	176.75 ± 1.67^{b}	192.02 ± 1.23^{a}
Cortisol (ng/ml)	2.9 ± 0.11^{b}	3.36 ± 0.12^{a}

Values are means ± standard error. Means inside the same row of diverse litters are essentially distinctive at $p \leq 0.05$.

Table 7. Variation of some blood serum antioxidant enzymes of Nile tilapia on exposure to *Streptococcus* infection in Kafer El sheikh Governorate, Egypt

Items	Non infected	Infected
Glutathione peroxidase (GPX) (u/ml)	16.6 ± 1.21^{b}	21.6 ± 1.11^{a}
Catalase(CAT) (u/ml)	12 ± 0.^{06b}	19 ± 0.03^{a}
Superoxide dismutase (SOD) (u/ml)	200.62 ± 0.22^{b}	282.58 ± 0.11^{a}

Values are means ± standard error. Means within the same row with different litters are significantly different at $p \leq 0.05$.

DISCUSSION

Streptococcal infections have been frequently reported in the freshwater and saltwater fish that have been cultivated in several regions of the world, particularly tilapia species, and several *Streptococcus* species were involved (Chang and Plumb, 1996). The most effective *Streptococcus* species in fish are *S. iniae, S. difficile, S. agalactiae, S. parauberis, S. dysgalactiae and S. Shiloi* (Eldar et al., 1995; Mata et al., 2004; Netto et al., 2011). *S. agalactiae* belongs to group B streptococci, which may be either haemolytic (Evans et al., 2002) or non-haemolytic (Finch and Martin, 1984) .All eight strains of *S. agalactiae* isolated from tilapias are non-haemolytic. *S. agalactiae* is really the only streptococcal species which belongs to the Lancefield serotyping sero-group B (Devriese et al., 1991).

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Water quality parameters

Natural conditions encompassing the cultural areas could influence the water quality and introduce stress to the cultivated fish. This inevitably diminishes the immune status, and activates bacterial disease which leads to infection (Amal et al., 2015).

The results of the analysis of water quality parameters shown in table 2 revealed that the mean value of pH, dissolved oxygen, unionized ammonia and nitrite were 8.2 ± 0.73, 7.44 ± 0.54, 0.05 ± 0.008 parts per million (ppm) and 0.00 ppm, respectively. Our results indicated that some parameters of water quality, like water pH, dissolved oxygen, and nitrite, were within the recommended range for tilapia cultivation, but ammonia was higher than the recommended range for tilapia culture. These results were similar to those studied by Zamri-Saad et al. (2014) and lower than those reported by Ali et al. (2008).

The ammonia in water samples is assumed to originate from the feces of fish and excessive feed provided to the fish. Tilapias can live in pH extending between 5 and 10, but grow best at pH levels between 6 and 9 (Popma and Masser, 1999). But on the other hand, a low pH of the water contributed to behavioral changes, damage to the gill epithelial cells, and a reduced nitrogen excretion efficiency, and causes high mortality (Sammut, 2001).

Non-ionized ammonia is the most poisonous fish parameter in the aquaculture and contaminated water (Zhao et al., 1997; Harris et al., 1998; El-Shafai et al., 2004). In fresh water, the toxic levels for free ammonia in short-term exposure were usually between 0.6 and 2 mg/l, while others consider 0.1 mg/l as the highest tolerable concentration (Pillay, 1992).

The present study also identified the significant parameters of water quality that affected the presence of the bacteria in cultivated fish. Although each sampling site has a different parameter of water quality related to the presence of bacteria, ammonia and nitrite are two of the major important contaminants that threaten the health of aquatic organisms, particularly in freshwater, and have since been described as the most important parameters, that there are strong associations with the presence of bacteria at all sampling.

Popma and Masser (1999) recorded that metabolism, growth, and resistance disease were impaired if Dissolved Oxygen declines over an extended period and tilapias predisposed to streptococcosis. In addition (Swann, 1992; El-Sayed, 2006) studied that, the concentration of dissolved oxygen higher than 5 ppm is necessary for good growth of tilapia. Nitrite is extremely toxic to tilapia as it disrupts the fish's physiological function and results in growth retardation, as reported by El Sayed, (2006)**.** Nitrite could inactively enter the circulatory system as a nitrous acid and diffused unconditionally through the layers of fish gills. Upon entering the circulation system, nitrite oxidizes the iron within the hemoglobin particle from ferrous state (Fe2+) to ferric state (Fe3+) and the resulting product is called methemoglobin. Because methemoglobin is unable to reversibly combine with oxygen, exposure to nitrite can lead to significant respiratory trouble since even the blood's oxygen-carrying capacity is difficult (Boyd and Tucker, 1998).

Isolation and identification of *Streptococcus* species

In table 3, data revealed that *Streptococcus* species were isolated from 100 diseased tilapia fish, since 38 samples showed an incidence of 38%, while 20 water samples showed 8 positive *Streptococcus* isolation with an incidence of 40%. These findings were lower than those mentioned by Asencios et al. (2016) and El-refaee (2005), who isolated *Streptococcus* species with the percentage of 100 % and 39.8 %, respectively from tilapia fish. The recorded results were higher by El-Rouhy (2002) in Sharkia and Ismailia (21.8%), Zeid (2004) in El-Mansoura city (18%), and Huang et al. (1991) in marine water fish (17.3%). Also, the recorded results were higher than reported by Badran and Eissa (1991), who mentioned that incidence of *Streptococcus* was 1.7% in tilapia. The incidence of *Streptococcus* was reported by El-Bouhy and Megaheed (1994) 9.2% and 10% in cultivated *Oreochromis niloticus* by Ebtsam (2002). The different percentage of streptococcal infection could be attributed to different fish species and different environmental factors as well as the use of manure and waste water beside chicken and duck houses over fish ponds.

The tables 4 and 5 of the present study showed the identification of *Streptococcus* species isolated from positive tilapia fish samples and water. The proportions of fish and water samples were 65.78% and 87.5% for *Enterococcus faecalis*, 39.47% and 25% for *Streptococcus pyogenes*, 15.78% and 25% for *Enterococcus faecium*, 13.15% and 37.5 % for *Streptococcus agalactiae*, and finally 7.89 % and 12.5% for *Streptococcus iniae* respectively. The percentage of isolation of *Enterococcus faecalis* and *Streptococcus pyogenes* from fish was found to be higher than those reported by Abou El-gheit (2005), Amal (2009), Khafagy et al. (2009) and Osman et al. (2017). The *Enterococcus faecalis* isolated from tilapia fish with the percentages of 7.5%, 45%, 5%, and 23.76% and Abou El-gheit (2005) isolated *Streptococcus E. pyogenes* with a percentage of 16%, while the percentage of isolation of *Enterococcus faecium* ,*Streptococcus agalactiae* and *Streptococcus iniae* from fish were lower than the percentage which reported by Asencios et al. (2016), who isolated *Streptococcus agalactiae* from fish at 56.3%, but Amal (2009) isolated *Streptococcus iniae* with 32.4%, while the results of the present study was similar to Delphino et al. (2019), who isolated *Streptococcus agalactiae* from fish with 16% and Hernández et al. (2009) with 17.5% from fish. Although Saleh et al. (2019) isolated *Streptococcus iniae* with 13.3% from fish.

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Diagnostic techniques to detect pathogenic bacteria responsible for the streptococcosis of fish are usually based on the cultured technique, which takes many days to reach a final diagnosis leading to an increased disease outbreak potential. PCR can target unique genetic sequences of microorganisms and has been previously developed to detect pathogenic fish bacteria using a primer specific that is specific for a gene segment of a particular bacterium (Mata et al., 2004).

In the present study, the multiplex PCR test for the detection of streptococcosis caused by *E. faecalis, S. agalactiae,* and *E. faecium* was proposed, which could cause great losses and could be a threat to the fish farmers. All primers were used to detect these pathogenic bacteria isolated from infected fish cultured (Ke et al., 2000; Jackson et al., 2004). The m-PCR was accurate and specific for the detection of representative pure isolates of *E. faecalis, S. agalactiae, and E. faecium* with the detection limits.

Molecular identification

In the present study, PCR was used to identify the isolates using species-specific primers. The majority of the isolates from the previously mentioned specimens were *E. faecalis* (6 of 6) with the percentage of 100%, followed by *S. agalactiae* (3 of 6) with the percentage of 50%, and *E. faecium* (2 of 6) with the percentage of 33.3%. However, Ouissal et al. (2015) detected *E. faecium* from European sea bream as the most frequently isolated species of *Enterococcus* with the percentage of 24.32%, followed by 18.91% to *E. faecalis*, while Qasem et al. (2008) found that most of the isolates from fishes and wastewater samples (9 out of 17) with the percentage of 52.94 % were identified as *S. agalactiae.*

By detecting warm-water streptococcosis, the sensitivity threshold for the identification of *S. iniae, S. difficilis, S. parauberis,* and *L. garvieae* was between 2.5×10^3 and 1.2×10^4 cells/g tissue (Mata et al., 2004). Although the sensitivity of m-PCR to the detection of specific bacteria from fish tissue was not fulfilled, the m-PCR analysis in this study allowed the detection of *S. agalactiae* (5 of 6) with the percentage of 83.3% and *E. faecalis* (2 of 6) with the percentage of 33.33%, directly from the infected tilapia organs (Itsaro et al., 2012).

Biochemical parameters associated with *streptococci* infection

The present experiment was conducted to diagnose of *Streptococcus* infection using the estimation of water quality parameters and biochemical parameters, since diagnostic techniques for the detection of pathogenic bacteria responsible for fish streptococcosis are usually based on the cultured technique, which requires many days to reach a proper diagnosis leading to the increased potential for a disease outbreak. In tilapia fish, biochemical changes were studied to identify the variable parameters for the determination of healthy and infected fishes.

In table 6 the results of the present study revealed that there were insignificantly decreased in total protein and albumin but there was significant decreased in globulin, also there were significant increase in total cholesterol, urea, creatinine levels, and AST, ALT, GPX, CAT, SOD activities compared to non-infected fish. These results were similar to recorded by Adel and Shalaby (2004) and Yu et al. (2010), who revealed that decrease of serum total protein could be due to increased protein breakdown as a stimulator of corticosteroid hormones, which enhances proteins breakdown to provide amino acids and gluconeogenesis to provide glucose to cope with the increase in energy demands to balance stressful condition. Elevated Creatinine and urea levels may also be associated with reduced kidney function (Zotti et al., 2008) and as an indication of gill and kidney dysfunction (Adham et al., 2002; Yang and Chen, 2003). The increase in ALT and AST levels was similar to studies by Chen et al., (2004), Abuseliana et al., (2010), Bin and Xiao-jin, (2010) and Khalil et al., (2011). The increased level of these parameters due to infection were suggested to be due to the severe damage of viscera organs such as liver and kidney. There were no changes in alkaline phosphatase enzyme between infected and uninfected fishes, these differed from those reported by chen et al. (2011), who studied that alkaline phosphatase was significantly increased 12 hours post-infection with *streptococcus iniae*. Elevated cholesterol and glucose levels could be associated with the stress response and metabolism. These results were consistent with previous findings from Alsaid et al. (2014) and a similar increase in blood glucose levels had been detailed by Evans et al. (2006) taken after exposure of (*O. niloticus*) Nile tilapia to unionized ammonia. These elevated glucose levels may be due to the decrease in insulin levels, since insulin has a greater influence on protogenic and lipogenic pathways (El- Naggar et al., 1998). Also, the results of the present experiment revealed that there was a significant increase in cortisol levels in an infected group than in the uninfected group. These results were similar to those of Qiang et al., (2016), which increased the level of ammonia serve as acute stress and secrete a high amount of cortisol to facilitate the synthesis of glucose and degradation of fat.

In table 7, the results of the present research showed a significant increase in GPX, CAT, and SOD activities compared to non-infected fish. These results were consistent with those examined by Harikrishnan et al. (2012). In addition, malondialdehyde and SOD are example of biomarker for oxidative stress besides catalase (CAT) enzymes, Glutathione Peroxidase (GPx) and Glutathione Reductase (GR). When fish are under stress conditions, the level of Reactive Oxygen Species (ROS) also increases. Defense mechanisms to fight the ROS overload were found in many mammalian species and fish. Thus, fishes are mainly being used as bio indicators for environmental changes (Beutler,

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1984). Catalase is an essential antioxidant defense component, protects fish from oxidative stress by converting the hydrogen peroxide into oxygen and water (Atli and Canli, 2007). SODs are enzymes which then catalyze the dismutation of superoxide (O^{2-}) radicals into either conventional atomic oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is a by-product of oxygen metabolism and causes many types of cell damage if not regulated (Garry, 2011; Tohru and Masuko, 2011).

CONCLUSION

Tilapia has become a perfect host for *Streptococcus* infections. *Streptococcus* (*S. agalactiae* and *S. iniae*) are exceptionally pathogenic since they can infect many spices of fish around the world. Streptococcosis has been found to cause millions of economic losses in aquaculture worldwide. Tilapia farmers should be advised and trained on the proper management of tilapia fish to avoid the spread and outbreak of disease. Furthermore, water quality parameters play a vital role in tilapia farming. In particular, an ideal parameter for water quality should be preserved to avoid "stress'' in fish, which could lead to infection events. The diagnosis of diseased and carrier fish could be achieved using fast and accurate molecular techniques and measurement of fish biochemical parameters. Also, the present study demonstrated that the m-PCR assay was a sensitive and specific diagnostic tool for simultaneous detection of fish streptococcosis caused by *E. faecalis*, *S. agalactiae*, and *E. faecium* from fish and water isolates, however only a limited range of isolates could be detected directly from multiplex PCR from fish tissues and water. Although chemotherapy was not really suggested, proper management, and immunization could be aspects of the system to prevent and control streptococcosis.

DECLARATION

Acknowledgments

All authors are very grateful to all members of Animal Health Research Institute, Kafr El sheikh branch.

Competing interests

The authors declare that they have no competing interests.

Author's contribution

Hala AM Abd El-Hady and Mayada AM Abou Zeid found a research idea, planned the study design, performed data, the bacteriological and_molecular_examinations, and drafted the manuscript. Gehan IE Ali shared the research's idea, shared designed work, and shared a collection of blood samples during the experiment and helped the manuscript preparation. All authors have read and approved the final manuscript.

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To cite this paper: Ali GIE, Abd El-Hady HAM, and Abou Zeid MAM (2020). Rapid Detection of Streptococci in Cultured Tilapia Fish Using PCR and Chemical Analysis. *World Vet. J.*, 10 (1): 286-296. DOI: https://dx.doi.org/10.36380/scil.2020.wvj37

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 297-305, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj38

Contribution of Veterinary Sector to Control COVID-19 Pandemic in Nepal

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ORIGINAL ARTICLE
pii: S232245682000038-10
Received: 28 Jul 2020
Accepted: 09 Sept 2020

ABSTRACT

Nepal has been identified in a high-risk zone by the World Health Organization (WHO) for the Corona Virus Disease of 2019 (COVID-19). Nepal's first official reported case was confirmed on 13 January 2020 and so far, 24,000 cases have tested positive with 91 deaths. The imposition of lockdown has begun since March 24 and it was extended until 22 July. Reverse transcriptase Polymerase Chain Reaction and rapid diagnostic tests are used by the Government of Nepal to identify COVID-19. Among the worst-hit sectors are tourism as well as travel and the farming industries, the later contributes to the employment of around 65% of the population and a significant share of the national economy. The loss of commercial sectors, such as dairy, feed, and poultry industries are estimated to be in billions of rupees. One shortcoming of resource poorly managed countries, such as Nepal is the lack of interdisciplinary coordination. Over half a decade long professional existence and crucial contributions in Nepal's overall development, veterinarians are just beginning to be recognized as essential service providers. Since veterinarians of Nepal have the experience of eradicating cattle plague dealing with Avian Influenza and Swine Flu among others, they study and seem to be mentally prepared to handle pandemics. Currently, five veterinary diseases diagnostic laboratories with RT-PCR facilities are serving as COVID-19 diagnosis labs. Veterinarians from Nepal are serving in innovating newer tools as well as performing, supervising, evaluating, reporting diagnosis, and actively in pursuit of establishing the One Health (OH) approach. The sincere and patriotic role played by veterinarians has pressurized the government of Nepal to recognize veterinary service as an essential commodity. In this regard, universities should take the lead and bring veterinarians in policy and planning as well as decision making. The Tribhuvan University is responsible to have the initiative and Agriculture and Forestry University to deliver the missed opportunities. The 17 sustainable development goals that are to be achieved by 2030 are not possible without a multi-sectoral approach and veterinarians are best suited in this regard. It also needs to be realized that veterinarians are better positioned to handle emerging and re-emerging zoonotic diseases and capable of combating the present situation of COVID19 pandemics. Reiterating the fact that OH approach should not only be in paper and there is no alternative but combined utilization of scarce resources for healthy animals, humans, and the environment with the veterinary profession at its core.

Keywords: COVID-19, Essential services, One Health, SARS-CoV-2, Veterinary

INTRODUCTION

On 31 December 2019, a number of cases were reported to suffer from pneumonia with an unknown origin in Hubei Province, Wuhan, People's Republic of China (PRC) (Wang et al., 2020). World Health Organization (WHO) named it Corona Virus Disease of 2019 (COVID-19) on 11 February 2020. Alarm bells rang and this outbreak was declared as a public health emergency of international concern on 30 January and WHO waited until 11 March to announce it as a pandemic (Hau and Shaw, 2020). In this vein, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was an unprecedented agent that causes COVID-19 and is still under investigation. Similar to other pathogenic agents, it starts spreading from animals to humans and this animal-to-person spread was suspected after the initial outbreak in December among people who had a link to a large wet market in Wuhan, China. Victim "zero" is notably under serious investigation. Unless, WHO comes to a logical conclusion, the etiology for this unknown disease was identified as a novel type of coronavirus on 7 January, and the International Committee on Taxonomy of Viruses named it as SARS-CoV-2 on 11 February (Singhal, 2020). The virus is changing dimension, evolving and rush for mass scale vaccine production or declare disease free status is becoming a tricky situation so is the effort to establish real origin of this microbe.

Regarding SARS-CoV-2, patients might be afflicted with symptoms, such as cough, shortness of breath or difficulty breathing, fever, chills, muscle pain, sore throat, and other less common symptoms (e.g., nausea, vomiting or diarrhea) during the first 2-14 days (Singhal, 2020). Currently, 216 countries and territories around the world have reported around 20.7 million positive cases and over 751 thousand fatalities among which the residents of the India,

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United States of America, Brazil, Spain, Italy, the United Kingdom, France are at high risks (WHO, 2020). According to the World Organization for Animal Health, the transmission of COVID-19 is mainly due to human-to-human contact and there is no evidence supporting the fact that animals are playing any significant epidemiological role in the disease transmission. Food and Agriculture Organization (FAO) also stated that animal products consumed mostly for food are safe to eat when they are well-cooked. According to recent studies which are few in number, there is a slight risk of COVID-19 transmission from animals to people but, it can still be transmitted from people to animals on some occasions. Among different types of animal species, dogs, cats, minks, and tigers have tested positive but there is not enough scientific evidence to identify the source of SARS-CoV-2 or to explain the original route of transmission to humans (Singhal, 2020).

Sad stories of people throwing away (killing) their pets for the fear of the virus and abandoning aged and diseased pets, as well as the pain and plight of the community animals, are heart-wrenching. Mink farms bred by the fur industry in Holland had to be shut down due to the fear of human infection to SARS-CoV-2 (Akhmetzhanov et al, 2020). Likewise, the governments of Canada and the United States locked down big commercial meat processing plants for the fear of the COVID-19 outbreak. Detection of virus in frozen food items is also being reported (Togoh, 2020), though the Beijing scare in June on imported salmons was brushed aside by WHO said that there is no evidence that the disease spreads by food or food products. On tracing the chopping board and faulty handling was blamed but that was after the shelves were emptied. Likewise, just recently Chinese city of Shenzhen raised the alarm bells again by announcing that chicken wings imported from Brazil tested positive for coronavirus (Gan, 2020). Though, it is unusual for salmons to carry the virus, chicken do suffer from corona virus inflicted disease called the infectious bronchitis disease (Poudel et al., 2020) but it is not the same as COVID-19.

Understanding the risk of human or animal exposure to the infected SARS-CoV-2 animals or their products is essential for preventing virus spread and prioritizing research in this field an urgent task. Protecting food systems, and preparation and adoption of the national OH investigations and mitigation measures equally important (El Masry et al., 2020). Boycotts, restricted transportation and trade of agriculture products citing COVID-19 precaution is another concern.

In Nepal, the first case of COVID-19 was reported on 13 January in a 32-year-old Nepalese student studying at Wuhan University of Technology, Wuhan, China (Bastola et al., 2020). As of 12 August, there were a total of 24,432 COVID-19 cases, out of whom 16728 were recovered and 91 were dead (MoHP, 2020). An epidemiological study estimated 2.25% prevalence and 0.5% case-fatality rate of COVID19 among the tested population in Nepal (Dhakal and Karki, 2020). Nepal has been on a nationwide lockdown since 24 March, it was then eased a bit but the lockdown was again extended until 22 July 2020. It is notable that Nepal evacuated its citizens residing in Wuhan on 15 February and had them quarantined for two weeks (Asim et al., 2020). The imposition of lockdown has a significant effect on various sectors in Nepal, especially the veterinary sector. As reported, organized commercial sectors, more specifically dairy, feed and poultry industries, have been hit hard and lost billions of local currencies. With this background in mind, the present article highlights the major impacts of COVID19 on the veterinary sector and suggestion on how it has to be given due role head for the agriculture-dependent economy of Nepal.

Scenario of COVID-19 in Nepal

Nepal is a landlocked country having an area of 147.181 square kilometers. It is bordered with China in the North (1,414 kilometers border with PRC's autonomous region of Tibet, in the Himalayas) and India on the other three sides. A revised map was issued amid threats of encroachment from two great nations, between which Nepal is sandwiched. The two most populous nations in China and India are emerging as hotbeds for virus spread which threatens Nepal with a high risk for the pandemic and also one of the least prepared (Dhakal, 2020). Quarantine facilities and intensive care units are getting crowded and this means that patients afflicted with COVID-19 have to wait long for empty beds (Poudel, 2020). At the time of manuscript revision on 12 August, there were a total of 24,432 cases with 16,728 recovery and 91 deaths (MoHP, 2020, Figure 1), but globally 161 Nepalese died due to COVID-19 (NRNA, 2020).

Returning migrants cause the surge of Covid-19 cases and keep the disease on an upward trend for some time. Remittance is the backbone of Nepal's economy and its sheer contribution to the financial situation of Nepal has been estimated $8.79 billion in the fiscal year of 2018-2019 (MoLESS, 2020). For decades, India has been the largest destination for Nepalese immigrants, however, Middle East, Malaysia, South Korea, Japan, Europe, North America are also on the list (MoLESS, 2020, Figure 2). Among the Nepalese immigrants, a large number of people who returned from India continue to test positive, and accordingly faced severe hardship. The lockdown of over four months failed to prepare the country for the arrival and quarantine of the returnees in the border. Open border with India also proved to be disadvantageous as migrants (i.e., both Nepalese citizens and Indians) could easily sneak in and out from farmlands and river beds. Although public schools and other facilities were hastily used to house the migrants, the lack of sincere efforts and failure of resource mobilization were the major complaints from the majority.

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Given the global economic turmoil, it is likely that many of these migrants would stay in their home country since the appeal for their manual and semi-skilled type of labor gets dismissed or the job vacancies are filled with locals. There is no doubt that the lack of basic facilities in the hastily prepared makeshift camps, and government bogged down by surging cases is thinking beyond containing the spread. Waste management of these camps is a good example of how much we have learned from this pandemic and the concept of OH. The excessive use of protective materials without any education for safe disposal using the three R's (Reduce, Reuse, and Recycle) is intriguing. Although there is a need for a structured change in waste management (i.e., both household and medical waste), Nepal's system of waste collection and safety have not moved beyond providing masks and latex gloves. Some changes, such as hand washing and social distancing, may have become a new normal, but again it is yet to be assessed if the pre, during, and post COVID-19 behavioral changes are significant.

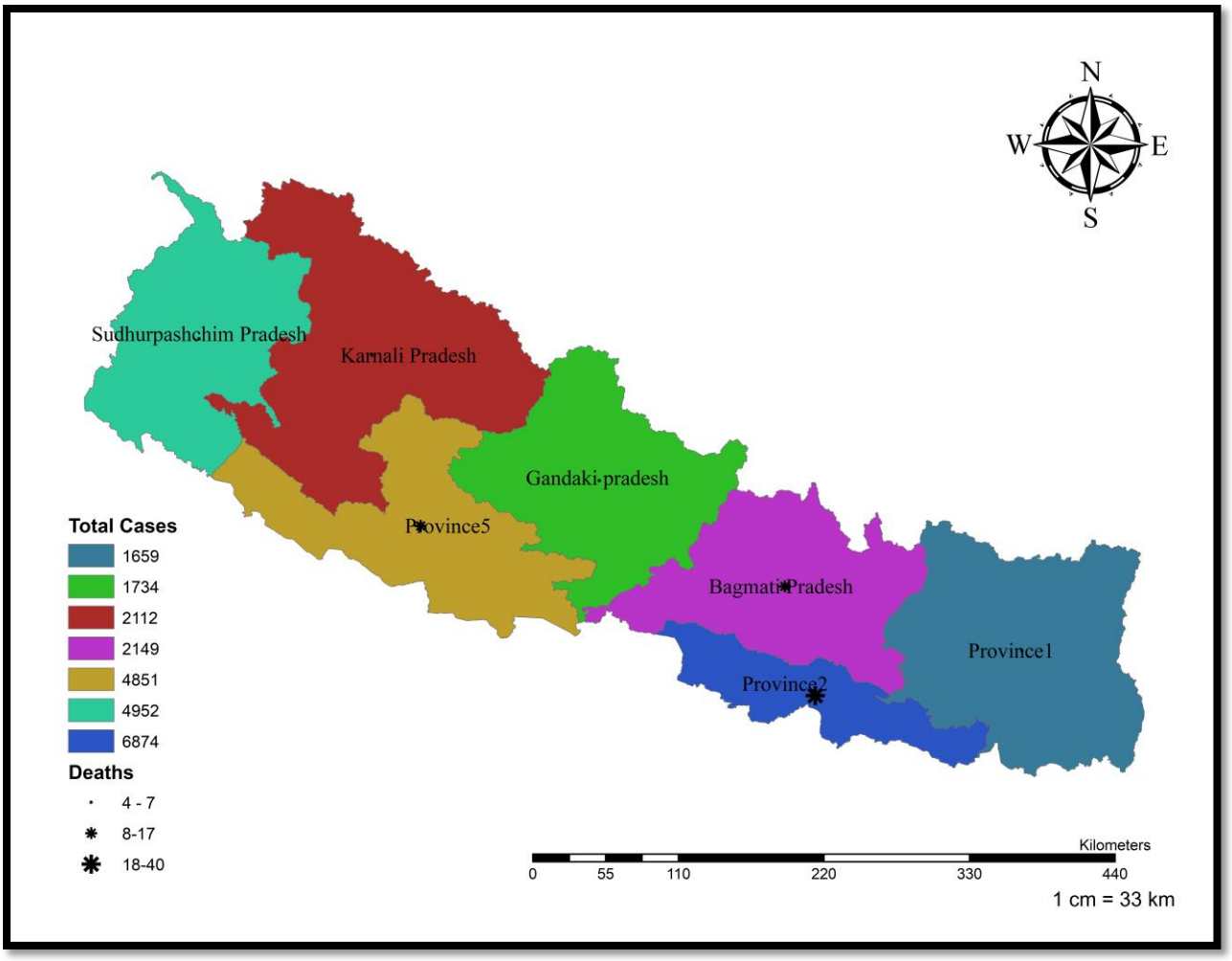

Figure 1. Provincial map of Nepal showing province wise COVID-19 cases and deaths on 10 June 2020. The high Himalayan regions have remained relatively safe while the south bordering India and cities close to the border have seen maximum cases.

Veterinary medicine in the global scenario

Tremendous efforts from multi-sectoral stakeholders are made to tackle the multifaceted viral pandemic. The frontline workers are risking their lives for the safety of their citizens and those in the background are busy working to find a solution to this current problem and likely new ones. Besides, the new experience of detecting and dealing with the novel coronavirus in animals and success in treatment and vaccination (Pedersen et al., 2019), veterinarians are also involved in the discovery of medicine and important immunological compounds. Various veterinary institutes in the USA and Australia are carrying out different research related to SARS-CoV-2 (Table 1). The experience of having managed threats for pandemics, such as avian Influenza (Horimoto and Kawaoka, 2001) and other crises have prepared veterinarians and veterinary laboratories to detect SARS-CoV-2 via reverse transcription polymerase chain reaction (RT-PCR). Veterinarians are also providing personal protective equipment and ventilators to tackle with COVID-19. However, as real inside stories emerge from these diagnostic facilities veterinarians and veterinary technicians are given secondary roles and responsibilities. The planning of pandemic control, approach in community spread intervention have

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the most hearing from politicians, medical personals, administration and very little from veterinarians-which is not how it is supposed to be. Virus in community- its sewage system, its garbage bins or littered dumps now full of masks and snot, cough filled tissues, scavenging animals, interacting pets it is a complex cycle.

Table 1. The COVID-19 research activities undertaken by various veterinary institutes in the international arena

SN	Research Activities	Working Institute	Reference
1	Developing a treatment protocol of Feline Infectious Peritonitis in cats experimentally infected with feline enteric coronavirus and conducting clinical trials for the treatment of COVI-19	University of California, Davis, School of Veterinary Medicine	Pedersen et al. (2019)
2	Coronavirus testing, development of sample diagnostic kits for SARS-CoV-2 and working for the development of COVID-19 vaccine	Louisiana State University, School of Veterinary Medicine	LSU (2020)
3	Investigation on the SARS-CoV-2 spike protein and mechanism for fusing with host cell which can lead to vaccine and drug development	Cornell University of Veterinary Medicine	Millet and Whittaker (2014)
4	Research for treatment of COVID-19 using Ivermectin. (Parasiticide, used in veterinary medicine). Ivermectin impedes SARS-CoV-2 in vitro	Victorian Infectious Diseases Reference Laboratory, Royal Melbourne Hospital, The Peter Doherty Institute for Infection and Immunity	Caly et al. (2020)

Veterinary sector in Nepal

Veterinary is an important profession in Nepal with around 65% population engaged in agriculture. The mass immigrant returnees are deemed to livestock agriculture as their main occupation for survival. The livestock sector along with fisheries contribute to 12.5% of the national total Gross Domestic Product (GDP) and the poultry sector counts for 4% GDP (FAO, 2014). Agriculture as a whole has been a hard-hit sector, and poultry as the most organized sector has incurred a daily loss above Rs 220 million due to the lockdown in the country (Dhungana, 2020). With a network of 10 veterinary laboratories, 48 veterinary hospitals, 753 animal service centers, and other private animal hospitals and clinics in Nepal, most had to close down due to the imposition of lockdown. The lockdown in Nepal has a detrimental effect on all sectors, especially dairy, egg, and meat sectors where a large number of multiple species were under husbandry practice (Table 2, Krishi Dairy, 2019).

Table 2. Nepal's diverse species both in the wilderness and in husbandry practices

Domestic Ruminants	Number	Other Animals	Number
Cattle	7376306	Pig	1435369
Buffalo	5277819	Chicken	72245732
Sheep	800749	Duck	404670
Goat	11647319	Rabbit	75740
Yak/Chauri	69978	Horse	58091

Except for some selected veterinary hospitals (e.g., the central veterinary hospital, some district-level veterinary hospitals, and expert centers), Veterinary Teaching Hospital of the Institute of Agriculture and Animal Science as well as Tribhuvan University of Nepal were open to provide their services. The Nepal Veterinary Association (NVA) tried to set up telemedicine centers and requested the help of charities to feed community animals that were left starving. Likewise, one notable achievement of NVA during this lockdown was to obtain an interim verdict (on 6 May 2020) from the Supreme Court of Nepal to the GoN to enlist veterinary services as an essential service. On World Veterinary Day 2020, NVA organized a webinar with the purpose of global public awareness towards COVID-19 situation with the major focus of Nepal on three different topics, a) development of COVID-19 vaccine, b) medical aspects of COVID-19, inter sectoral collaboration, and COVID-19 epidemiology with a global focus, and c) the regional and national scenario of COVID-19. Difficulties faced by veterinarians to deliver their services due to blanket lockdown was also realized by NVA and the strong leadership said enough of it. After two months of lockdown, on 21 May, the Supreme Court of

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Nepal ordered GoN enlisted veterinary services as an essential sector which allows veterinarians to carry out their work during lockdown and other administration restrictions (My Republica, 2020).

There is no doubt that we have no other options but to evolve together with these emerging and reemerging diseases. The effects of global politics and insecurity over the pandemics, its economics, trade barriers and restrictions will be remain for long time (Sanford et al., 2016). In this regard, New Zealand, Hong Kong are ideal example that we can never say that we have contained the virus, hence OH approach intended mitigating it in collective community changes in behavior is an envisioned approach for the future (Kaphle, 2020). Even in this crisis, veterinary diagnostic facilities that are playing important roles in combating COVID-19 still have areas to work upon beyond routine protocol screening. Overwhelmed and stretched resources are obvious but veterinarians can be pooled for beyond lab works too. In Nepal, five well equipped veterinary laboratories are supporting the government's testing of COVID-19 (MoHP, 2020, Figure 3). This disease has a negative effect not only on peoples' lives but also on stray animals. Due to the closure of butcher shops and restaurants, as well as low waste scavenging, approximately 26,000 street dogs in Kathmandu and much more in the country are starving and no effective steps have been taken by the GoN (Ojha and Lama, 2020). However, various animal welfare organizations, such as Sneha's Care, Animal Nepal, and Kathmandu Animal Treatment Centre, Street Animal Welfare (SAW) and volunteer individuals have taken the responsibility to feed community animals, including dogs, cattle, monkeys on daily basis (Ojha and Lama, 2020).This situation is due to the lack of a separate government body and institutional framework for the enactment of OH approach in Nepal (Acharya et al., 2019).

The OH is an approach for designing and implementing programs, policies, legislation, and research in which multiple sectors involved in public health, animal health, and eco-system-related health issues communicate and work together to achieve better public health outcomes (Acharya et al., 2019; Poudel et al., 2020; figure 4). In such a catastrophic situation of COVID-19 where there is no vaccination and treatment, OH approach is necessary to stand against this disease, and in nations with poor health facilities, such as Nepal, the government should put more emphasis on such approaches (Acharya et al. 2019). Although, several action steps, including nationwide lockdown, restriction of foreigners, practice of isolation, and quarantine, and sample test by rapid diagnostic test and RT-PCR are done by the government to control the pandemic COVID-19 after around three months of lockdown, there are less than 1500 PCR tests per day which are insignificant in the present context. In this situation, GoN and MoHP are unable to fully utilize the veterinary sector resources which could be an effective way to increase sample testing of COVID-19. The optimum utilization of manpower related to the medical and veterinary field, increase in the number of RT-PCR tests. The people following preventive measures can help to combat COVID-19. GoN should realize the importance of the veterinary sector and implement the OH approach effectively to win the battle against COVID-19 in Nepal.

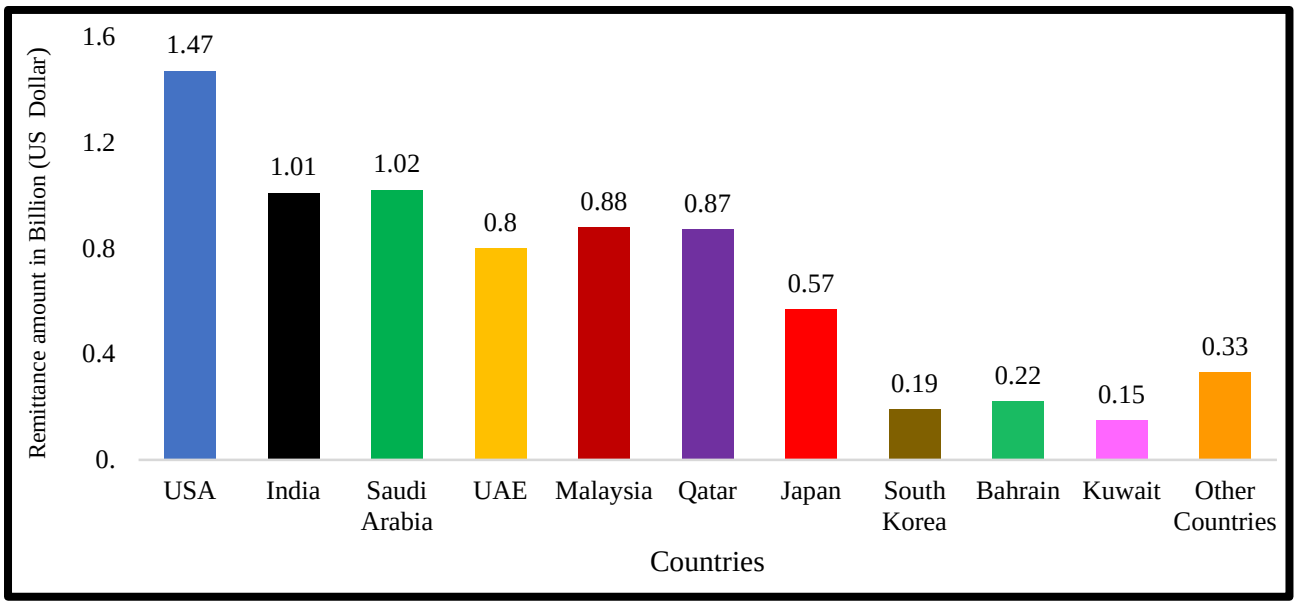

Figure 2. Remittance is the backbone of Nepal's economy. The current global recession is bound to hit Nepal hard and the return of Nepalese from abroad is both a challenge and an opportunity to kick start productivity in the country. Hard-working, patriotic, and now more experienced foreign countries returned migrants are likely to be a huge asset if the government's policy and plan can engage them in productive sectors. Bar graph showing remittance by destination in 2017-18.

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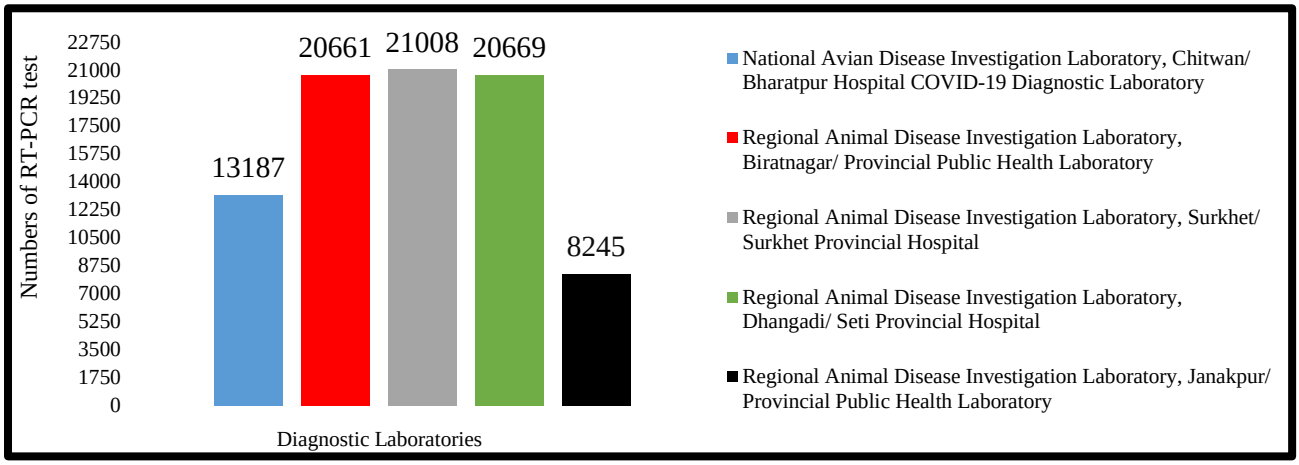

Figure 3. Bar graph showing the total number of reverse transcription-polymerase chain reaction tests of COVID-19 conducted in veterinary laboratories of Nepal on 12 August 2020.

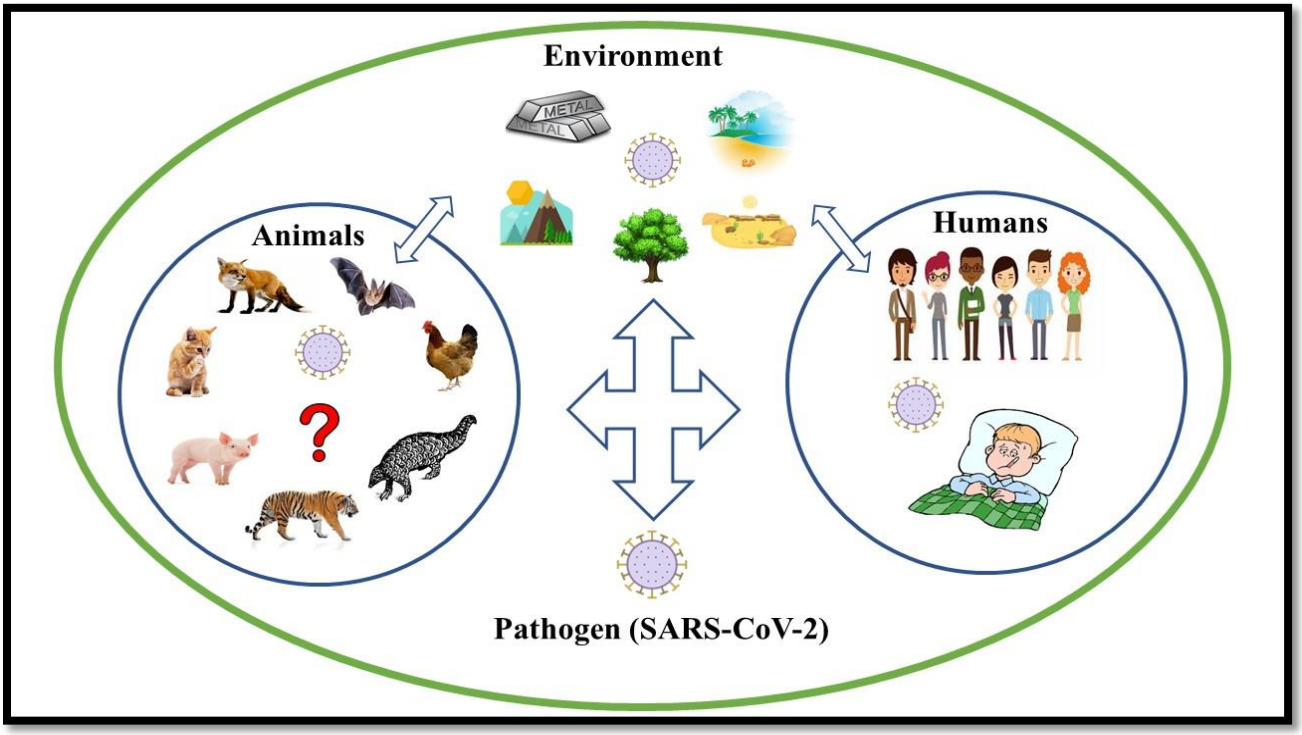

Figure 4. Interrelation of animals, humans, environment, and pathogen in the context of COVID-19

CONCLUSION

Across the world, there is debate on whether lockdown is going to continue or life returns to its normal form. Political affiliations, as well as ethnic and geographical dimensions, are dissected over how one is perceiving the threat and its handling by their respective governments. Even satirical bibliography has emerged in response to comments by the politicians and health officials at national and international levels-vodka or spice as prevention approach. The COVID-19 is rapidly spreading throughout the country and massively affecting the daily life of human beings. In case the government ignores the veterinary services as an essential sector, it may face economic and animal health challenges. Although avian and animal disease laboratories conduct the diagnostic test of SARS-CoV-2, there still is a long way to go for an effective health approach in Nepal. Veterinarians can play a crucial role in controlling such emergencies, however, there is no alternative for OH and one world approach. Conservationists alarmed that the coronavirus pandemic could spark a surge in ocean pollution-adding to a gloomy situation of plastic waste that already threatens marine life. Coronavirus waste, such as disposable protective materials and latex gloves can be seen littered along roadside and streams ending up floating like jellyfish across sea beds. Safely managing medical and domestic waste is crucial to successfully containing the disease and as drainage gets clogged, community animals ingest or play around with them we are inviting trouble. Plastic recovery from bellies of community cows that is the national animal of Nepal is frequently

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reported in kilograms and it looks like field veterinarians have added risk to deal with. We have not learnt from the past and this unsustainable COVID-19 waste management is bound to hurt the achievement of sustainable development goals by 2030 (You et al., 2020). There is no doubt that lockdown and minimized human activities could have a positive impact on the environment. This pandemic has led to environmental benefits, such as cleaner air, lower carbon emissions, and respite for wildlife. Reduced human mobility during the pandemic can reveal critical aspects of our impact on animals and provide important guidance on how to share space on this crowded planet (Christian et al., 2020). As vaccine development rush continues, some already declared (Figure 5), the traffic is getting crowded. Remarkable progress has been made in the process of COVID-19 vaccine development with the human trials stage achieved just 67 days after the outbreak. The WHO report on status of the vaccine development process is encouraging (Figure 6, Mackintosh et al., 2020). Once rich nations vaccinate their citizens, poorer countries may receive it as grants or may be reversed to establish safety and completion of phase 3. However, we as veterinarians will have roles in making sure the vaccines have no spillover effects in animals and vice versa. Now, the question is whether we can capitalize on this momentum and achieve collective community led transformation? Veterinarians and the veterinary sector in Nepal like those of other countries are hard hit by this COVID-19 pandemic and yes, they are integral part of the economy recovery and community healing strategies. The frontline warriors are working tirelessly and under no spotlight. With an established multi-sectoral roles and responsibilities, the reseahcers have to reformulate policies for achieving sustainable development goals by 2030. Nepalese veterinarians are aware and very much ready, but is the government even thinking in that direction is the big question.

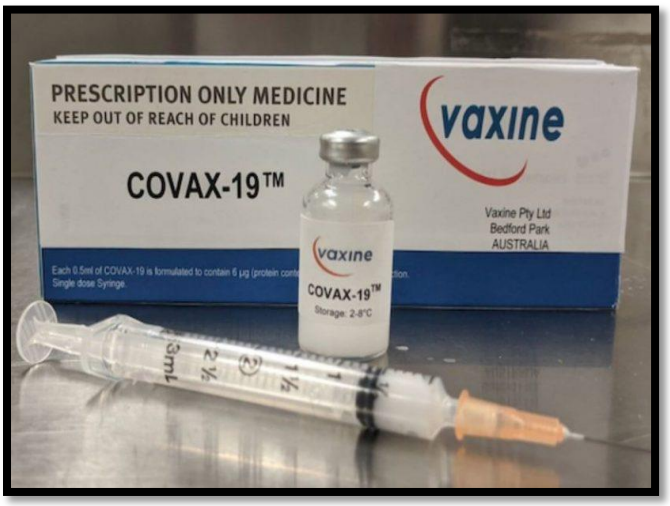

Figure 5. Discovery of the vaccine for COVID-19, many nations have declared that they have successfully tested the vaccine and ready for mass production. This advertisement is one such example from Australian company. Some multi-billionaires were already ahead of the game and preparing facilities in the anticipation of a possible pandemic. Nations and institutions were also on their mark as people currently see the announcement about the success of certain stages in the production process. There are also claims about sabotage, espionage, and signs of healthy and unhealthy competitions. This vaccine is reported in news to be leading the race but it faces stiff competition.

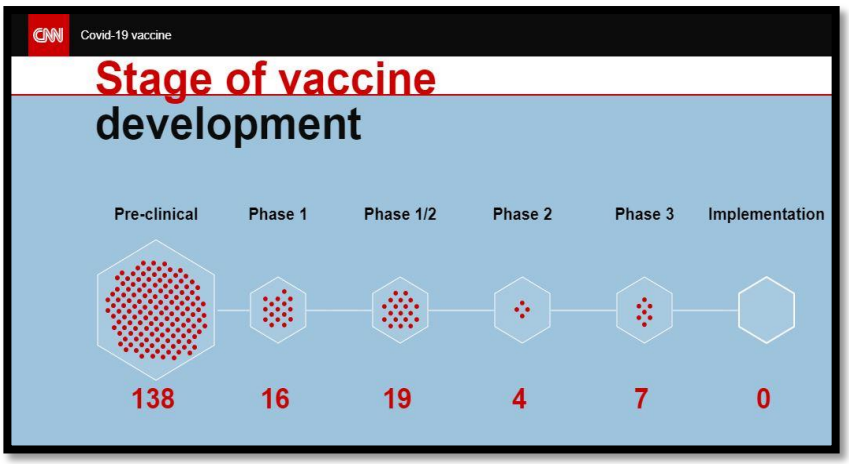

Figure 6. Multi billion dollars are spent by nations, universities, foundations, multinational companies among other players. The race to be the first to develop is seen with Russia, China, Australia, India rushing ahead, though blame of cutting corners and ignoring safety standards is also making news (Whatever, the figure retrieved from CNN shows that there is a healthy pool of interest and investment and some already close to the implementation stage).

DECLARATIONS

Author's contribution

Deepak Subedi conceptualized the study. Deepak Subedi, Anil Gautam, Ashwani Khanal, and Suman Bhandari wrote the manuscript. Deepak Subedi and Krishna Kaphle reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare no conflict of interest.

Acknowledgments

To all frontline workers in the challenge against the pandemic COVID-19.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 306-311, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj39

Histopathological Features of Listerial Rhombencephalitis in Dairy Calves in Kazakhstan

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ORIGINAL ARTICLE
pii: S232245682000039-10
Received: 01 Aug 2020
Accepted: 09 Sept 2020

ABSTRACT

Listeriosis is an infectious disease with zoonotic potential and can cause high fatality rates in animals and humans. Although the available evidence demonstrates the significance of post-mortem histological evaluation in the appropriate diagnosis, there is no sufficient evidence addressing histopathological alterations observed in calves clinically suspected of listeriosis. The current study aimed to carry out a post-mortem analysis of the dairy calves with clinical suspicion of listeriosis and determine a condition that contributed to death using histopathological evaluation. The tissue samples were obtained from 16 dairy calves with the age range of one day to one month, which died presumably due to listeriosis on several livestock farms in the Almaty region of Kazakhstan. The calves had symptoms of neurologic dysfunction prior to death. Necropsy and collection of brain histological specimens were performed, followed by procedures of fixation, dehydration, paraffinization, sectioning, staining with hematoxylin, and eosin along with Levaditi's method, and the microscopic examination. The histopathology findings were consistent with infection by *Listeria monocytogenes* and localized to the brainstem leading to the diagnosis of listerial rhombencephalitis.

Keywords: Brain, Calves, Histopathology, *Listeria monocytogenes*, Listeriosis, Rhombencephalitis

INTRODUCTION

A facultative anaerobic bacterium *Listeria monocytogenes* is the causative organism of listeriosis (Todd and Notermans, 2011) and is disseminated through the oral route predominantly (Gelbíčová et al., 2016). Ruminants and monogastric animals may cause listeria infection in humans, constituting a particular hazard to individuals with various immune system vulnerabilities, including infants, organ transplantation patients, and others. The mortality rate for listeriosis is up to 75% in humans and up to 100% in animals. Animals under three years of age are more susceptible to the disease (Chlebicz and Śliżewska, 2018). Moreover, this intracellular pathogen is capable of permeating the placental barrier (David and Cossart, 2017), which poses substantial risks to human and animal fetuses. For instance, the transmission during pregnancy may lead to congenital infection (St Edmunds et al., 2008). As a reflection of this data, a survey of 1500 dairy farmers conducted by Erdogan et al. (2001) showed the highest within-herd incidence of clinical listeriosis in dairy calves and replacement heifers. According to a study performed on 20 dairy farms in the northern hemisphere, the average yearly prevalence of *L. monocytogenes* was more than twice as high among calves compared to cows (Bandelj et al., 2018). Concerning the nervous form of listeriosis affecting the central nervous system (CNS), such as rhombencephalitis, a retrospective cohort study revealed that the mean age of sheep and cattle with neurolisteriosis was 39 months (Giles et al., 2017).

The methods usually carried out to confirm the diagnosis of bovine listeriosis are histopathology and culture tests (Radostits et al., 2007). The advantages of histopathology include low cost and ability to observe the tissue reaction (Gupta et al., 2009). Inherent operator variability is among its limitations (Fernandez et al., 2005). *Listeria monocytogenes* can be detected by silver impregnation staining techniques (Topalovski et al., 1993; Luca et al., 2015; Wilson et al., 2015). Their advantage is high sensitivity (Rabilloud, 2012), whereas the imperfection is poor suitability for quantification (Weiss et al., 2009). As for the present study, brain tissues were stained by Levaditi's silver impregnation method. Molecular tests frequently used for the identification of pathogenic material are immunohistochemistry and polymerase chain reaction (PCR). Due to the quantitativeness of the latter, it is less sensitive to interobserver variability (Furrer et al., 2015). Immunohistochemistry allows the identification of specific antigens within tissue sections (Duraiyan et al., 2012). One of its drawbacks is the complexity of the interpretation of results owing to different specificity and sensitivity of commercial antibodies (Furrer et al., 2015). Besides, as Kim et al. (2016)

To cite this paper: Ibazhanova AS, Kenzhebekova ZhZh, Nurgazy BO, Khussainov DM, Namet AM, Alimov AA and Orynkhanov KA (2020). Histopathological Features of Listerial Rhombencephalitis in Dairy Calves in Kazakhstan. *World Vet. J.,* 10 (3): 306-311. DOI: https://dx.doi.org/10.36380/scil.2020.wvj-39

has remarked, more sensitive methods spawn increased background signal aside from the target signal. Interestingly, Peters et al. (1995) stated that the clinical diagnosis of listeric encephalitis was confirmed in eight of eleven cerebrospinal fluid samples from ruminants by histological and/or bacteriological examination, whereas PCR allowed detecting *L. monocytogenes* in only one of the samples.

The authors point out a considerable role of postmortem inspections, including histological examination, in a correct diagnosis for listeriosis. At the same time, it must be recognized that no ample evidence addressing histopathological alterations observed in calves with clinical suspicion of listeriosis is available. Therefore, the aim of this cross-sectional study was to carry out a post-mortem analysis of the dairy calves' tissues and determine a condition that contributed to their death using histopathological evaluation.

MATERIALS AND METHODS

This study was carried out at the Department of Biological Safety at Kazakh National Agrarian University, Kazakhstan between 2018 and 2019. The test materials were tissue samples obtained from 16 dairy calves ranging in age from one day to one month, which died presumably due to listeriosis, displaying symptoms of neurologic dysfunction prior to death on several livestock farms in Almaty region of Kazakhstan. The provisional diagnosis was made by rural veterinarians with higher education in the veterinary field. Clinical manifestations included throwing head back, muscle tremors, circling, gait abnormalities, and recumbency. On the day of the calves' death, in order to establish its cause, they were delivered to the department by farm workers. The above cranial neuropathies allowed narrowing the diagnosis to rhombencephalitis (Bradshaw and Venkatesan, 2016). Necropsy and collection of brain histological specimens were performed. The pathological specimens were routinely fixed in 10% neutral buffered formalin and Carnoy's solution. Paraffin and cryostat 5-10 µm sections were stained with hematoxylin-eosin. Levaditi's technique was used to detect *L. monocytogenes* in the samples (Drury et al., 1967). The slices were examined under Zeiss Axiostar plus microscope (Zeiss Inc., Göttingen, Germany) and micrographs were captured by means of a microscope-mounted Leica digital camera (Leica Camera AG, Wetzlar, Germany), at magnifications of 150x and 300x.

Ethical approval

All procedures involving the animals were carried out in conformity with Directive 2010/63/EU and were approved by the ethics committee of Kazakh National Agrarian University (Kazakhstan).

RESULTS

According to the results of the histopathological assessment, in the medulla oblongata and pons Varolii, accumulations of lymphocytes, histiocytes, and neutrophils were observed. The blood vessels were hyperemic and thickened 5 to 319 times (16.5 - 1052.8 µm). Thus, in the region of the medulla oblongata adjacent to the fourth cerebral ventricle, we noted necrotic foci, lymphoneutrophilic pleocytosis, neuronal damage (chromatolysis, acute swelling, vacuolization, pycnosis, translucent cell formation), diapedesis, inflammatory cell infiltrates around blood vessels, perivascular and pericellular edema. The damaged neurons were surrounded by glial cells (Figure 1).

Lymphoneutrophilic pleocytosis, edema, and necrotic foci of approximately 105 µm (the largest foci were up to 1300 µm) were found underneath the ependyma of the fourth ventricle as well. In the necrotic foci, *Listeria* were Levaditi-stained black (Figure 2). The disease was confirmed by Levaditi's staining in all sixteen calves.

In the cerebellum, many Purkinje cells were edematous, their nuclei shriveled or poorly stained, the vessels were congested, and hemorrhages were found, along with cellular infiltrates comprised of lymphocytes, neutrophils, and histiocytes. These histopathologic changes are concordant with infection by *L. monocytogenes* and localized to the brainstem, which therefore led to the diagnosis of listerial rhombencephalitis.

DISCUSSION

The predilection of *L. monocytogenes* for brainstem is widely acknowledged (Xu et al., 2019). In the current study, histopathological examination of specimens from dairy calves with neurological manifestations revealed cerebral tissue inflammation enveloping hindbrain structures, which is characteristic of rhombencephalitis (Oevermann et al., 2010). Cell populations involved in the intracerebral immune response against *L. monocytogenes*-induced lesions overlap those reported in animals (Haligur et al., 2019). In addition to the already asserted axonal pathway of *L. monocytogenes* into the CNS, the transneuronal spread was hypothesized for this infectious agent (Henke et al., 2015). The observed neuronal necrosis phenomenon is a common sequel of encephalitis, although is not yet explained explicitly. It is alleged to be caused by metabolites of infiltrating neutrophils or neuronal infection engendered by *L. monocytogenes* (Precht et al., 2018).

To cite this paper: Ibazhanova AS, Kenzhebekova ZhZh, Nurgazy BO, Khussainov DM, Namet AM, Alimov AA and Orynkhanov KA (2020). Histopathological Features of Listerial Rhombencephalitis in Dairy Calves in Kazakhstan. *World Vet. J.*, 10 (3): 306-311. DOI: https://dx.doi.org/10.36380/scil.2020.wvj-39

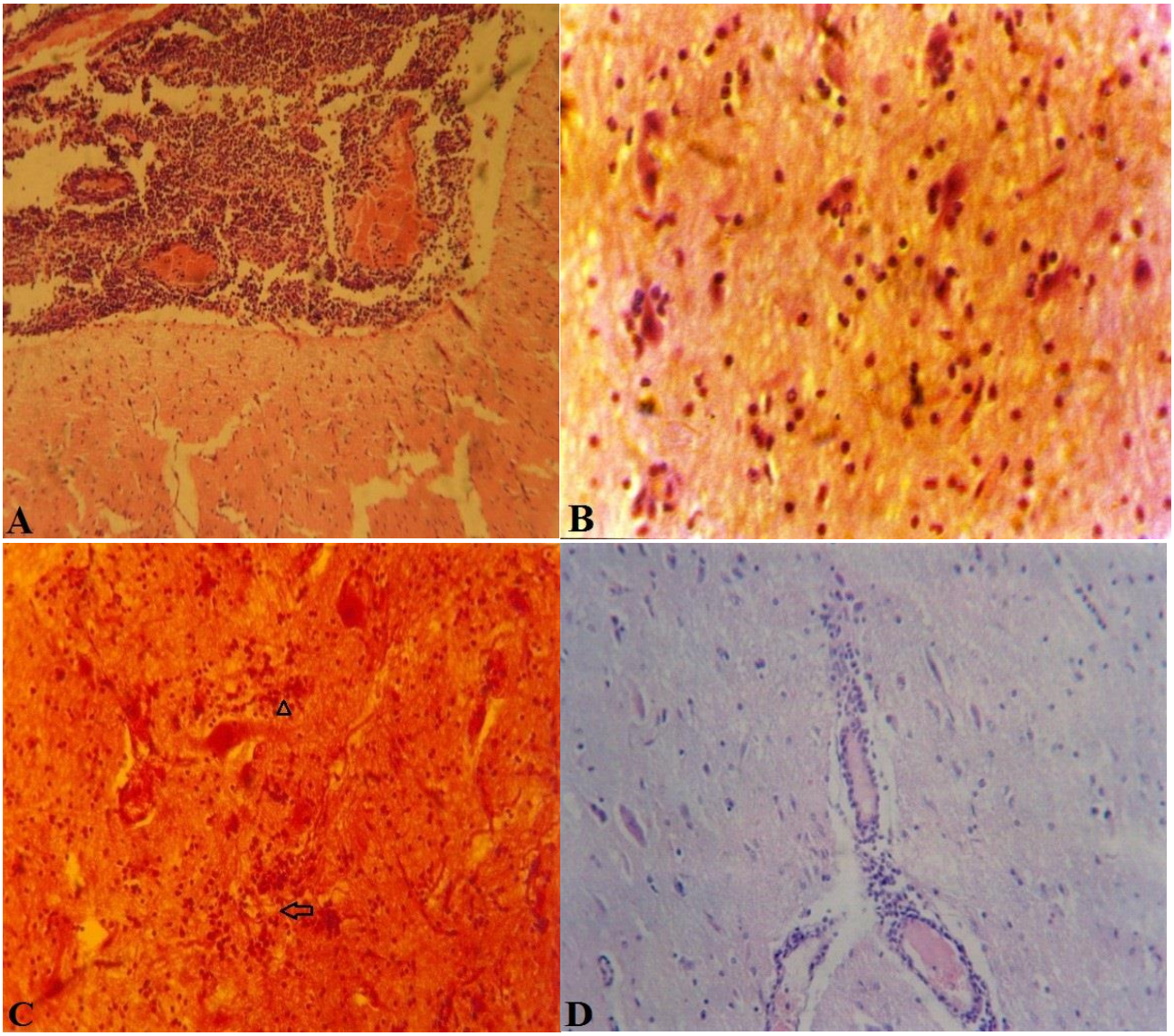

Figure 1. Histological features of listerial rhombencephalitis in the medulla oblongata of calves' brain that died with neurological signs. **A:** multifocal and coalescing foci of necrosis and hemorrhage, with lymphocytes, neutrophils and histiocytes. Hematoxylin and Eosin (H&E) stain, 150×; **B:** neuronophagia. H&E, 300×; **C:** neuronal dystrophy (arrow) and glial cell proliferation (arrowhead). H&E, 150×; **D:** inflammatory perivascular cuffs. H&E, 150×

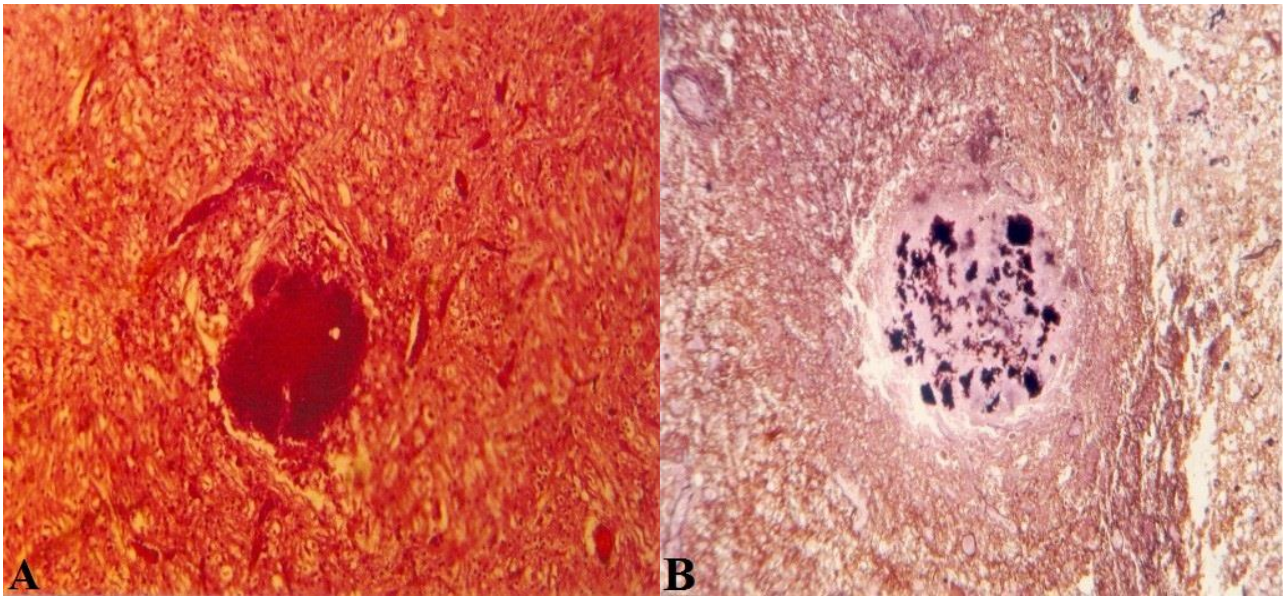

Figure 2. Histological features of listerial rhombencephalitis in the fourth cerebral ventricle of calves that died with neurological signs: **A:** necrotic focus. H&E, 150×; **B:** *Listeria monocytogenes* (black) in necrotic focus. Levaditi stain, 150×

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Another complication associated with *L. monocytogenes* infection is ventriculitis. In our case, the fourth ventricle was found edematous. However, we have not managed to retrieve any research publication containing information on similar alterations in the fourth ventricle of animals with listeriosis. Inter alia, dilatation of the fourth ventricle, as well as inflammation of its choroid plexus and ependyma have been previously described by other authors in human listeriosis of various forms (Engelen-Lee et al., 2018; Liang et al., 2019).

Perivascular infiltration of inflammatory cells was detected in the medulla sections from the calves' brain, since neuroinvasive *L. monocytogenes* infection triggers the inflammatory cascade. In particular, it recruits miRNA-155 which increases inflammation of the cerebral tissue via a variety of inflammatory cells, such as interferon γ-secreting lymphocytes, invading the brain and activating microglia during the adaptive immune response (Zhang et al., 2018).

The evidence confirms the extension of the brainstem encephalitis to the cerebellum (Coombs, 2017), which is in compliance with the data obtained in the current research. Nonetheless, this phenomenon is rarely reported in cattle, but, as revealed by Guldimann et al. (2015), *L. monocytogenes* can propagate in all microglia, including even those of the hippocampus. An experiment on ruminant rhombencephalitis (Rocha et al., 2013) indicated the presence of microabscesses and mononuclear perivascular cuffing in the white matter of bovine cerebellum.

The limitation of the current study is in the lack of an immunoassay or PCR analysis. Despite the prevalence of these diagnostic techniques, they also have a number of disadvantages, such as cross-reactivity (Favrot, 2015; Sakamoto et al., 2018; Ahmed et al., 2020). Furthermore, histopathological assessment is pathognomonic and is still exploited in some researches for confirmation of listeriosis (Teixeira et al., 2011; Precht et al., 2014).

CONCLUSION

This research provided an opportunity to investigate histopathological manifestations of listerial rhombencephalitis in dairy calves. The results indicate that *L. monocytogenes* produced destructive changes in parenchymal cells and brain microvascular system of the investigated animals. Based on the characteristic clinical signs and the obtained findings during histopathological examination, severe multifocal rhombencephalitis associated with *L. monocytogenes* infection was diagnosed.

DECLARATIONS

Acknowledgments

Authors are grateful to Sergey Sergeevich Kozhevnikov for the translation of this manuscript from Russian into English.

Authors' contribution

A. Ibazhanova and Zh. Kenzhebekova performed the histopathological examination (including necropsy and sample collection) and manuscript writing. B. Nurgazy supervised the laboratory procedures and participated in interpretation of results. A. Namet and K. Orynkhanov provided a critical review. D. Khussainov and A. Alimov prompted a number of literature sources for introduction and discussion sections.

Competing interests

The authors have not declared any conflict of interest.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 312-319, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus, and Amniotic Fluid at Birth and their Associations with Calf Birth Weight

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ORIGINAL ARTICLE
pii: S232245682000040-10
Received: 24 Jul 2020
Accepted: 02 Sep 2020

ABSTRACT

Amniotic fluid is a dynamic complex mixture that carries components contributing to the regulation of fetal development. The present study aimed to measure the levels of trace elements, such as Fe, Zn, Cu, Mg, Se, and Mn in amniotic fluid, maternal serum, and venous umbilical cord serum at delivery. The study further investigated the relationships among levels of elements in amniotic fluid (AF), maternal serum (MS), and venous umbilical cord serum (VUCS) in order to assess the possibility of monitoring abnormal fetal growth. Blood samples were obtained from 30 pregnant female camels at delivery and the corresponding VUCS and AF were examined. The Fe, Zn, Cu, Mg, Se, and Mn were analyzed on the atomic absorption spectrophotometer. Concentrations of trace elements, Fe, Zn, Cu, Mg, Se, and Mn in VUCS were significantly higher, compared to MS or AF. The trace elements, Fe, Zn, Cu, Mg, Se, and Mn were present in significantly lower concentrations of AF than in MS or VUCS. Neonatal birth weight did not correlate with MS levels of the trace elements. However, neonatal weight correlated positively with venous cord serum Fe, Se, and Zn levels. There was a scarcity of correlation between maternal and fetus trace elements in the pregnant camels. In conclusion, AF could even be the result of simple filtration of maternal blood. Evaluation of selected trace element levels in MS did not appear to be useful within the assessment of fetus growth. The findings of this study indicated an active transport for Fe, Zn, Cu, Mg, Se, and Mn between pregnant camels and fetus.

Keywords: Calf birth weight, Dromedary camel, Placental barriers, Pregnancy, Trace element

INTRODUCTION

Newborn calves of dromedary camels show weakness and low birth weight in a high proportion, leading to economic loss. Calf birth weight was very variable, and depended on the gender of the fetus, breed, parity (Freetly et al., 2000; Nagy and Juhász, 2019) and the nutritional state of the mother (Zachara et al., 1986), especially in the late pregnancy period that fetal requirements increase for energy, protein and minerals. Usual fetal development depends on suitable store of trace elements, including iron (Fe), zinc (Zn), and copper (Cu), magnesium (Mg), and selenium (Se) and manganese (Nandakumaran et al., 2016). The viable role of trace minerals in fetal development and their growth was recently suggested as well as their relationship to calf birth weight (Graham et al., 1994). Deficiency of trace minerals such as Fe and Zn can retard the fetal growth (Mitchell et al., 1998). Likewise, Se (Black, 2001; Mitchell et al., 1998) and Cu deficiency (Mills and Davies, 1979) were implicated as possible factors that could impair fetal development. Lower levels of the trace minerals such as Cu, Fe, and Zn were stated in aborted fetus, suggesting a possible role for these minerals in fetal growth and development (Graham et al., 1994). Pregnancy placed a considerable burden on the homeostasis of trace elements in mammals (Black, 2001), whereby the physiology of pregnant animals, and the requirements of the growing fetus changed (Faye and Bengoumi, 1994). There is evidence that camels were susceptible to trace element disorders similar to those of other ruminants (Faye et al., 1992). There were several reports of clinical mineral defects in camels (Faye et al., 1992; Faye and Bengoumi, 1994; Zong-Ping et al., 1994; Chuka Ozegbe, 2005), and their prevalence and importance were likely to be misjudged as signs of subclinical deficiency may not be detected over a long period of time.

In all mammalian species, amniotic fluid (AF) builds up early, and then decreases with the growth of the embryo (Suliburska et al., 2016). The role of the trace elements contained in AF is not clear. It was suggested that this fluid could be an important source of fetal nutrition (Abdelrahman and Kincaid, 1993). Each fetus was completely dependent on its mother via the placenta for the supply of trace elements (Perveen et al., 2002). The

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sensitivity of the fetus to the absorption of trace elements was not only strongly influenced by the uptake by the mother, the stage of pregnancy and the placental transport, but also by the inherent ability of the fetal organs to accumulate reserves (Black, 2001). The transport of trace elements from the pregnant animal to the fetus varied during pregnancy, and it gradually increased during this period of time (Tibary and Anouassi, 1997). Some trace elements were transported across the placenta in an active process, while others appeared to be transported passively (Romeu et al., 1986). Camels have an epitheliochorial type of placenta in which the fetal membranes do not invade the endometrial layer of the uterus (Tibary and Anouassi, 1997). The mechanisms involved in transplacental transport of trace elements from the pregnant camel to the fetus are still not well known. In order to more fully understand the transfer of trace elements from dam to fetus; concentrations of the trace elements in maternal, venous umbilical cord serum and amniotic fluid were investigated. Although some data exist for a number of species, including rabbits (Kriesten Schmidtmann et al., 1986), rats (Romeu Alemany et al., 1986), mice (McArdle and Erlich, 1991), sheep (Langlands Bowles et al., 1982), cattle (Van Wouwe et al., 1991) and humans (Hurley, 1976; Zhou et al., 2019), references were few for camels. The purpose of present study was to determine the element concentrations of Fe, Zn, Cu, Mg, Se and Mn in maternal, fetal cord blood and amniotic fluid at birth. Moreover, correlations between elements in fetal cord serum, amniotic fluid and maternal blood were established. Present study aimed to be a contribution for a better understanding of the mechanism of trace element transport from the pregnant dam to the fetus, and of the effect of certain elements on birth weight.

MATERIALS AND METHODS

Ethical approval

All institutional and national guidelines for the care and use of animals were followed according to the guidelines approved All procedures involving the care and the use of the animals were approved by the ethics committee of the faculty of veterinary medicine, institutional animal care and use Committee, Aswan University, Egypt.

Animals and study area

The present study was carried out on 30 pregnant camels (camels' dromedaries) during September 2016 to February 2018 in a private farm in Daraw village, Aswan province, Egypt. Camels were free from brucellosis and tuberculosis (with average age: 7 to 10 years; weight: 450 to 550 kg), which were vaccinated against Camel pox, Brucella and Rift Valley Fever. All the animals were raised under the semi-intensive system in which they were fed with barely and alfalfa hay with very limited grazing, and with common salt supplements, and had free access to drinking water. The camels were housed in an open yard. The pregnant camels were selected in consultation with cameleer who recorded their mating history, and the pregnancy was confirmed by rectal palpation.

Sampling

Ten milliliters of blood was collected from each pregnant camel immediately after delivery via jugular vein and from the umbilical cord vein of newborn while the placenta was still not separated. The umbilical cord vein contained a serum which was enriched with all necessary nutrients from the maternal blood within the placenta to supply the fetus. The blood samples were allowed to clot, and centrifuged at 3000 round per minute (rpm) for 15 minutes; the serum was separated and stored at – 20 °C for further analysis. During the first stage of labor, the amino-chorionic sac (transparent, a vascular amnion) appeared within the vulva with the fetus parts were visible within the water bag, a ten- milliliter syringe fitted with a twenty-gauge needle was wont to collect a sample of amniotic fluid which was turbid, yellowish and watery by penetrating the amnion, taking care to avoid contamination from blood or allantois fluid. Following rupture of the amnion, a sample of cord blood was obtained from umbilical cord vein. Calf birth weights were measured before colostrum was taken. All the collected blood serum and amniotic fluid samples were analyzed for Fe, Zn, Cu, Mg, Se and Mn by using an atomic absorption spectrophotometer (Shimadzu, Model AA-6601, Japan).

Statistical analyses

All obtained data were analyzed using Statistical Package for the Social Sciences (SPSS) version 25 (Armonk, NY: IBM Corp). Differences in the element contents in maternal, venous umbilical cord serum and amniotic fluid were statistically analyzed with Analysis of variance (ANOVA). Pearson's correlation was used to examine the relationships between each of trace elements and calf birth weight. Likewise, correlation of trace element levels among maternal, venous umbilical cord serum and amniotic fluid were analyzed. Mean values in the same row with different letters were statistically significant, and the highest values were represented with the letter (a). Statistical significance was declared at the $p \leq 0.05$ level and the data were presented as the mean ± Standard Error (SE).

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RESULTS

Trace element contents in maternal, venous umbilical cord serum and amniotic fluid

Iron

As shown in table 1, the mean levels of iron were 0.66 ± 0.12 parts per million (ppm) in maternal serum, 0.37± 0.09 ppm in amniotic fluid and 2.98 ± 0.66 ppm in venous umbilical cord serum, respectively. The venous umbilical cord serum iron levels were significantly higher than those in maternal serum ($P < 0.05$) and amniotic fluid; and, the maternal serum iron levels were also significantly higher than those in amniotic fluid ($P < 0.05$).

Zinc

Zinc levels in the maternal serum, venous umbilical cord, and amniotic fluid were 0.78 ± 0.10 ppm, 1.08 ± 0.29 ppm and, 0.53 ± 0.09 ppm respectively. The venous umbilical cord had a significant higher concentration of zinc than maternal serum and amniotic fluid ($P < 0.05$). Beside, zinc levels were also significantly higher in the maternal serum than in the amniotic fluid ($P < 0.05$).

Copper

The mean copper levels in maternal and cord serum and amniotic fluid were 0.75 ± 0.08 ppm, 0.91 ± 0.14 ppm and 0.61 ± 0.09 ppm; respectively. There was a significant difference between copper levels in maternal, cord and amniotic fluid ($P < 0.05$).

Magnesium

The mean levels of magnesium in maternal and venous umbilical cord serum and amniotic fluid were 7.21 ± 0.75 ppm, 8.42 ± 0.46 ppm and 6.78 ± 0.44 ppm respectively. The mean magnesium levels in both maternal and venous umbilical cord serum were significantly higher than those in amniotic fluid ($P < 0.05$).

Selenium

As shown in table 1, the mean levels of selenium were1.06 ± 0.24 ppm, 0.79 ± 0.15ppm and 0.66 ± 0.06 ppm in venous umbilical cord serum, maternal serum and amniotic fluid, respectively. The content of selenium in amniotic fluid was significantly lower than that in both maternal and venous cord serum ($P < 0.05$); and maternal serum selenium levels were also significantly lower than those in venous umbilical cord serum ($P < 0.05$).

Manganese

The mean manganese levels in maternal serum, amniotic fluid and venous umbilical cord serum were 0.27 ± 0.15ppm, 0.18 ± 0.04 ppm and 0.52 ± 0.13ppm respectively (Table 1). Mean manganese levels in maternal serum were significantly lower than that in venous umbilical cord serum ($P < 0.05$). Besides, magnesium levels were also significantly higher in the maternal serum than in the amniotic fluid ($P < 0.05$).

The correlation between maternal serum and amniotic fluid trace element contents

Several elements in AF were effectively influenced by those in maternal serum (Table 2). Concentrations of Fe, Cu, Mg, Se and Mn in AF were positively correlated with those in maternal serum (r: 0.09, 0.08, 0.01, 0.41 and 0.12), respectively. In contrast, there was a negative correlation of Zn contents between AF and maternal serum (MS) with r: - 0.19.

The correlation between maternal and venous umbilical cord serum trace element contents

Maternal serum concentrations of Fe, Cu and se were negatively correlated with those in venous umbilical cord serum (VUCS) (r: - 0.04, - 0.11 and - 0.07), respectively, but there were positive correlations of Zn, Mg and Mn contents between MS and VUCS with r: 0.01, 0.07 and 0.08 respectively (Table 3).

The relationship of trace elements levels in maternal serum, Venus umbilical cord serum and amniotic fluid with calf birth weight

The mean birth weight of the camels was 25.5±0.08 kg. Pearson correlation showed a significant positive correlation ($P < 0.05$) amongst VUCS levels of Fe, Zn and Se and calf birth weight (Table 4). Fe level in the AF showed a significant negative correlation (table 5) with calf birth weight ($P < 0.05$). No significant correlation ($P > 0.05$) could be shown between calf birth weights and levels of trace elements in maternal serum samples (Table 6).

Table 1. Element levels in maternal sera, venous umbilical cord sera and amniotic fluid of camels at parturition

Parameter (ppm)	Maternal serum (n=30)	Amniotic fluid (n=30)	Cord serum (n=30)
Fe	0.66 ± 0.12^{b}	0.37 ± 0.09^{c}	2.98 ± 0.66^{a}
Zn	0.78 ± 0.10^{b}	0.53 ± 0.09^{c}	1.08 ± 0.29^{a}
Cu	0.75 ± 0.08^{b}	0.61 ± 0.09^{c}	0.91 ± 0.14^{a}
Mg	7.21 ± 0.75^{b}	6.78 ± 0.44^{c}	8.42 ± 0.46^{a}
Se	0.79 ± 0.15^{b}	0.66 ± 0.06^{c}	1.06 ± 0.24^{a}
Mn	0.27 ± 0.15^{b}	0.18 ± 0.04^{c}	0.52 ± 0.13^{a}

Data are expressed as mean values ± Standard error (SE); the number of studied samples in each fluid is shown in parentheses. ^{a,b,c} Mean± SE in the same row with different superscripts are significantly ($P<0.05$) different. n: the number of studied samples.

To cite this paper: Essawi WM and Gouda HF (2020). Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus and Amniotic Fluid at Birth and their Association with Calf Birth Weight. *World Vet. J.*, 10 (3): 312-319. DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

Table 2. Correlation between concentrations of trace elements in maternal sera versus amniotic fluid of camels at parturition

Parameter (ppm)	Pearson correlation Coefficient (r)	p Significant correlation at p= 0.05
Fe	0.09	0.65
Zn	-0.19	0.31
Cu	0.08	0.69
Mg	0.01	0.97
Se	0.41	0.03
Mn	0.12	0.53

Table 3. Correlation between concentrations of trace elements in maternal sera versus venous umbilical cord sera of camels at parturition

Parameter (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	-0.04	0.84
Zn	0.01	0.94
Cu	-0.11	0.55
Mg	0.07	0.70
Se	-0.07	0.71
Mn	0.08	0.60

Table 4. Relationship between trace elements concentrations in maternal serum and calf birth weight

Elements (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	-0.23	0.23
Zn	0.11	0.57
Cu	-0.09	0.76
Mg	-0.15	0.43
Se	0.11	0.56
Mn	-0.02	0.92

Table 5. Relationship between trace elements concentrations in amniotic fluid and calf birth weight

Parameter (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	-0.43	0.02
Zn	0.07	0.70
Cu	0.12	0.58
Mg	0.19	0.32
Se	-0.35	0.87
Mn	0.15	0.44

Table 6. Relationship between trace elements concentrations in venous umbilical cord sera and calf birth weight

Parameters (ppm)	Pearson correlation Coefficient (r)	Significant correlation at p= 0.05
Fe	0.37	0.04
Zn	0.37	0.04
Cu	-0.24	0.21
Mg	-0.11	0.56
Se	0.48	0.01
Mn	0.24	0.20

DISCUSSION

To the best of knowledge, the current study is first study applied This was often the primary study to characterize the trace elements profiles in MS, VUCS and AF of pregnant dromedary camel , and to investigate the correlations between the concentration of the elements levels in MS, VUCS and AF, and calf birth weight. whether the correlations between element levels in MS, VUCS and AF exists or not. There were two main reasons to study the physiological relationships between blood trace elements profiles in the dams and the body weight of their newborn. First, a dam that calves a heavy calf had a greater risk of dystocia. Second, a calf which was too weak at delivery may have more problems of vitality. For good intrauterine development, the fetus needs a sufficient amount of nutrients and trace elements that can only be obtained from the mother's blood via the placenta (Rossipal et al., 2000). In the present study, the function of placenta as a fetal-maternal barrier in the transfer of some minerals from dam to fetus during gestation was well investigated. In this study, the significant

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higher concentration of Zn and Mn in VUCS than in MS was proved. Present study was in agreement with previous ones comparing the levels of metal elements in maternal and cord blood (Baig et al., 2003; Nandakumaran, 2016; Zhou et al., 2019) in human. It was supposed that Zn and Mn may cross the placenta via active transport, and the fetus exerts specific demand for Zn and Mn. However, other clarifications for the higher Mn concentrations in cord blood was also suggested, such as lower or restricted removal of Mn by fetus or inability of the fetus to use Mn (Widdowson et al., 1974).

The demand for trace elements increased rapidly during gestation, and this may result in a decline in maternal and/or fetal stores (Al-Saleh et al., 2004). In the present study, venous umbilical cord serum Fe, Cu, Se and Mg concentrations were significantly higher than those in the corresponding dam serum.

Iron was the most vital element within the blood which contributes to hemoglobin composition, and was additionally essential for the successful development of the fetus. In the present study, levels of Fe in VUCS were higher than those in MS. This finding was in accordance with that reported previously in human (Rallis and Papasteriadis, 1987) and in ovine (Gooneratne and Christensen, 1989), and indicated active transport of this element across camel placenta. This trend of changes during gestation in camel appeared too compatible with the observations in the pregnant cattle, where a decrease in placental transfer of Fe appeared to occur in the last third of pregnancy (Richards, 1999).

There was constant increase in Cu deposition throughout the fetal period and, therefore, an increasing demand for Cu by the fetus (Eltohamy et al., 1986). In this study, the level of Cu in VUCS was higher than that in MS, whereas another study showed inverse results (Zhou et al., 2019).This interaction implied that fetus has a capacity to sequester maternal Cu, even when the dam is Cu deficient (Graham et al., 1994). According to Seboussi et al. (2010) and Eltohamy et al. (1986), Cu concentration in serum decreased at the end of pregnancy due to active transfer from hepatic storage of the dam to its fetus. A significant correlation between Se and Cu was observed in camels receiving a selenium supplementation (Koller et al., 1984).

In the present study, the levels of Se in VUCS were higher than that in MS. This may be due to that selenium level which may readily crosses the camel placenta as observed in bovine (Wooten et al., 1996) and human (Baig et al., 2003). As shown in the present study, Mg levels in VUCS were significantly higher than that in MS, which was in accordance with the recent study in human (Nandakumaran et al., 2016). The results showed that the concentration of trace elements in AF was found to be lower than MS. These data suggested that trace elements in AF may be derived from blood. The role of trace elements contained in AF was not clear. Because AF was swallowed by the fetus, it was suggested that this fluid may be an important source of certain trace elements for fetal nutrition (Wooten et al., 1996).

In the current study, we did not observe a correlation between the concentration of the various elements under investigation in the maternal serum and the birth weight of the calf. Maternal serum levels of the various elements studied did not correlate positively or negatively with calf birth weight. Birth weight of the calf was one of the basic issues to judge pregnant camel management, and to expect the possibility of newborn thickness or mortality. According to various earlier studies (Barhat et al., 1979; Al Mutairi, 2000; Bissa, 2002; Nagy and Juhász, 2019), birth weight of camel calves varied from 19 to52 kg. The calf birth weight in the present study ranged between 18 and 45 kg, and the absence of a correlation of trace element levels with calf weight led to assume that the levels of these elements in MS were not useful values for the assessment of fetal weight. However, Fe levels in the AF were found to correlate negatively with calf birth weight while levels of other elements did not reveal any significant correlation. Interestingly, Fe, Zn and Se levels in the VUCS were found to have a positive correlation with calf birth weight. Amongst the variation factors, breed, parity and weight of the pregnant dam, sire, and the year and month of birth were reported to effect calf birth weight significantly (Barhat et al., 1979; Al Mutairi, 2000; Bissa, 2002; Nagy and Juhász, 2019). To the best of knowledge, no study was demonstrated an association between trace elements concentrations and camel calf birth weight. In cow, Graham et al. (1994) reported that fetal size increased as fetal Cu increased, and was less than or equal to maternal Cu. Gooneratne and Christensen (1989) showed that neither maternal nor fetal Mn were correlated with fetal size. Clearly, further studies are necessary to examine the effects of these trace elements on calf birth weight.

Several elements in AF and VUCS were effectively influenced by those in MS (Table 2 and 3). For the VUCS, the results showed a positive correlation between concentrations of Zn, Mg and Mn in VUCS and those in MS. In contrast, there were negative correlations of Fe, Cu and Se contents between VUCS and MS. On the other hand, concentrations of Fe, Cu, Mg, Se and Mn in AF were positively correlated with those in MS, while, a negative correlation of Zn contents between AF and MS was reported. There was no significant correlation between all minerals in MS and VUCS or AF. Unfortunately, there were no many previous studies reported regarding this aspect in camel. Mitchell et al. (1998) reported a positive correlation among maternal and fetal Cu, maternal and fetal Mn, and maternal and fetal Zn. In contrast, there was a lack of correlation between maternal

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and fetal Fe in cow. A positive correlation between maternal and fetal trace elements suggested fetal dependence on the dam for its supply of nutrients, including trace elements. A lack of correlation suggested fetal independence. Mechanisms regulating interactions between nutrients were still poorly described Mechanisms regulating retention, excretion or interactions between nutrients at sites of cell transport or storage proteins were needed further investigations, but studies such as presented here can direct future research toward biochemical descriptions of nutrient interactions.

CONCLUSION

The present results indicated a lively placenta transport of Fe, Zn, Mg, Mn, Cu and Se appear to be exchanged actively between dam and fetus. Fe, Zn, Cu, Mg, Se and Mn exchanged passively between dam and amniotic fluid. Evaluation of Fe, Zn, Cu, Mg, Se and Mn in maternal serum did not appear to be useful within the assessment of fetal growth**.**

DECLARATION

Acknowledgements

The authors thank all pastoralists in Daraw Village, in Aswan province, Egypt and every one members of the laboratory belonging to Aswan University for his or her help during the study.

Competing interests

The authors declare that they have no conflict of interest exists.

Author`s contribution

Walaa M. Essawi collected the samples, designed the experiment, performed laboratory analyses and participated in the preparation of the manuscript. Hagar F. Gouda contributed to data analysis and prepared the manuscript (writing and revision). All authors approved the final version of manuscript before publication.

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To cite this paper: Essawi WM and Gouda HF (2020). Trace Elements Profiles of Pregnant Camels (*Camilus dromedaries*), Fetus and Amniotic Fluid at Birth and their Association with Calf Birth Weight. *World Vet. J.*, 10 (3): 312-319. DOI: https://dx.doi.org/10.36380/scil.2020.wvj40

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 320-324, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj41

Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions

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ORIGINAL ARTICLE
pii: S232245682000041-10
Received: 23 Jul 2020
Accepted: 05 Sep 2020

ABSTRACT

Modern pig farming worldwide has been facing substantial economic loss due to perinatal mortality which is mainly associated with the farrowing process. Therefore, the present study aimed to identify factors affecting the farrowing duration in natural farrowing sows in the intensive indoor conditions. In total, 210 farrowing sows in 4 commercial farms were included in the study. The association between potential risk factors and farrowing duration was analyzed by using general linear models. Two final models demonstrated that the number of total born piglets, number of stillborn and mummified piglets, litter weight, and average birth weight were significantly associated with farrowing duration. Among the four factors, average birth weight had a negative association with whereas the other three factors had positive associations with farrowing duration. Two models explained about 19.1-19.5% variation of the farrowing duration. The results also demonstrated that the number of total born piglets, stillborn, and mummified piglets were more important than litter weight and average birth weight in explaining the variation of farrowing duration.

Keywords: Birth weight; Farrowing duration, Sow, Stillbirth, Total born

INTRODUCTION

Modern pig farming has been confronted with substantial economic loss and animal welfare problem due to perinatal mortality (van Dijk et al., 2005). A majority of stillbirth (66.8%) occurs during expulsive stage of farrowing process, and further, about 16.4% of stillbirths occur shortly postpartum (Leenhouwers et al., 2003) with a similar disease etiology (Rangstrup-Christensen et al., 2017). Prolonged farrowing duration increases the risk of umbilical cord rupture and asphyxia (Mota-Rojas et al., 2006), which predisposes piglets to stillbirth (Borges et al., 2005; Canario et al., 2006), reduces postnatal viability by delaying first contact with udder, and lowers growth rate and survival rate over 10 days postpartum (Herpin et al., 1996) and before weaning (Rootwelt et al., 2013). Thus, farrowing duration is clearly an important indicator for survival of piglets.

Effects of various factors on farrowing duration has been investigated with controversial results (van Dijk et al., 2005; Motsi et al., 2007; Bjorkman et al., 2018). The disparity in results may be attributable to the difference in farrowing conditions (Oliviero et al., 2010), sow breeds (van Dijk et al., 2005), the increase in litter size over time (Koketsu et al., 2017), and number of factors that were investigated in different studies (van Dijk et al., 2005; Motsi et al., 2007).

The aim of this study was to evaluate effects of various factors including parity, gestation length, number of total born piglets, litter weight, average birth weight, presence or absence of stillborn and mummified piglets in a litter, and number of stillborn and mummified piglets in a litter on farrowing duration of sows raised in intensive indoor farrowing conditions.

MATERIALS AND METHODS

Ethical approval

This study was performed according to all ethics and animal rights of Vietnam National University of Agriculture, Vietnam.

Animals

Data were collected from 210 Landrace x Yorkshire crossbred sows raised in four commercial farms in three provinces (Hung Yen, Bac Giang and Thai Binh) in the North of Vietnam. All sows were artificially inseminated with semen from Duroc boars. During gestation, sows were fed 1.8-4.0 kg of commercial feed containing 13-17% crude

To cite this paper: Hoai Nam N and Sukon P (2020). Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions. *World Vet. J.*, 10 (3): 320-324. DOI: https://dx.doi.org/10.36380/scil.2020.wv41

protein and metabolizable energy of 2900-3100 Kcal/kg (Hi-Gro 566, 567S, Charoen Pokphan, Vietnam). All sows were vaccinated against classical swine fever, porcine respiratory and reproductive syndrome, foot and mouth disease, porcine circovirus disease and Aujeszky's disease. Sows were kept in individual gestation crates sized about 60 cm x 220 cm. About a week before estimated farrowing date, sows were removed to farrowing crates with the size of 180 cm x 220 cm. The sow area, with a slatted floor and similar size as gestation crates, was located in the middle of the farrowing crates. Water was provided *ad libitum* through a nipple drinking system.

Data collection

This study was conducted during June to November, 2019. In each farm, data were collected by 2 to 3 veterinarians who had been trained on data collection. Signs of milk letdown, vulva swelling, mucous secretion were monitored carefully to detect the time of parturition. Data including sows number, parity, gestation length, number of total born (NTB), number of live-born, number of stillborn, number of mummified, litter weight, average birth weight (ABW), and duration of farrowing were recorded. Gestation length was calculated as the interval from the first insemination to the date of farrowing. The NTB was calculated as sum of number of live-born, stillborn and mummified piglets. Live-born and stillborn piglets were weighted individually. Litter weight was the sum of weight of individual piglet in a given litter. Average birth weight was calculated by dividing litter weight by the number of live-born and stillborn piglets. Farrowing duration was the interval between the first and the last piglet expulsion. None of the sows were induced for parturition. Any sows that were administered oxytocin during farrowing or needed manual extraction were excluded from observation, and data of such sows were not used in any analysis.

Statistical analysis

General Linear Models (GLM) were used to determine factors that affected the farrowing duration. Farrowing duration was transformed by using natural logarithm to obtain normal distribution. Risk factors were farms, parity, gestation length, NTB, litter weight, ABW, presence or absence of stillborn and mummified piglets in a litter (PSM), and number of stillborn and mummified piglets in a litter (NSM). Initially, each factor was included in the GLM as a sole explanatory variable for farrowing duration. Only variables significant at $P \leq 0.25$ (Hosmer and Lemeshow, 2000) in the univariate analysis were further analyzed in combination with others to find out suitable models. The NTB and litter weight ($r = 0.817$, $P < 0.01$), PSM and NSM ($r = 0.651$, $P < 0.01$) were highly correlated, thus they were analyzed in different models. Models were built up by considering the pairs of variables (NTB and NSM, NSM and litter weight, litter weight and PSM), followed by adding the third variable if the added variable conferred a significant effect signaling by a P-value <0.05. All statistics were carried out in Statistical Package for the Social Sciences (SPSS) version 22 (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY).

RESULTS

Descriptive statistics

In total, data from 210 litters were included in this study. The average farrowing duration was 215 ± 122 minutes. Of the 210 litters investigated, 64.8% (136/210) had at least one stillborn or mummified piglet, and 61.0% (128/210) of litters had at least one stillborn piglet. Thirty-nine out of 210 litters (18.6%) had at least one mummified piglet. Totally, 2995 piglets were born from 210 sows, among them 2685 (89.6%), 251 (8.4%) and 59 (2.0%) were live-born, stillborn and mummified piglets, respectively. Descriptive statistics of some reproductive traits of studied sows are presented in Table 1.

Table 1. Descriptive statistics for some reproductive traits of 210 investigated sows from four farms in the North of Vietnam in 2019.

Parameter	Mean ± SD	Minimum	Maximum
Parity	4.6 ± 2.5	1	10
Gestation length (days)	115.3 ± 1.8	110	120
Number of total born piglets	14.3 ± 3.3	4	24
Number of live-born piglets	12.8 ± 3.2	4	22
Number of stillborn piglets	1.2 ± 1.5	0	11
Number of mummified piglets	0.3 ± 0.6	0	4
Litter weight (kg)	19.2 ± 4.6	4.4	34
Average birth weight (kg)	1.36 ± 0.19	0.84	1.96
Farrowing duration (minutes)	215 ± 122	22	1226

SD: standard deviation

To cite this paper: Hoai Nam N and Sukon P (2020). Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions. *World Vet. J.*, 10 (3): 320-324. DOI: https://dx.doi.org/10.36380/scil.2020.wv41

Factors influencing the farrowing duration

At the initial stage of GLM, the log-transformed farrowing duration had no significant correlation with farms and gestation length (Table 2). In the next step, parity and PSM were rejected from final models due to their nonsignificant effect. Finally, two models were built to explain the variation of farrowing duration (Table 3). The first model contained three variables including NSM, litter weight and ABW (R^2 = 0.191) and the second model consisted of two variables including NTB and NSM (R^2 = 0.195). Univariate analysis showed that the most explanatory variables were NTB and NSM with R^2 = 0.134 and 0.137, respectively. The NTB, NSM, and litter weight positively correlated with farrowing duration. By contrast, ABW was negatively associated with farrowing duration.

Table 2. Univariate analysis of factors associated with log-transformed farrowing duration in 210 sows from four farms in the North of Vietnam in 2019

Variables	Significance	Inclusion in the multivariate analysis
Farms	0.283	Excluded
Parity	0.202	Included
Gestation length	0.560	Excluded
NTB	< 0.001	Included
Litter weight	0.001	Included
ABW	0.005	Included
PSM	0.008	Included
NSM	< 0.001	Included

NTB: number of total born piglets; ABW: average birth weight; PSM: presence or absence of stillborn and mummified piglets; NSM: number of stillborn and mummified piglets.

Table 3. Multivariate analysis of the factors associated with log-transformed farrowing duration in 210 sows from four farms in the North of Vietnam in 2019

Model	Parameter	Regression coefficient	95% CI	Significance
Model 1	Intercept	5.421	4.981 - 5.861	< 0.001
	NSM	0.075	0.039 - 0.110	< 0.001
	Litter weight	0.022	0.008 - 0.036	0.002
	ABW	-0.504	-0.831 - (-0.176)	0.003
Model 2	Intercept	4.638	4.381 - 4.895	< 0.001
	NTB	0.036	0.018 - 0.055	< 0.001
	NSM	0.072	0.036 - 0.108	< 0.001

NSM: number of stillborn and mummified piglets; ABW: average birth weight; NTB: number of total born piglets; CI; confidence interval; Model 1 conferred a R^2 = 0.191; Model 2 conferred a R^2 = 0.195.

DISCUSSION

The farrowing duration has been reported to range between 130 and 396 minutes (van Rens and van der Lende, 2004; van Dijk et al., 2005; Oliviero et al., 2010; Bjorkman et al., 2017). The result of present study was very close to the findings obtained by Motsi et al. (2007) who demonstrated that average farrowing duration of 136 sows was 217 ± 160 minutes. The incidence of stillbirth at litter level in the present study (61.0%) was higher than the results reported by Cozler et al. (2002) and Borges et al. (2005). However, the stillbirth rate (8.4%) was still in the range of previous reported results (Lucia et al., 2002; Borges et al., 2005; Vanderhaeghe et al., 2010; Nam and Sukon, 2020).

The nonsignificant effect of parity on farrowing duration in this study confirmed the findings by several other works (Fahmy and Friend, 1981; van Dijk et al., 2005; Motsi et al., 2007; Oliviero et al., 2010). However, it did not agree with results found by Bjorkman et al. (2017) who reported that parity was positively correlated with farrowing duration. The disparity in results among studies may be due to the difference in methods of analysis. Whereas Bjorkman et al. (2017) evaluated effects of parity on farrowing duration without adjustment for other factors, the present study and others (van Dijk et al., 2005; Oliviero et al., 2010) did that in combination with other variables. The nonsignificant effect of parity on farrowing duration in the present study may be attributable to relatively high proportion of high parity sows. Sows at higher parity might have been more strictly selected based on their reproductive criteria including the farrowing duration. Thus, the selection may exert their effect on the heterogeneity of farrowing duration among different parities, and may thereby alleviate the effect of parity on farrowing duration.

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This study failed to detect any significant effect of gestation length on farrowing duration. This finding confirmed the results of Oliviero et al. (2010). Some authors found a positive correlation between these two criteria (Fahmy and Friend, 1981), by contrast, others found a negative association (van Dijk et al., 2005). Gestation length had a negative correlation with litter size (Sasaki and Koketsu, 2007; Rydhmer et al., 2008), thus it was expected to have a negative association with farrowing duration. However, in the current study, such association between gestation length and litter size did not exist, giving a possible explanation for the nonsignificant association between gestation length and farrowing duration.

It is not clear whether long farrowing duration induces stillbirth or stillbirth increases farrowing duration (van Dijk et al., 2005). Many studies found a positive association between these two factors (Borges et al., 2005; van Dijk et al., 2005; Canario et al., 2006). Also, Motsi et al. (2007) substantiated that PSM was positively correlated with farrowing duration. By contrast, current study failed to detect any significant effect of PSM on farrowing duration. Firstly, it was possible that the association between PSM and other factors such as litter weight ($r = 0.269$, $P < 0.01$) and ABW ($r = -0.168$, $P < 0.05$) alleviated the effect of PSM on farrowing duration when they were put in the same models. Secondly, using PSM as an explanatory variable for farrowing duration erroneously implies that all litters with stillborn and/or mummified have equal number of these piglet types. Thus, NSM may be a better factor than PSM for explaining variation of farrowing duration because it represents the quantity of stillborn and/or mummified piglets. Indeed, this study found that increased NSM elevated farrowing duration. This result confirmed many previous findings by several authors (van Dijk et al., 2005; Oliviero et al., 2010; Bjorkman et al., 2017). A possible reason for the finding was that only live piglets could actively move through the pelvic canal (Taverne and van der Weijden, 2008), therefore, dead piglets could not participate into the farrowing process to obtain a right position, resulting in increased farrowing duration (van Dijk et al., 2005).

Positive association between farrowing duration and NTB in this study corroborated previous findings (Fahmy and Friend, 1981; van Dijk et al., 2005). Another study found the association between number of live-born, instead of NTB, and farrowing duration (Motsi et al., 2007). In the current study, the number of live-born was not included in the analysis because this factor did not confer practical significance for plans to reduce farrowing duration and stillbirth. On one hand, increased NTB increased litter weight ($r = 0.817$, $P < 0.01$), and as the consequence, the litter weight also positively correlated with the farrowing duration. However, this result was not in the agreement with previous study that detected no association between litter weight and farrowing duration (Motsi et al., 2007). It was not a surprise because that study did not find any correlation between NTB and farrowing duration. On the other hand, increased NTB resulted in decreased ABW ($r = -0.267$, $P < 0.01$), therefore, this study found a negative association between ABW and farrowing duration. This finding was in contrast to the previously published results that demonstrated a positive association between ABW and farrowing duration (Motsi et al., 2007). Bigger piglets, in comparison with their small littermates, may have thicker placenta, and they need more time to break the placenta to start their journey through uterus towards the birth canal (van Rens and van der Lende, 2004). They are also subjected to higher friction on the walls of the birth canal (Motsi et al., 2007). Collectively, it takes longer time for heavier piglets to be delivered. However, as mentioned above, ABW was negatively associated with NTB, and it seemed that, in comparison with ABW, NTB had a stronger effect on farrowing duration. Indeed, in this study the NTB explained 13.4% variation of farrowing duration compared with 3.7% by ABW. Therefore, increased ABW reduced farrowing duration.

CONCLUSIONS

This study demonstrated that the number of total born piglets and the number of stillborn and mummified piglets in a litter were the most important factors affecting duration of farrowing of sows in an intensive system. Litter weight and average birth weight also affected farrowing duration but to a lesser extent.

DECLARATIONS

Acknowledgements

The authors are grateful to Luong Van Dung, Nguyen Thi Thu, Nguyen Thi Ngan, Nguyen Thi Hong Nhung, Tran Van Hoa, Nguyen The Nam, Hoang Thi Quynh, Nguyen Thi Sinh, Dang Van An for their assistance in data collection.

Competing interests

The authors declare that there is no conflict of interests.

Authors' contribution

NHN collected data. NHN and PS conceived and designed the study, analyzed data and wrote the manuscript. Both authors approved the final manuscript.

To cite this paper: Hoai Nam N and Sukon P (2020). Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions. *World Vet. J.*, 10 (3): 320-324. DOI: https://dx.doi.org/10.36380/scil.2020.wv41

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To cite this paper: Hoai Nam N and Sukon P (2020). Associated Factors for Farrowing Duration in Sows with Natural Parturition in Intensive Conditions. *World Vet. J.*, 10 (3): 320-324. DOI: https://dx.doi.org/10.36380/scil.2020.wv41

2020, Science line Publication
World's Veterinary Journal
World Vet J, 10(3): 325-331, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj42

Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains

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ORGINAL ARTICLE
pii: S232245682000042-10
Received: 28 Jun. 2020
Accepted: 10 Aug 2020

ABSTRACT

Vibrio species are the most common and serious pathogens in fish and shellfish marine aquaculture worldwide. The present study aimed to determine the prevalence of *Vibrio* spp. in seabass and seabream in fish markets, especially streptomycin-resistant strains that have great public health importance. A total of 30 seabass (*Dicentrarchus Labrax*) and 30 seabream (*Sparus aurata*) were purchased from fish markets at Kafr El Sheikh Governorate and subjected to bacteriological examination. The PCR assay was used for the detection of virulence genes (*tdh* and *trh*), aminoglycoside resistance gene (*aadA1)*, and *toxR* gene. The results indicated that the total prevalence of *Vibrio* spp. was 26.66%, including *V. parahaemolyticus* (8.3%), *V. alginolyticus* (8.3%), *V. mimicus* (3.3%), *V. harveyi* (5%) and *V. vulnificus* (1.6%). The *toxR*, *trh*, and *aadA1* genes were found in all *V. parahaemolyticus* isolates while *tdh* gene was found in 80% of isolates. Antimicrobial sensitivity test of *V. parahaemolyticus* isolates showed sensitivity to ciprofloxacin, norfloxacin, cefotaxime, and chloramphenicol. *Vibrio parahaemolyticus* isolates were resistant to ampicillin, erythromycin, streptomycin, and gentamycin. The present results indicated that good hygienic measures should be taken to avoid infection with *Vibrio* species, especially *V. parahaemolyticus* that can pose a great risk to human health.

Keywords: Antibiotic resistance, Seabass, Seabream, Streptomycin, *Vibrio parahaemolyticus*.

INTRODUCTION

Vibrio genus contains Gram-negative, halophilic, rod-shaped, non-spore forming, oxidase-positive bacteria, which widespread in the coastal and estuarine environments (Austin and Austin, 2007). *Vibrio parahaemolyticus* is the most recorded pathogenic species of *Vibrio* genus and affects persons who consume improperly cooked or raw seafood **(**Raissy et al., 2015). This foodborne bacteria is reported as the main cause of seafood-borne illness in Egypt and many other countries around the world such as United States, Malaysia, Thailand, Korea, China, and Japan (Yoon et al., 2008; Iwahori et al., 2010; Abdel-Azeem et al., 2016**)**. Infection with *V. parahaemolyticus* may cause acute human gastroenteritis, the major symptoms of which are headache, diarrhea, abdominal pain, and in some cases, septicemia (Broberg et al., 2011; Wang et al., 2015; Su and Liu, 2017). In coastal areas of the world, like Japan, *V. parahaemolyticus* has been regularly recognized as the main cause of sporadic cases of gastroenteritis (Qadri et al., 2005; Wang et al., 2017). In China, about 322 gastroenteritis outbreaks due to *V. parahaemolyticus* infection were reported from 2003 to 2008 (Wu et al., 2014). Multiplication of *V. parahaemolyticus* is related to water temperature and season (Deepanjali et al., 2005; Angela et al., 2006), with the highest prevalence in summer due to the higher salinity of water than other seasons (Zulkifli et al., 2009).

The pathogenicity of bacteria depends mainly on some virulence factors and virulence genes, which act together as major orchestrators. The most virulence genes leading to pathogenicity of *V. parahaemolyticus* are hemolysin genes (*tdh* and *trh*) (Hiyoshi et al., 2010). Molecular epidemiological studies demonstrated a clear relation between the hemolysin genes and disease-causing ability of *V. parahaemolyticus* (Kishishita et al., 1992; DePaola et al., 2003; Vongxay et al., 2008; Chao et al., 2009; Han et al., 2015; Hasrimi et al., 2018**)**. These two genes were recorded in the most isolates from clinical cases of *V. parahaemolyticus* infections **(**Bej et al., 1999; Rojas et al., 2011). The *tdh* and *trh* genes encode virulence factors of thermostable direct hemolysin (TDH), and TDH-related hemolysin (TRH), respectively, which are involved in important pathogenic activities, such as enterotoxicity, hemolytic activity, cytotoxicity and cardiotoxicity (Shirai et al., 1990; Osawa et al., 1996).

The *toxR* gene is a pandemic marker gene for all *V. parahaemolyticus* strains either pathogenic or nonpathogenic one, and it was recorded in some other *Vibrio* species (Kim et al., 1999). The sequence of *toxR* gene can be used for molecular identification of *V. parahaemolyticus* (Yung et al., 1999; Hubbard et al., 2016). The *aadA1* and *aadA2* encode

To cite this paper: Adel M. El-Gamal and Engy F. EL-Bahi (2020). Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains. *World Vet. J.,* 10 (3): 325-331. DOI: https://dx.doi.org/10.36380/scil.2020.wvj42

aminoglycoside adenyl transferase and confer resistance to streptomycin, already are detected in *Vibrio* species isolates (Dalsgaard et al., 2001).

Cooking and frying of marine fish reduce the count of *V. parahaemolyticus.* After cooking (in oven 120 ºC for 35 min), the percentage reduction in total count of *V. parahaemolyticus* was 98.2%, while after frying for 10 min at 190 °C, *V. parahaemolyticus* was completely destroyed and the percentage reduction was 100% (Saad et al., 2015); or even boiling at 64 °C for more than 90 seconds can kill *V. parahaemolyticus* (ICMSF, 1996). The bacteria will be removed by using high cooking temperature, although the toxin might remain in the foodstuff depending on the processing conditions (Raissy et al., 2015). The current study aimed to determine the prevalence of *Vibrio* spp., especially streptomycin-resistant strains, in seabass and seabream in fish markets of Kafr El Sheikh Governorate, Egypt.

MATERIALS AND METHODS

Samples collection

Thirty seabass and 30 seabream with a weight range of 100-250 g were purchased from fish markets at Kafr El Sheikh Governorate from February to August 2019. All samples were transferred in ice box to Animal Health Research Institute, Kafr El Sheikh laboratory, Egypt.

Bacteriological examination

Bacteriological examinations were done according to ISO/ TS 21872-1 (2007) and ISO/ TS 21872-2 (2007).

Samples preparation

After skin sterilization with alcohol, the muscles above the lateral line were removed, 25 g of each fish sample were mixed with 225 ml of alkaline saline peptone water (APW, pH 8.6) in a Stomacher bag. After that, these mixtures were incubated at 37 °C for 8-16 hours.

Isolation of *Vibrio* species

After the incubation period, the upper layer of the alkaline saline peptone water (APW) enrichment broth was inculcated on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, UK), and then these plates were incubated at 37 °C for another 18-24 hours. After that, growing colonies were used for further screening tests including Gram staining, oxidase and catalase tests.

Biochemical identification

Suspected colonies of *Vibrio* spp. on TCBS media and positive oxidase test were subjected to further identification by Microbact GNB kit (Oxoid, UK).

Polymerase chain action

Suspected isolates of the *V. parahaemolyticus* were examined by using PCR for the detection of virulence genes (*tdh* and *trh*), *toxR* gene, and *aadA1* gene. DNA extraction were performed according to the manufacturer's recommendations by using the QIA amp DNA Mini kit (Qiagene, Germany, GmbH). Oligonucleotide primers were supplied from Metabion (Germany). The primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan) using an Applied Biosystem 2720 thermal cycler. Primers used and PCR conditions are presented in Table 1. The products of PCR were separated by electrophoresis on 1% Agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5 V/cm. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Antimicrobial susceptibility test

Antimicrobial disk susceptibility test were performed as described by the Clinical and Laboratory Standards Institute (CLSI, 2012).

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target genes	Primers Sequence (5'-3')		Amplified segment (base pair)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
toxR	F	*GTCTTCTGACGCAATCGTTG*	368	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Kim et al., 1999
	R	*ATACGAGTGGTTGCTGTCATG*							
aadA1	F	*TATCAGAGGTAGTTGGCGTCAT*	484	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Randall et al. 2004
	R	*GTTCCATAGCGTTAAGGTTTCATT*							
trh	F	*GGCTCAAAATGGTTAAGCG*	250	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Mustapha et al., 2013
	R	*CATTTCCGCTCTCATATGC*							
tdh	F	CCATCTGTCCCTTTTCCTGC	373	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 40 sec.	72˚C 7 min.	
	R	*CCAAATACATTTTACTTGG*							

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RESULTS AND DISSCUSION

Vibrio spp. commonly inhabit the marine environments and can be found in the fresh water (Sujeewa et al., 2009). Seafood may be a vehicle for most of the bacterial pathogens such as *Vibrio* spp. (Huss, 1997). Various outbreaks of bacterial disease associated with seafood consumption have been reported (Friesema et al., 2012). Recently, *V. parahaemolyticus* recoded as an important species causing seafood infection associated with gastroenteritis illness in humans.

Table 2 shows that the total incidence of *Vibrio* spp. isolated from the examined seabass and seabream samples is 26.66% (16 out of 60 samples). Raissy et al. (2015) and Azwai1 et al. (2016) recorded nearly similar results (22% and 22.9%, respectively). however, the result of present study is lower than that recorded by Pal and Das (2010), Saad et al. (2015), Abdel-Azeem et al. (2016), Fri et al. (2017), and Hemmat et al. (2018).These differences may be due to difference in the type of examined fish, difference in the method of bacterial isolation, or difference in the hygienic state of fish sources. Additionally, the difference in results can be attributed to difference in season of sampling, as *Vibrio* spp. has been reported to have higher concentrations in summer seasons due to higher water salinity levels than other seasons (Zulkifli et al., 2009). As presented in table 2, several *Vibrio* strains were isolated from examined seabass and seabream, including *V. parahaemolyticus* (8.3%), *V. alginolyticus* (8.3%), *V. mimicus* (3.3%), *V. harveyi* (5%) and *V. vulnificus* (1.6%). *Vibrio cholera* was not detected in the studied samples. The examined seabass fish were more infected with *V. parahaemolyticus* than the examined seabream fish which may be due to the hygienic state of each fish source.

Similarly, Saad et al. (2015) isolated *V. parahaemolyticus* (10%), *V. fluvialis, V. vulnificus, V. alginolyticus , V. mimicus,* and *V. damsel* from *Tilapia nilotica* and *Mugil Cephalus*. Hemmat et al. (2018) isolated *V. parahaemolyticus* (12%), *V. mimicus, V. alginolyticus*, *V. cholera*, *V. vulnificus,* and *V. fluvialis* from *Oreochromis niloticus*, *Mugil Cephalus*, shrimp and crab. Raissy et al. (2015) isolated *V. harveyi* that was the most frequent species isolated, followed by *V. parahaemolyticus* (3.5%), *V. mimicus, V. vulnificus,* and *V. alginolyticus* from some marine fish and shrimps. Fri et al. (2017) isolated *V. fluvialis, Vibrio vulnificus,* and *V. parahaemolyticus* (5.45%) from dusky kop fish and sea water. Pal and Das (2010) isolated *Vibrio parahaemolyticus* with a high prevalence (35%) from shrimp, prawn, bhetki, pamfret and hilsa. According to the Egyptian Organization for Standardization and Quality Control (EOSQC, 2005), any seafood products must be free from *V. parahaemolyticus*.

As shown in table 3 and figure 1, all examined *Vibrio parahaemolyticus* isolates were positive for *toxR* gene. This result support finding of Yung et al. (1999); Pal and Das (2010), who reported that *toxR*-targeted PCR protocol can be used for *V. parahaemolyticus* detection. Also, all examined *Vibrio parahaemolyticus* isolates were positive for *aadA1* gene (Figure 2). Taviani et al. (2008) stated that *aadA1* gene is responsible for antibiotic resistance against aminoglycoside group including streptomycin in *Vibrio* spp. isolates from shellfish and other marine fish.

Pathogenicity of *V. parahaemolyticus* is conferred either by *tdh*, and/or *trh* (Yamaichi et al., 1999**)**. As shown in table 3, all examined *V. parahaemolyticus* isolates were positive for *trh* gene (figure 3), and 80% were positive for *tdh* gene (Figure 4). The results did not match with that reported by Rojas et al. (2011) who detected *tdh* gene in 10.5% of *V. parahaemolyticus* isolates, while *trh* gene was not found. Also, Pal and Das (2010) recorded *tdh* gene in 35% of *V. parahaemolyticus* isolated from fish samples while *trh* gene was found only in 1.7% of *V. parahaemolyticus* isolates. Wang et al. (2017) recorded the virulence genes; *tdh* and *trh* with 87.9% and 3.7% of examined *V. parahaemolyticus* strains, respectively. Fri et al. (2017) recorded *trh* gene as 9.46% in examined *V. parahaemolyticus* strains, while Wong et al. (2000) recorded only one *V. parahaemolyticus* isolate (1.4%) harboring *trh* gene, but did not detect *tdh* gene among the examined *V. parahaemolyticus* isolates.

Antimicrobial susceptibility test showed that *V. parahaemolyticus* isolates were sensitive to ciprofloxacin, norfloxacin, cefotaxime, and chloramphenicol while they were resistant to ampicillin, erythromycin, streptomycin, and gentamycin (Table 4). These results indicate that the examined strains were resistant to most members of the aminoglycoside group, which may be due to the fact that *aadA1* gene was detected in all examined *V. parahaemolyticus* isolates. This result is nearly similar to that recorded by Rojas et al. (2011), who reported that *V. parahaemolyticus* had resistance to streptomycin and ampicillin with intermediate susceptibility to gentamicin.

Table 2. Prevalence of *Vibrio* species in examined seabass and seabream fish, Egypt.

***Vibrio* spp.**	**Number of positive samples**		
	Seabass (n=30)	**Seabream (n=30)**	**Total (%)**
V. parahaemolyticus	1	4	5 (8.3)
V. alginolyticus	3	2	5 (8.3)
V. mimicus	0	2	2 (3.3)
V. harveyi	1	2	3 (5)
V. vulnificus	1	0	1 (1.6)
Total	6	10	16(26.6)

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Table 3. Distribution of virulence genes among examined isolates of *Vibrio parahaemolyticus* isolated from seabass and seabream fish.

Sample No.	*toxR*	*tdh*	*trh*	*aadA1*
1	+	+	+	+
2	+	-	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+

Table 4. Results of agar disc diffusion test of *Vibrio parahaemolyticus* isolated from marine fish

Antibiotic	Disc symbol & concentration (µg/disc)	Result
Norfloxacin	Nor (10)	S
Erythromycin	E (15)	R
Ampicillin	AMP (10)	R
Amoxicillin + clavulinic acid	AMC (30)	S
Cefotaxime	CTX(30)	S
Doxycycline	DO (30)	R
Streptomycin	S(10)	R
Sulpamethazol + Trimethoprim	SXT(25)	R
Chloramphenicol	C (30)	S
Gentamycin	CN(10)	R
Ciprofloxacin	Cip (5)	S

S: Sensitive R: Resistant

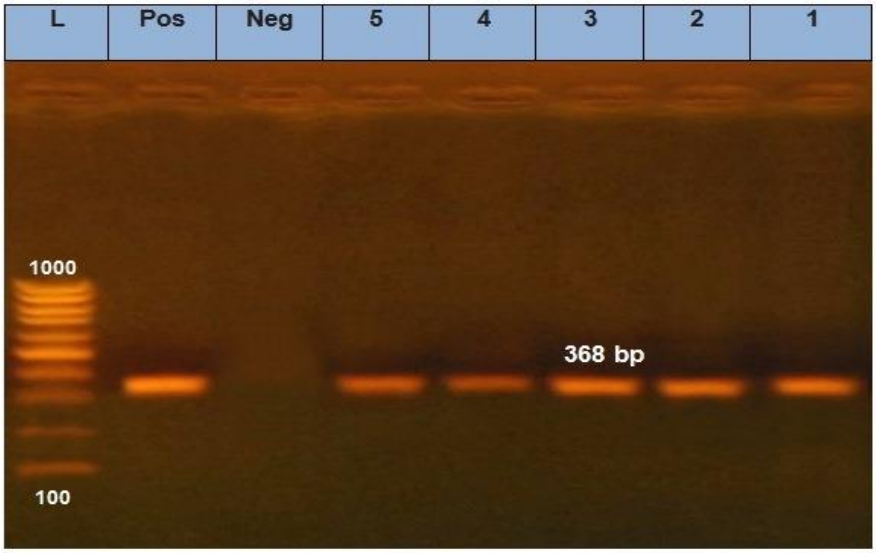

Figure 1. Agarose gel electrophoresis of PCR amplification of *toxR* gene (368 bp) of *Vibrio parahaemolyticus.* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1-5: Positive samples.

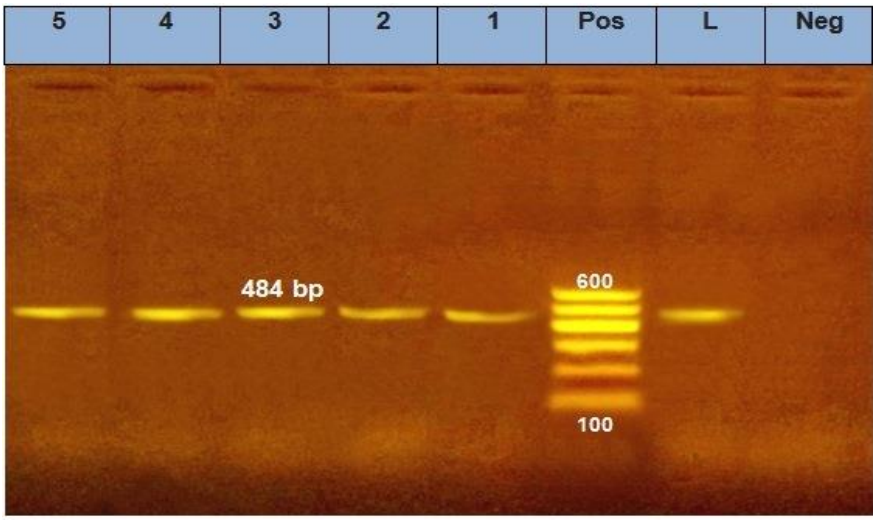

Figure 2. Agarose gel electrophoresis of PCR amplification of *aadA1 gene* (484 bp) of *Vibrio parahaemolyticus.* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1-5: Positive samples.

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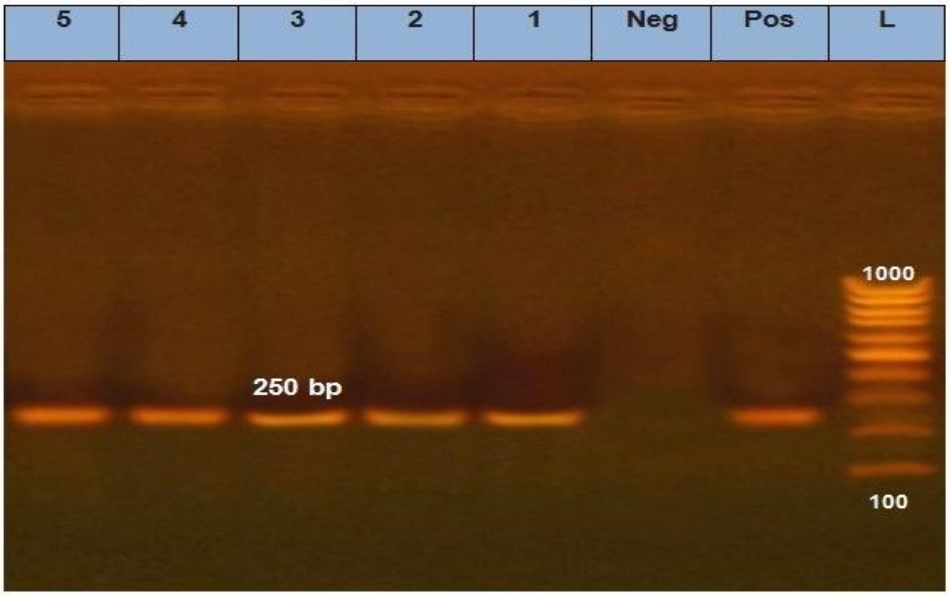

Figure 3. Agarose gel electrophoresis of PCR amplification of *trh* gene (250 bp) of *Vibrio parahaemolyticus.* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1-5: Positive samples.

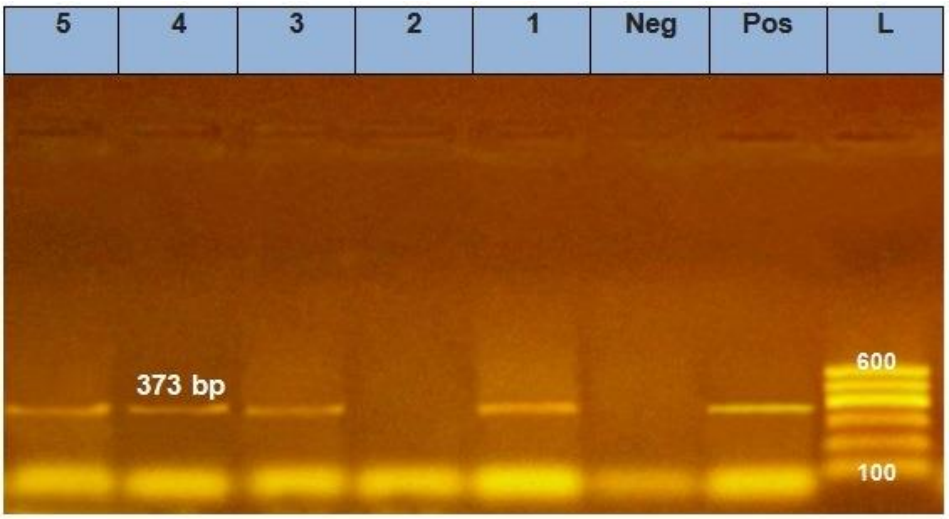

Figure 4. Agarose gel electrophoresis of PCR amplification of *tdh* gene (373bp) of *Vibrio parahaemolyticus*. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1,3,4 and 5: positive samples, Lane 2: negative sample.

CONCLUSION

Vibrio spp. especially *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, and *V. vulnificus* are commonly isolated from seabass and seabream fish, which affects persons who consume improperly cooked or raw seafood. Most of these bacteria have antibiotic resistance genes that pose a great risk to human health; therefore, good hygienic measures should apply to avoid such infections.

DECLARATIONS

Acknowledgments

The authors would like to thank all member of Animal Health Research Institute, Egypt, for their kindly help during the investigation and paper preparation.

Authors' contributions

All authors participated equally in study design, data collection, data analysis, writing, and approving the final manuscript**.**

Competing interests

The authors declare that they have no competing interests.

To cite this paper: Adel M. El-Gamal and Engy F. EL-Bahi (2020). Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains. *World Vet. J.,* 10 (3): 325-331. DOI: https://dx.doi.org/10.36380/scil.2020.wvj42

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To cite this paper: Adel M. El-Gamal and Engy F. EL-Bahi (2020). Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains. *World Vet. J.*, 10 (3): 325-331. DOI: https://dx.doi.org/10.36380/scil.2020.wvj42

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 332-337, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj43

Effect of Dietary Dried Fennel and Oregano and Thyme Supplementation on Zootechnical Parameters of Growing Rabbits

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ORIGINAL ARTICLE
pii: S232245682000043-10
Received: 17 Jul 2020
Accepted: 29 Aug 2020

ABSTRACT

The objective of this study was to analyze and compare the effects of fennel, oregano, and thyme dietary supplements on the feeding of rabbits. In this regard, 96 weaned rabbits (30-day-old), white New Zealand, were divided into 4 groups and submitted to the following dietary treatments: Control diet, F diet (Control diet + 5% *Foeniculum vulgaris*), O diet (Control diet + 5% Origanum compactum), and T diet (Control diet + 5% *Thymus capitatus*). The essential oils of the above mentioned aromatic plants were extracted and were analyzed using a gas chromatograph coupled to a mass spectrometer. The treatment of fennel, oregano, and thyme had no beneficial effects on the growth performance of the rabbits but reduced the mortality rate. The phenylpropanoid and the phenolic monoterpenes were the major components of *Foeniculum vulgaris*, *Origanum compactum*, *and Thymus capitatus* essential oils. The aromatic plants and their active compounds can be used as additives in rabbit nutrition.

Keywords: Essential oil, Fennel, Growth performance, Oregano, Rabbit, Thyme

INTRODUCTION

The ban on antibiotics as growth promoters in feed by the European Commission (EC) in January 2006 due to cross and multiple bacterial resistances (Huyghebaert et al., 2011) has led to the search of new practices to decrease rabbit health problems. The use of aromatic plants to reduce the rate of health incidents and death from diarrhea and to increase production efficiency was one alternative additive tried in growing rabbits (Efterpi et al., 2012).

Foeniculum vulgare was used for bacterial, fungal and viral infections. It has anticolitic activity and was indicated in the treatment of stomatitis, spastic gastrointestinal disturbances, abdominal cramps and flatulence (Badgujar et al., 2014; Al-Snafi, 2018). *Origanum compactum* has been traditionally used to treat diarrhea (Ennabili et al., 2004) and *Thymus capitatus*is widely used as a stomachic (Megdiche-ksouri et al., 2015). The antibacterial and antifungal activities of the essential oils of oregano and thyme were established by several studies (Bounatirou et al., 2007; Bouhdid et al., 2009; El Ouariachi et al., 2011; Bouyahya et al., 2016; El Jalel et al., 2018). The aim of this study is to examine the effects of *Feoniculum vulgaris and Origanum compactum* and *Thymus capitatus*as natural feed supplements on growth performance in weaned rabbits.

MATERIAL AND METHODS

Medicinal plants

The areal parts of tree aromatic and medicinal plants *Foeniculum vulgaris*, *Origanum compactum* and *Thymus capitatus* were collected in northern Morocco. Identification of Aromatic plants was executed by Professor Bakkali, a specialist in botany, in the Laboratory of Biotechnology and Biomolecular Engineering. Afterwards, the leaves were separated and dried at room temperature for two weeks in the absence of light and then stored in sealed paper bags until their use for analyses.

Animals and experimental procedure

A total of 96 weaned rabbits (30 days old; white New Zealand (900 ± 100 g initial weight), were divided into four groups and submitted to the following dietary treatments (Table 1). The first group was control diet, the second group was F diet (Control diet + 5% *Foeniculum vulgaris* leaves), the third group was O diet (Control diet + 5% *Origanum compactum* leaves) and the last group was T diet (Control diet + 5% *Thymus capitatus* leaves). Rabbits were kept in standard cages with 6 animals per cage (2010/63/EU Official Journal of the EU 2010) in a building with temperatures between 15 and 20 °C. The length of daily illumination was 16 hours. The rabbits had access to feed and water *ad libitum*. Body weight of rabbits and feed consumptions were measured every week during the experiment as well as mortality rates.

To cite this paper: Benlemlih M, Barchan A, Aarab A, Bakkali M, Arakrak A and Laglaoui A (2020). Effect of Dietary Dried Fennel and Oregano and Thyme Supplementation on Zootechnical Parameters of Growing Rabbits. *World Vet. J.,* 10 (3): 332-337. DOI: https://dx.doi.org/10.36380/scil.2020.wvj43

Table 1. Ingredients and chemical composition and nutritive value of diets for male and female white New Zealand rabbits

Ingredients	(%)	Chemical composition	Control diet	Fennel diet	Oregano diet	Thyme diet
			(g/100g)	(g/100g)	(g/100g)	(g/100g)
Bran	28.5	Dry matter	89.9	90.02	89.7	89.7
Corn	9.5	Ash	7.7	8.22	7.9	7.3
Soy bean meal	9.5	Crude protein	20.3	20.5	19.9	19.7
Sun flower meal	14.2	Ether extract	5.8	5.6	5.8	6.1
Alfalfa	33.75	NDF	30.5	29.9	30.7	30.6
Vegetable oil	2.8	ADF	17.5	17,5	18.4	18.1
Premix[1]	0.6	ADL	4.6	4.5	5.1	4.7
Salt	0.5	**Digestible energy** (Kcal/Kg)	2522	2549	2502	2528
DL Methionine	0.1					
L-Lysine	0.2					
Dicalcium phosphate	0.25					
Calcium carbonate	0.1					

[1] One kilogram of Premix provides: 1000000 IU vit.A, 300000 IU vit. D, 2 g vit. E, 0.4 g vit. K, 0.075 g vit. B1, 0.4 g vit. B2, 1.218 g vit. B3, 0.099 g vit. B5, 0.083 g vit. B6, 0.190 g vit. B9, 0.030 g vit. B12, 0.005 g Biotin, 0,2 g Cuivre, 4 g Fer, 5 g Zinc, 0.012 g Iode, 0.012 g Selenium, 0.020g Cobalt, 6 g Manganese, 57 g Choline chloride and QSP calcium. Premix contained 50 ppm of Salinomycin; NDF: neutral detergent fibre; ADF: aciddetergent fibre; ADL: detergentlignin.

Chemical analysis

Chemical analyses of diets were calculated with Spanish foundation for the development of animal nutrition (Fundación Española para el Desarrollo de la Nutrición Animal, FEDNA) table of composition and nutritive values of diets. The essential oils were extracted via steam distillation for 2 hours using a Clevenger-type apparatus. The supernatant was separated by decantation after adding 50% NaCl. The essential oils were stored in sealed glass vials at 4◦C prior to analysis. Gas chromatography–mass spectrometry (GC-MS) analysis was performed using a gas chromatograph (Trace GC ULTRA; Thermo Scientific, Waltham, MA, USA) coupled to a mass spectrometer (Polaris Q MS with ion trap; Thermo Scientific) in the electron impact (EI) ionisation mode (70 eV) in the 50–350 m/z range. The analysis was carried out using a VB-5 methyl polysiloxane at 5% phenyl) (Thermo Scientific) column (30m × 0.25mm, film thickness 0.25 µ m) using a temperature program of 40–300◦ Catarate4◦C min−1. Injector temperature was set at 220◦C. Helium gas was used as the carrier gas at a constant flow rate of 1.4 mL min−1. Diluted samples (1% in n - hexane; Sigma–Aldrich, Steinheim, Germany) of 1.0 µL were injected in the split mode to allow better identification of compounds. The analysis was repeated twice for each sample. The constituents were identified by comparison of their retention indices and mass spectra with those in the computer library (NIST MS Library Search, v.6.0) and with literature data.

Statistical analysis

The results were quoted as mean ± standard deviation (SD), statistical evaluation of the results was performed by one-way ANOVA with the level of significance set at $p < 0.05$ and Square test for mortality.

RESULTS

Live weight, growth rate, feed intake, feed conversion rate and the mortality of rabbits during the experiment are presented in table 2. In general, no significantly differences in feed intake or feed conversion were observed in rabbits fed with different diets but the group of rabbits fed with the fennel supplemented diet appeared to eat bigger quantities of feed and presented a higher body weight when compared to the control group of rabbits at two weeks after weaning. The body weight varies significantly ($P<0.05$) at the age of slaughter and the thyme group presented the best body weight and the higher growth rate in the last week of experience. Mortality rate was significantly ($P<0.05$) lower for rabbits fed with fennel or oregano or thyme supplemented diets when compared to those fed with the control diet during growing period.

Essential oils yields expressed in relation to dry weight plant material are shown in table 3. The yield of the essential oils, based on the dry weight of the samples, was 1.89%, 2.92% and 1.96 % for *Foeniculum vulgaris*, *Origanum compactum* and *Thymus capitatus* respectively. The yield of essential oil of *Origanum compactum* (around 3%) was higher than the other aromatic plants. The chemical composition of essential oils of *Foeniculum vulgaris*, *Origanum compactum* and *Thymus capitatus* is presented in table 3. The essential oils of *Foeniculum vulgaris* was dominated by

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anethol (83.29%) and followed by limonene (14.40%). The carvacrol (68.99%) was the main compound in *Origanum compactum* oil followed by thymol (18.67 %). The *Thymus capitatus* oil was dominated by carvacrol too (95.25%).

Table 2. Effects of dietary supplementation of fennel oregano and thyme in growth performance and mortality of 30 days old white New Zealand rabbit

Indices	Days	Group				
		Control	Fennel	Oregano	Thyme	P value
Body weight (g) ± SD	30	580±60 [a]	570±90[a]	570±47[a]	600±79[a]	0.146
	37	800.3±165[a]	911.4±218[a]	651.1±139[a]	711±72[a]	0.176
	44	920±156[a]	1080±155[a]	850±92[a]	981.3±80[a]	0.118
	51	1300.5±74[a]	1206±82[a]	1129.3±117[a]	1 220±106[a]	0.152
	58	1750±37[b]	1438.8±91[a]	1 460±101[a]	1 630±68[b]	< 0.001
	70	2209±103[bc]	1979±155[a]	2068.6±72[ab]	2304.9±28[c]	0.002
Feed intake (g .d^{-1}) ± SD	30	47.9±4[b]	30.4±4[a]	35,4±10[a]	30.4±4[a]	0.006
	37	50±14[a]	62.5±20[a]	72.2±25[a]	36.7±4[a]	0.075
	44	66.6±10[a]	76.5±12[a]	65±10[a]	68.9±5[a]	0.422
	51	91.9±6[a]	91.6±6[a]	90,3±10[a]	78.6±9[a]	0.095
	58	107.5±22[a]	92.6±6[a]	106.7±8[a]	108.3±12[a]	0.374
	70	108.1±9[b]	93.8±6[a]	112.0±8[b]	112.5±6[b]	0.013
Growth rate (g .d^{-1}) ± SD	30-37	37.5±2[a]	45.4±33[a]	14.7±19[a]	14.8±8[a]	0.167
	37-44	15.7±4[a]	31.0±13[a]	32.9±7[a]	35.5±7[a]	0.063
	44-51	49.0±19[b]	18.1±13[a]	39.9±4[b]	34.1±4[ab]	0.022
	51-58	64.2±6[c]	33.1±5[a]	47.3±3[b]	58.6±6[c]	< 0.001
	58-70	38.3±5[a]	45.1±4[a]	50.7±2[bc]	54.0±3[c]	< 0.001
Feed conversion ratio ± SD	30-37	2.41±0.7[a]	2.56±0.7[a]	2.45±1[a]	2.23±1.1[a]	0.983
	37-44	3.19±0.5[a]	3.18±2[a]	2.29±1.2[a]	1.80±0.2[a]	0.788
	44-51	2.36±0.4[a]	2.83±1[a]	2.04±0,1[a]	2.03±0,2[a]	0.383
	51-58	2.60±0.5[ab]	3.75±0.2[c]	3.26±0.5[bc]	2.41±0.3[a]	0.028
	58-70	2.96±0.4[b]	2.05±0.2[a]	2.13±0.2[a]	1.96±0.1[a]	0.002
Mortality (%) ± SD	30 -58	63.63[b]	33.33[a]	41.6[a]	41.6[a]	< 0.05
	30 -70	68.18[b]	37.5[a]	41.6[a]	41.6[a]	

SD: Standard deviation; Mortality ratesare analysed using a $\chi 2$ test; The values with different superscript letters in a row are significantly different ($p<0.05$).

Table 3. Chemical composition of essential oils of *Foeniculum vulgaris, Origanum compactum* and *Thymus capitatus*

Component \ Concentration			Peak area %	
	Retention Time (min)	Foeniculum Vulgaris (%)	Origanum Compactum (%)	Thymus Capitatus (%)
Myrcène	6.55	-	0.44	-
Para cymene	7.19	-	2.53	-
cis-Ocimene	7.73	-	-	-
α-Pinene, (-)-	7.75	0.12	-	-
γtérpinene	7.79	-	3.98	-
α-Pinene, (-)-	8.28	-	-	-
Linalol	8.51	-	1.09	-
Camphene	8.74	-	-	-
α-Phellandrene	9.70	-	-	-
Terpinene -4-ol	9.79	-	0.58	-
α-Pinene, (-)-	11.01	-	-	-
dl-Limonene	11.01	14.40	-	-
Thymol	11.38	-	18.67	-
Carvacrol	11.55	-	68.99	-
1-8 cineol	11.68	-	-	-
Fenchone	12.99	-	-	-

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Fenchone	13.00	1.32	-	-
β-Caryophyllene	13.08	-	1.08	-
α -Campholene Aldehyde	15.63	-	-	-
Borneol	16.47	-	-	-
Isopulegyl acetate	17.81	0.12	-	-
α-Fenchylacetate	18.23	0.76	-	-
Trans Anethol	20.07	-	-	-
Trans Anethol	20.10	83.29	-	-
Carvacrol	20.69	-	-	95.25
Caryophyllene	24.24	-	-	1.49
Caryophyllene	24.89	-	-	-
Tetradecamethylcycloheptasiloxane	27.26	-	-	-
3,5-Diethylphenol	37.95	-	-	0.74
1,15-Dihydrohexadecamethyloctasiloxane	38.85	-	-	0.91
6-Acetyl-2,2-dimethyl-8-(3-methyl-2-butenyl)-2H-1-benzopyran	39.37	-	-	-

DISCUSSION

Weaning of the young rabbits is the most critical period; it influences their health, their growth and mortality rate. Aromatic plants have been found to possess many biological activities with their natural compounds that why they are demanded in food.

Omer et al. (2013) found that adding 0.5% fennel seed with 0.5% oregano leaves as feed additives improved performance parameters of rabbits. Abdullah et al. (2009) also observed that supplementation of broiler diets with 1, 2 and 3 g/Kg of fennel seeds improved weight gain. These findings were according to present experimental results about 5% of fennel. This aromatic plant helps to improve the appetite with the anethole and limonene, active compound in the essential oil of present *Feniculum vulgaris.* Anethole has digestive stimulating and appetizing effects (Cabuk et al., 2003). That could explain the bigger quantity consumed and the best weight gain by this group in the first weeks. Badgujar et al. (2014) reported that essential oil of fennel reduces intestinal gas and regulates the motility of smooth muscles.

According to Ayala et al. (2011) dietary supplementation with dried oregano improved rabbit performance. Cardinali et al. (2015) showed that supplementation with 0.2% oregano can improve productive performance and carcass quality in rabbits. Giannenas et al. (2005) also found that dehydrated oregano plants (5g/kg) exerted a growth promoting effect when incorporated in chicken diets. However, other researchers found that dietary oregano essential oil exerted no growth-promoting effect on rabbits (Botsoglou et al., 2004). The dietary inclusion of dried oregano (10 or 20 g/kg diet) had also no effect on the performance parameters of laying quail (Christaki et al., 2012). These results are in agreement with present observations that indicated oregano did not positively affect body weight gain and feed intake in rabbit. Oregano had higher essential oil yield than thyme; this last aromatic plant did not affect performance parameters of rabbit but body weight was better at age of slaughter.

Contradictory results on the effects of thyme or its essential oil are reported for rabbits. In some cases, growth performance improved (Dalle Zotte et al., 2014; Gerencser et al., 2014; Abdel-Wareth et al., 2018), but in others there was no effect (Soultos et al., 2009; Gerencser et al., 2012). Ocak et al. (2008) indicated also that thyme did not significantly affect the growth performance of broilers.

The crude protein and digestible energies of all dietary treatments were in the superior range recommended for growing rabbits, which contributed to high rate mortality. Present results showed that supplementing the diet with fennel or oregano or thyme reduced the mortality rate approximately 40% compared to the control diet. Low mortality rate of rabbit repoted by using sage and oregano extracts (Szaboova, 2007). The treatment with fennel seeds and thyme or their essential oil had also a beneficial effect on the mortality rate (Benlemlih et al., 2014). There is an evidence to suggest that aromatic plant with components of the essential oil may have a role in combating bacterial diseases in rabbit and reducing mortality. Placha et al. (2013) observed that dietary inclusion of 0.5 g/kg of thyme essential oil was able to limit the colonization of coliforms in the caecum. Reduced counts of coliforms and clostridia, *E. coli* and *S. aureus* was presented by Benlemlih et al. (2014) during the application of dried fennel and thyme to rabbit. The essential oil of present aromatic plants contains a high percentage of phenolic compounds such as carvacrol and thymol, the most important antibacterial properties (Sokovic, 2010). Koné et al. (2016) observed a small positive effect of polyphenols in reducing bacterial microflora of rabbit meat. It was also mentioned that thymol possess antibacterial effect against various gram positive and negative bacterial strains (Meeran et al., 2017). Anethole and limonene present in fennel oil are responsible for its antimicrobial effects (Gulfraz et al., 2008; Vimal et al., 2013). The antispasmodic activity has also

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been shown for thymol and carvacrol (Keyhanmanesh and Boskabady, 2012). There are some evidences that major components of present aromatic plants have a different biological activity and have potential health effects in rabbit lifespan.

CONCLUSION

It could be concluded that supplementing rabbit diets with 5% of thyme has a significant beneficial effect on performance. Fennel, oregano and thyme had potential health effects and reduced mortality rates. Aromatic plants and their active compounds can be tried as additives substances for rabbit nutrition and improving health performance.

DECLARATIONS

Acknowledgments

The corresponding author thanks National Center for Scientific and Technical Research for the research grant. The authors would like to thank the members of the rabbit company Lapinord for co-operation in this experiment and we gratefully thank the reviewers for their comments on this manuscript.

Competing interests

The authors declare that they have no competing interests.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 338-361, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus,* Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies

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ORIGINAL ARTICLE
pii: S232245682000044-10
Received: 22 Jul 2020
Accepted: 14 Sept 2020

ABSTRACT

Bulk tank milk analysis was referred to as a useful and appropriate diagnostic tool to evaluate milk quality and mastitis pathogens in cattle dairy herds. Out of the total number of 150 pooled tank milk samples collected from 150 cattle dairy farms, 13 locally field *Staphylococcus aureus* isolates were detected and confirmed phenotypically by culturing, gram staining, biochemical, and molecular identification to be *Staphylococcus aureus* in the overall herd by the prevalence of 8.6%. Isolation and identification of *Salmonella* Typhimurium field isolates from bulk tank milk samples revealed that 20 locally field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemical and molecular identification to be *Salmonella* Typhimurium in the overall herd by the prevalence of 13.3%. The results of total bacterial plate count (cfu/ml) revealed that the geometric mean of 150 dairy farms was 3.2×10^8 cfu/ml. The geometric mean of somatic cell count (SCC)/ml in Bulk tank milk samples of 150 cattle dairy farms were 556.7×10^3. The geometric mean value of *Staphylococcus aureus* count in this study was 3.7×10^3 cfu/ml. Serological identification of the 20 isolates revealed that they were *Salmonella* Typhimurium. The study provided various risk factors that had a clear and effective role in determining the level of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard plate count, and Somatic cell count in bulk tank milk. The PCR amplification with (*hlg*) gene-specific primers revealed a product with an approximate size of 937 bp. (*hlg*) gene found in 13 (54%) *Staphylococcus aureus* isolates. The PCR identification of *sopB* (*SigD*) virulence gene for *Salmonella* Typhimuriym revealed a product with an approximate size of 517 bp. *SopB* gene found in all *Salmonella* Typhimurium isolates (100%). Phylogenetic and partial gene sequence analysis of (*hlg*) *Staphylococcus aureus* gene of Egyptian isolated strain showed a great identity with the different *Staphylococcus aureus* strains uploaded from the gene bank. Phylogenetic analysis of *Salmonella* Typhimurium (*sopB*) virulence gene of Egyptian isolated strain indicated a great homology with the different *Salmonella* Typhimurium strains uploaded from the gene banks. The results of the present study emphasize the importance of more efficacious preventive programs for controlling the mastitis and bacteriological quality of bulk tank milk and monitoring mastitis economic losses.

Keywords: Mastitis, PCR, Phylogenetic analysis, Risk factors, *Salmonella* and *Staphylococcus aureus*

INTRODUCTION

The using of continuous and regular methods for monitoring and assessing udder health is extremely useful for improvements of udder health status, milk quality premiums programs. The regular assessment of bulk tank milk (BTM) analysis is regarded as one of the foremost significant tools to give insight and perception from the udder health status, and also the proper application of sanitary and hygienic measures in dairy herds, (Jayarao and Wolfgang, 2003). Bulk tank milk analysis is one of the most important diagnostic method for assessment of milk quality and mastitis pathogens (Godkin et al., 1993; Riekerink et al. 2006). There are numerous diagnostic tests commonly used to quantify and assess the quality of BTM, such as Somatic cell count (SCC) and standard plate count (SPC) (Cicconi-Hogan et al., 2012). The SPC assesses the bacterial count in the milk, and estimates the number of aerobic bacteria present per milliliter of milk. SPC is considered one of the foremost important tools to assess management and milk quality, as reported and described in previous studies and milk quality management recommendations (Schroeder, 2009). A high bulk tank SPC can be a consequence of bacteria from dirty milking equipment, milk from cows with subclinical or clinical mastitis, or contamination from dirty udders (Murphy and Boor, 2000). The regulatory cut-off for SPC was 100,000 cfu/mL (Cicconi-Hogan et al., 2012). Quantifying and determining of bulk milk SCC is an internationally recognized diagnostic tool to determine the quality of the milk and also the udder health status of the cattle within the herd. Many management practices were related to higher bulk milk SCC (BMSCC) (Schukken et al. 2003). Milk borne pathogens including *Salmonella* and *Staphylococcus aureus* were identified by several researches in BTM with various prevalence rates from dairy farms (Ruzante et al., 2010; Cicconi-Hogan et al., 2012). *Salmonella* was thought to be one of the main important

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and significant food borne bacterial diseases worldwide. Risk factors related to incidence of *Salmonella* in BTM was not previously identified. Fecal contamination related to poor milking hygiene was considered one of the most important causes of bulk tank contamination with *Salmonella* (Van Kessel et al. 2004). The effector protein genes *sopB* were located in numerous regions of the *Salmonella* chromosome, and were present in a wide range of *Salmonella* serotypes, supposing that this effector protein may play vital virulence functions (Mirold et al., 2001). *Staphylococcus aureus* was regarded as one in all of the most important contagious mastitis pathogens in dairy cattle, and was related to large economic losses (Halasa et al., 2007; Hogeveen et al., 2011; Keefe, 2012). Hemolysins produced by *Staphylococcus aureus* are very vital virulence factor, with cytotoxic action responsible and answerable for lysing erythrocytes, and it results in worsening of clinical signs during blood stream infections (Duan et al., 2018). The *Staphylococcus aureus* gamma-hemolysin contains two polypeptides, whereas the gamma-hemolysin locus (*hlg*) has three open reading frames (Cooney et al., 1993). Phylogenetic methods can be used to analyze nucleotide sequence data in such a way that the order of descent of related strains can be determined (Hall and Barlow, 2006). So the aim of this study is to quantify and determine the prevalence of *Salmonella* Typhimurium and *Staphylococcus aureus* in the BTM in cattle dairies, identifying the most important risk factors affecting the level of SPC, SCC, in BTM in cattle Dairies, identifying the most important risk factors affecting prevalence of *Salmonella* Typhimurium and *Staphylococcus aureus* in bulk tank milk in cattle dairies, molecular identification of *sopB* gene of *Salmonella* Typhimurium and *hlg* gene of *Staphylococcus aureus*, phylogenetic and gene sequence analysis of *sopB* gene of *Salmonella* Typhimurium and *hlg* gene of *Staphylococcus* aureus to give insight to the source and origin, molecular epidemiology and disease pattern of *Salmonella* Typhimurium and *Staphylococcus aureus* in Egypt's dairies.

MATERIALS AND METHODS

Ethical approval

The approval from the Institutional Animal Ethics Committee to hold out this study wasn't required as not contact and no invasive procedure on the animals were performed. However, current study was applied in accordance to the Institutional Animal Ethics of Animal Reproduction Research Institute (ARRI), Haram, Giza, Agricultural Research Center (ARC), and in accordance to the regulations and ethics of the European Union for the protection of experimental animals (2010/63/EU).

Study animals, study area and study design

A cross-sectional study was carried out from 2017 to 2019 in Delta region, Alexandria Road and upper and lower Egypt Districts from total number of one hundred and fifty (150) private dairy cattle farms with total population number of 8667 lactating cows belonging to Cairo, Giza, Qaluiobia, Sharkia, Monofia, Alexandria, Behera, Dakahlia, Benisuif, Fayoum, Sohag, Aswan and Asuit governorates.

Bulk tank milk sample collection and testing

A total number of 150 BTM samples were collected. The BTM samples were intended for checking the hygienic quality of raw milk collected in accordance to the standard methods described according to ISO707 (2008). After turning on the agitator for at least 10 minutes, 60ml BTM samples were collected from the top of the bulk tank using clean sanitized dipper. Samples were immediately refrigerated and transported to the laboratory in ice box, and examined within 24 hours after collection.

Microbiological examination of bulk tank milk samples

Preparation of serial dilution was carried out in accordance to methods described by APHA (1992) and APHA (2004). One ml completely mixed milk sample was transferred under aseptic conditions to 9ml of sterile ¼ strength Ringer's solution, and well mixed to urge 1/10 dilution. One ml from the first dilution was added to 9 ml of sterilized diluents to obtain tenth fold serial dilutions. The previous prepared dilutions were subjected to the subsequent microbiological examinations: The Total Bacterial Plate Count (TBPC) (cfu/ml) was assessed according to the methods descried by APHA (1992) and BAM on line (2009). Two ml of every previously prepared decimal dilution were inoculated into duplicate plates (one ml each), then 12 to 15 ml of Standard Plate Count Agar medium (Oxoid, CM0463) was added (cooled to 45 ± 1 °C) into each plate as well as control one. The plates were thoroughly mixed and incubated for 48 ±2 hours at 35 °C. Colony Forming Unit (CFU) per ml was calculated and recorded. The standard cut-off for SPC is 100,000 cfu/mL, in accordance with the European Union standards **(**Cicconi-Hogan et al., 2012). Analysis of BTM samples for SCC was performed according to the methods described by Zecconi et al. (2002). The SCC was quantified and measured using the electronic soma count 150, from Bentley (Chaska, MN 55318, United States). The standard cut off point of BSCC was 400, 000 cell/ml according to the regulations described by USDA (2011).

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Staphylococcus aureus count (cfu/ml) was assessed as reported by the methods illustrated by ISO 6888-1(1999) and ICMF (1986)**.** From each dilution, 0.1 ml was inoculated onto Baird parker agar plate. Inoculated plates were then incubated at 37 °C for 48 hours. Typical colonies of *Staphylococcus aureus* were enumerated, and therefore the average number per ml was calculated. Isolation and identification of *Staphylococcus aureus* was carried as stated in Quinn et al. (2002) and BAM on line (2009). Pure separated suspected colonies of *Staphylococcus aureus* were picked up from Baird-Parker agar plates, transferred to nutrient agar slants, and incubated at 37 °C for 24 hours. The isolated *Staphylococcus aureus* isolates were identified by Microscopic examination and Biochemical reactions. Biochemical tests used to confirm *Staphylococcus aureus* were coagulase test, catalase test, indole production, methyl red test, Voges-proskauer reaction, urease production, citrate utilization and sugar fermentation as stated in Toply and Wilsons (1993), colle et al. (1996), Harrigan (1998) and Quinn et al. (2002). Phenotypic characterization of some virulence factors was carried out to detect coagulase test according to methods described by Quinn et al. (2002), and hemolysis assay according to methods described by Koneman et al. (1997). Isolation and identification of *Salmonella* Typhimurium was carried out according to methods described by ISO-6579 (2002). Five ml of BTM samples were aseptically inoculated into 50 ml (1:10) of Preenrichment media (Buffer Pep-tone Water (BPW), and thoroughly mixed before being incubated at 37 °C ± 1 °C for 18 ± 2 hours. A volume of 0.1 ml was transferred to a tube containing 10 ml of the Rappaport Vassiliadis broth and then incubated at 41.5 ºC for 24 hours. From the enrichment culture, 10 µl were inoculated onto the surface of Xylose Lysine Deoxycholate (XLD), Hektoen Enteric and *Salmonella Shigella* agar plates, then incubated at 37ºC for 24 hours. *Salmonella* typical colonies were isolated, and further tested by standard biochemical methods and also serotyped using specific commercial sera. Microscopic identification of *salmonella* isolates films from suspected purified colonies were prepared, fixed and stained with gram's stain (Cruickshank et al., 1975). Biochemical identification of *Salmonella* isolates was carried out according to the methods described by ISO-6579 (2002). Purified isolates were examined by different biochemical reactions as oxidase, urea hydrolysis, H2S production on TSI, lysine decarboxylation, indole, methyl red test, Voges Proskauer, and citrate utilization tests.

Serotyping of *Salmonella*

Salmonella isolates were subjected to serological identification according to the methods described by Kauffman-White Scheme (Kauffmann, 1973) for determination of somatic (O) and flagellar (H) antigens.

Questionnaire and data collection

The study questionnaire sheet was designed according to the standard frame prepared by Dufour et al. (2010). The questionnaire sheets included questions related to hygiene of animals, environment and mastitis management. The questions were designed to be as closed as possible to avoid different interpretation between farmers.

Statistical analysis

All data analysis was carried out using the statistical studies. Association between the occurrence of infection and therefore the potential risk factors were studied using Chi-square $\chi 2$ and odds ratio (OR). A database and statistics system for epidemiology on microcomputers were used for performing $\chi 2$ Chi-square tests and odds ratio analysis. Answers to the questionnaire were transferred to Microsoft Excel, and grouped by their categorical response (e.g., Yes, No). Odds ratio was computed according to methods described by Thrusfield (2005). A P-value of < 0.05 and odds ratio of >1 and $\chi 2 > 3.82$ were regarded a significant association between the response and a category of the count.

Molecular identification of *Staphylococcus aureus* and *Salmonella* Typhimurium

All the identified *Staphylococcus aureus* and *Salmonella* positive isolates were examined by PCR for the presence of, *Staphylococcus aureus hlg* gene and *sopB* gene of *Salmonella*. The primers sequence and PCR product sizes are shown in table 1.

DNA extraction

DNA extraction from samples was done using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) accord to the manufacturer's instructions. Briefly, 200 µl of the sample suspension was used to be incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 minutes. After incubation, 200 µl of one hundred percent (100%) ethanol was once introduced to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted with a hundred (100) µl of elution buffer.

Oligonucleotide primer

Primers used were provided from Metabion (Germany) which are listed in table 1.

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PCR Design and amplification

Primers were employed in a 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of every primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was done in biosystem 2720 thermal cycler.

Analysis of the PCR products

1.5% agarose gel was used for separating PCR products. For gel analysis, 20 µl of the products was loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and therefore the data was analyzed by using computer software.

Phylogenetics and gene sequence analysis of, *sopB* gene of *Salmonella* Typhimurium and *hlg* Gene of *Staphylococcus aureus*.

It was performed in Elim biopharmaceuticals, Germany. Amino acids sequence analysis was done using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by Thompson et al. (1994) and Phylogenetic analysis was performed using neighbor joining and in MEGA6 (Tamura et al. 2013).

Table 1. Primer sequences, target genes, amplicon sizes and cycling conditions

Microorganism	Gene	Sequence(5'-3')	Amplified product	Reference
Salmonella	*sopB*	F-5' tcagaagRcgtctaaccactc-3'	517 bp	Huehn*et al.* 2010
		R-5'- taccgtcct cat gcacactc-3'		
S. aureus	*hlg*	F-5'- GCCAATCCGTTATTAGAAAATGC-3'	937 bp	Kumar *et al.*, 2009
		F-5'- CCATAGACGTAGCAACGGAT-3'		

S. aureus: *Staphylococcus aureus*

RESULTS AND DISCUSSION

Out of a total number of 150 pooled tank milk samples which were collected from 150 dairy cattle farms, 13 locally field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemical and molecular identification to be *Staphylococcus aureus* in overall herd prevalence of (8.6%) as shown in table 2. These results nearly agreed with Rysanek et al. (2007) (12.3%) and Neder et al. (2011) (11.7%), but disagreed with Sischo et al. (1993) (45%), Keefe et al. (1997) (70%), Stephan et al. (2001) (32.4%), Phuektes et al. (2003) (33%), Yagoub et al. (2005) Howard (2006) (57.1%), Miranda-Morales et al. (2008) (30%) (30%), USDA (2008) (43%), Olde Riekerink et al. (2010) (74%), Katholm et al. (2012) (97%), Khudor et al. (2012) (28.5%), Lee et al. (2012) (21.7%), Amal (2014) (40%), El-Gedawy et al. (2014) (17%) and Zecconi et al. (2020) (42 %).

Isolation and identification of *Salmonella* Typhimurium field isolates from bulk tank milk samples revealed that 20 locally field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemical and molecular identification to be *Salmonella* Typhimurium in overall herd prevalence of (13.3%) as shown in table 2, this finding agreed with Rohrbach et al. (1992) (8.9%), O'Donnell (1995) (0.36%), Steele et al. (1997) (0.17%), Karns et al. (2005) (11.8%), Van Kessel et al. (2008) (11%) and Abo-shama (2013) (14%) but disagreed with Hassan et al. (2000) (1.5%), Jayarao and Henning (2001) (6.1%), Murinda et al. (2002) (2.24%), Warnick et al. (2003) (1.1%),Van Kessel et al. (2004) (2.6%), Jayarao et al. (2006) (6%), Pangloli et al. (2008)(7%), Addis et al. (2011) (28.6%), Van Kessel et al. (2011) (48%), Tajbakhsh et al. (2013) (3.63%), El-Gedawy et al. (2014) (9%) and Sonnier et al. (2017) (18%). Serological identification of the 20 isolates revealed that they were *Salmonella* Typhimurium. The results of TBPC (cfu/ml) as shown in table 3, revealed that the geometric mean of 150 dairy farms was 3.2×10^8 cfu/ml.This finding agreed with (1.9×10^8 cfu /ml) Godefay and Molla (2000), (2.67×10^8 cfu/ml), Khin Zar Lin (2015), but disagreed with Abdallah (2002) (2.7×10^7 cfu/ml), Bonfoh (2003) (1.1×10^7 cfu/ml), Chye et al. (2004)(1.2×10^6 cfu/ml), Axelsson (2004) (2.4×10^4 cfu/ml) , El-Diasty and El-Kaseh (2009) (6.1×10^5 cfu/ml), Pantoja et al. (2009) (1.2×10^4), Uddin et al. (2011) (1.28×10^9cfu/ml), Hakem et al. (2012) (minimum count 3.7×10^5 and maximum count 4.9×10^5cfu/ml) ,Beli

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(2015) (3.89 × 10^6 cfu/ml), Meshref (2013), 3.62×10^7 ±1.37×10^7 cfu /ml) and Tasci (2011)(3.95×10^6 cfu /ml). *Staphylococcus aureus* is known as one of the foremost important causes of cattle mastitis, and it causes great economic losses in the dairy industry (Dufour et al., 2012). In term of *Staphylococcus aureus* count (SAC), the geometric mean value of SAC in present study was 3.7×10^3 cfu/ml as shown in table 3. These findings agreed with Stephan et al. (2001) (3×10^3 cfu/ml) but disagreed with Peles et al. (2007) (6.0 ×10^3 cfu/ml). The results in table 3 show that the geometric mean of SCCs/ml in BTM samples of 150 cattle dairy farms were 556.7×10^3 ; this result agreed with Erskine et al. (1987) (SCC 700×10^3 cells/ml), Fox et al. (2003) (533×10^3 cells/ml) and Pantoja (2009) (600×10^3 cells/ml), and however disagreed with Desmasures et al. (1997) (176×10^3 cells/ml), Secretaríade Agricultura Ganadería Pescay Alimentos (SAGPyA) (2005) (384×10^3 cells/ml), Rysanek et al. (2007) (240×10^3 cells/ml) in milk without pathogen, (330.8×10^3 cells/ml) with single pathogen and (367.6×10^3 cell/ml)with double pathogens, Vissio et al. (2008) (250×10^3 cells/ml). Bulk tank SCC could be a general indicator of the udder health in a herd, and it was also considered as an indirect index of milk quality (Schukken et al., 2003). Bulk tank SCC (BTSCC) was mainly known as one of the important tools to define the national and international regulative standards that control the hygienic milk production. The national standards for BTSCC differ from < 400,000 cells/ml (EU, Australia, New Zealand and Canada) to < 1,000,000 cells/ml (Brazil) (USDA, 2013). The USDA introduced a program that permits dairy processors to get an export certificate that confirm farm-level consent with the (400,000 cell/ml) limit adopted within the EU (USDA 2011).

Table 2. Point prevalence of *Staphylococcus aureus* and *Salmonella* Typhimurium in bulk tank milk in different localities

	Governorates													
	cairo	Giza	Qulobia	Sharkia	Monofia	Alexandria	Behira	Dkhalia	Benisuef	Fayoum	sohag	Aswan	Asuet	total
Poulation at risk	1520	644	671	729	841	718	284	502	620	618	549	510	461	8667
S.aureus	1	1	1	2	2	0	0	3	1	0	1	0	1	13
S.typhimurium	2	1	1	3	3	1	1	4	0	1	1	1	1	20

S. aureus: *Staphylococcus aureus: S.* Typhimurium: *Salmonella* Typhimurium

Table 3. Geometric mean value of Standard plate count, Somatic cell count and *Staphylococcus aureus* in bulk tank milk in different localities

Governorates	Geometric mean of SPC cfu /ml	Geometric mean of SCC ×10^3	Geometric mean of *S. aureus* cfu/ ml×10^3
Cairo	7.9×10^8	455	3.2
Giza	6.3×10^8	372	4.7
Qulobia	1.7×10^7	438	5.7
Sharkia	2.4×10^8	669	3.8
Monofia	6.9×10^8	352	7.2
Alexandria	1.7×10^8	552	3.4
Behira	1.2×10^8	620	5.6
Dkahlia	2.9×10^7	559	3.7
Benisuef	9.7×10^7	679	2.1
Fayoum	3.7×10^8	597	2.3
Sohag	2.4×10^7	567	3.1
Aswan	7.2×10^8	812	4.7
Asuet	3.7×10^8	778	2.7
Geometric mean value	3.2×10^8	556.7	3.7

SPC: Standard plate count; SCC: Somatic cell count

As shown in tables 4, 5 and 6 risk factors that were found to be associated with *Staphylococcus aureus*, *Salmonella* Typhimurium, SPC and SCC in bulk tank milk samples were Purchasing cattle or heifers as a risk factor has a significant association with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk as shown in table 4 (OR: 7.2, $\chi 2$: 92 and P < 0.00001, OR: 4.2, $\chi 2$: 44 and P < 0.0014, OR: 6.22, $\chi 2$: 78.7 and P < 0.000012 and OR: 3.14, $\chi 2$: 23.3 and P < 0.001 respectively). In spite of the actual fact that non lactating heifers have not yet been in connection with the milking equipment, they would still be infected with *Staphylococcus aureus* (Fox, 2009). Therefore, the purchase of *Staphylococcus aureus*-positive heifers was considered as one of the foremost vital risk factors for the introduction and spread of *Staphylococcus aureus* within the herd after they start lactation. The introduction of cattle was previously related to an increased risk of clinical salmonellosis. These animals were also carrier of *Salmonella*, and should contribute to extend *Salmonella* infection in the farm. These findings agreed with Nielsen et al. (2007). Cows purchased into the dairy herd were also infected with mastitis pathogens, and were a possible risk to other cows within the herd, and therefore affecting the level of bulk tank milk SCC and SPC. To prevent the introduction of mastitis pathogens when purchasing cows, a balance need is required to be found between minimizing the probability of buying

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an infected cow and maintaining sufficient choice within the population from which to pick replacements (Gunn et al., 2008). Quarantine and test all purchased incoming heifers for contagious mastitis as a risk factor recorded a significant association with *Staphylococcus aureus*, Salmonella Typhimurium SPC and SCC in bulk tank milk as shown in table 4 (OR: 4.92, χ2. 24 and P<0.00028, OR.: 3.92, χ2: 31.4 and P<0.0028, OR: 4.12, χ2. 42.8 and P<0.00024 and OR: 3.92, χ2:34.4 and P<0.00128 respectively).

Herds that were not quarantined bought animals which have been generally observed to be positive for *Staphylococcus aureus* in BTM than herds where quarantine was once applied. Intramammary infections brought by *Staphylococcus aureus* were usually sub-clinical; thus, *Staphylococcus aureus* can continue to be unnoticed and disregarded by producers if no testing was done. Moreover, testing newly bought heifers previous to introducing them into the farm would be critical and necessary for forbidding and diminishing the spread of *Staphylococcus aureus* and retaining the occurrence of recent infections to an occasional degree (DaCosta et al., 2016). Non applying quarantine policy in cattle dairy farms as a risk factor encompassed a significant association with *Salmonella* prevalence in bulk tank milk. Approximately half of the dairy herds purchased animals, and about half of the buying herds did not quarantine incoming animals (Nöremark et al., 2016). If purchase could not be avoided, quarantine is often unable to reduce the risks (Vanselow et al., 2007). Not applying fly control as a risk factor as shown in table 4 was clearly related to *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR:3.2, χ2. 22 and P < 0.0016, OR: 2.29, χ2: 25.4 and P < 0.0029, OR: 4.2, χ2. 47.2 and P < 0.00032 and OR: 3.2, χ2. 39 and P < 0.006 respectively).

Flies played a vital role in the transmission of *Staphylococcus aureus* between infected and uninfected heifers, and not applying fly control in cattle dairy farms was considered one of the most important risk factors for heifer's intramammary infection caused by *Staphylococcus aureus* (Capurro et al., 2010; Piepers et al., 2011; Anderson et al., 2012). Presence of biting flies that purpose teat lesions was related to a high level of *Staphylococcus aureus* mastitis suggesting that fly control should be included during a mastitis management plan (Ryman et al., 2013). Flies carry a variety of mastitis-causing organisms that may colonize in teat lesions, and affect SCC and SPC in bulk tank milk. One among the foremost probable suggested causes for the increased incidence of mastitis, and also the increased bulk tank SCC and SPC level was that the irritation of udders by flies, and also the spreading of microorganisms by these insects. Another report showed that presence of biting flies causing teat lesions was related to a high level of mastitis, and suggested that fly control should be included during a mastitis management plan (Ryman et al., 2013). Heifers from herds using fly control had a lower prevalence of mastitis in comparison with herds where no fly control was applied (Nickerson et al., 1995). Heavy fly infestation was known as one of the foremost important risk factors for *salmonella* in dairy cattle farms.Flies that feed on cattle manure played a critical role in *Salmonella* shedding in dairy farm environments (Holt et al., 2007; Thomson et al., 2017). Not milking cows with a high SCC/CM grouped separately as shown in table 4, were significantly related to *Staphylococcus aureus*, SPC and SCC in bulk tank milk (OR :2.72, χ2: 17 and P< 0.0058, OR.: 1.73, χ2: 12.7 and P < 0.03, OR: 5.93, χ2 : 46.9 and P < 0.00023, OR: 4.73, χ2; 42.8 and P < 0.00021 respectively) and not milking all incoming cattle separately or last as shown in table 4 (OR: 6.34, χ2 : 62 and P < 0.00002, OR : 3.22, χ2 : 42.5 and P < 0.0021 and OR: 4.34, χ2: 42.8 and P < 0.00045 respectively) as shown in table 4.

It is still preferable to milk cows with a high SCC/CM last and grouping separately (Barnouin et al., 2004). Cows in herds that did not milk cows with mastitis last were significantly more likely to have mastitis than those who did that. Failure to milk cows with mastitis last played a vital role in spreading of mastitis pathogens between cows by milker's hands leading to contagious mastitis, and also played a great role in affecting bulk tank SPC and SCC (FAO, 2014). High SCC cows and clinical mastitis cases should be milked last, employing a specific milking unit for these cows or rinsing, cleaning, or disinfecting the unit after these cows are milked, and before first-lactation cows are milked were significantly related to low bulk tank SCC (Dufour et al., 2011). Milking cows with mastitis in no specific order was another practice that significantly affected the *Staphylococcus aureus* presence in BTM. Riekerink et al. (2006) also observed lower incidence rates for *Staphylococcus aureus* in BTM belonging to herds with mastitis-affected cows milked separately from healthy cows. Teat cups were known as the foremost bacterial transmission tool (Benić et al., 2012), and therefore, cows affected with mastitis should be milked separately or after healthy animals (NMC, 2001; Zecconi, 2006; Arnold and Bewley, 2011; Middleton 2013). Not cleaning and disinfecting equipment and milking unit after milk infected cattle as a risk factor for *Staphylococcus aureus, Salmonella* Typhimurium SPC and SCC in bulk tank milk (OR: 6.98, χ2. 75 and P<0.000016, OR: 2.78, χ2. 29.4 and P<0.016, OR: 5.68, χ2: 59.4 and P < 0.000012 and OR: 4.72, χ2: 55.2 and P< 0.00013 respectively) as shown in table 4. Poorly cleaned and sanitized milking utensils were regarded as a very important source of the many microorganisms in bulk tank milk. Cleaning and sanitation of the milking equipment may well be considered as a critical point in the milking procedure, because a cleaning failure could influence the level of bacterial contamination of bulk tank milk (Bava et al., 2009). Cleaning and sanitation of the milking equipment were also identified as a risk factor related to high bacterial counts (Elmoselmany et al., 2009a; Jayarao et al., 2004).

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Table 4. Odds Ratio, Chi-square and p value for risk factors associated with *Staphylococcus aureus*, *Salmonella* Typhimurium, Standard plate count and Somatic cell count in bulk tank milk in Egypt dairies

Risk factors	*S.aureus*			*S.* Typhimurium			SPC			SCC		
	OR	$\chi 2$	P value	OR	$\chi 2$	P value	OR	$\chi 2$	P value	OR	$\chi 2$	P value
Purchasing cattle or heifers	7.2	92	< 0.00001	4.2	44	< 0.0014	6.22	78.7	P<0.000012	3.14	23.3	P<0.001
Not quarantining and Test all purchased incoming heifers for contagious mastitis	4.92	24	< 0.00028	3.92	31.4	< 0.0028	4.12	42.8	P<0.00024	3.92	34.4	P<0.00128
missed fly control	3.2	22	< 0.0016	2.29	25.4	0.0029	4.2	47.2	P<0.00032	3.2	39	P<0.006
Not milking Cows with a high SCC/CM grouped separately and milked last at each milking	2.72	17	< 0.0058	1.73	12.7	<0.03	5.93	56.9	P<0.00023	4.73	42.8	P<0.00021
Not milking all incoming cattle separately or last	6.34	62	<0.00002	2.12	22.3	< 0.023	3.22	42.5	P<0.0021	4.34	42.8	P<0.00045
Not cleaning and disinfect equipments and milking unit after milk infected cattle	6.98	75	<0.000016	2.78	29.4	< 0.016	5.68	59.4	P<0.000012	4.72	55.2	P<0.00013
7- Sanitary condition of the Barn, access alleys and Cubicle passages	2.72	20	< 0.014	3.22	30.5	< 0.002	3.12	32.5	P<0.0014	2.82	27.5	P<0.001
Not using Separate cleaned and sanitized calving paddocks or pens for cows and heifers	2.34	16	< 0.028	2.5	17.5	< 0.013	4.6	47.5	P<0.00023	3.16	37.6	P<0.0013
Not housing pregnant heifers with dry cows	4.35	32	< 0.00028	3.15	27.2	< 0.0018	2.25	22.3	P<0.028	2.21	20.5	P<0.024
Herds don't practice checking SCC records	4.27	59	< 0.000012	2.47	19.4	< 0.0032	5.12	79.1	P<0.000002	7.23	82	P<0.00000012

OR: odds ratio, $\chi 2$: chi square, SPC: Standard plate count, SCC: Somatic cell count, p value: probaplity value

Table 5. Odds Ratio, Chi-square and p value for risk factors significantly associated with *Staphylococcus aureus*, *Salmonella* Typhimurium, Standard plate count, Somatic cell count in bulk tank milk in Egypt dairies

Risk factors	*S.aureus*			*S.* Typhimurium			SPC			SCC		
	OR	$\chi 2$	P value	OR	$\chi 2$	P value	OR	$\chi 2$	P value	OR	$\chi 2$	P value
Cleanliness of the cows (udder and teats)	2.65	19	< 0.023	4.25	49	<0.00023	5.15	69.7	<0.000001	4.21	45.9	< 0.0012
Muddy bedding materials	2.25	16	< 0.013	3.15	36	<0.00015	5	66	< 0.000023	6.5	86	<0.0000003
Storage manure indoor	3.27	59	< 0.00123	4.17	62.7	<0.00002	6.21	89.7	< 0.000013	2.17	29.1	< 0.0021
Herd size	3	39	< 0.0003	4.21	49	<0.00001	3.2	37	< 0.0003	2.2	18.4	< 0.013
Not t applying pre and post-milking teat dipping	2.79	16	< 0.026	1.87	17.5	< 0.012	3.69	31.4	< 0.00013	2.69	21.4	< 0.0023
Not stripping milk regularly into strip cup to detect mastitis	2.27	19	< 0.015	1.37	9.2	< 0. 04	2.37	29.5	< 0.001	6.2	78	<0.0000001
Milking wet teats	2.42	24	< 0.0021	2.27	22	< 0.0021	5.1	72	<0.000002	7.1	92	< 0.0000001
Not using single paper towels or suitable woven cloths for teats drying	2.58	18	< 0.013	2.78	15	< 0.03	5.58	67	< 0.00004	7.4	98	< 0.0000013
Not replacing and sanitize teat cup Liners according to manufactures instructions	5.79	55	< 0.0006	2.9	20.82	< 0.003	6.26	86.4	< 0.00037	6.79	76.9	< 0.0000032
Not testing and service milking machine every 6 months or annually	6.24	45	< 0.0004	4.12	41.2	< 0.0002	5.26	68.2	<0.000001	4.24	48.9	< 0.00002
Not Wearing latex Gloves	2.32	18.9	< 0.038	7.1	90	<0.0000003	7.1	90	<0.0000003	7.4	93	<0.000000123

OR: odds ratio, $\chi 2$: chi square, SPC: Standard plate count. SCC: Somatic cell count. p value: probaplity value

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DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

Table 6. Odds Ratio, Chi-square and p value for risk factors significantly associated with *Staphylococcus aureus*, *Salmonella* Typhimurium, Standard plate count, Somatic cell count in bulk tank milk in Egypt dairies

Risk factors	*S.aureus*			*S.* Typhimurium			SPC			SCC		
	OR	$\chi 2$	P value	OR	$\chi 2$	P value	OR	$\chi 2$	P value	OR	$\chi 2$	P value
Not washing automatic Clusters with hot disinfectant after milking a cow with CM or a high SCC	2.43	18	< 0.024	2.12	15.7	< 0.021	4.23	48	< 0.00012	5	68.7	<0.00000024
Not applying Back flushing	5.98	59	< 0.00001	3.28	39.7	< 0.0012	7.28	98.4	< 0.0000021	6.12	88.2	< 0.000001
Dirty Bulk tank surface	7.5	85	< 0.000002	5.23	68.2	<0.0000001	6.7	88.3	< 0.000003	7.2	95.3	< 0.0000003
Claw pieces don't thoroughly brushed after each milking	3.5	41	< 0.0001	4.2	58	< 0.000002	6.7	78	<0.0000001	7	93	< 0.0000002
Gaskets and milk valves don't removed cleaned and brushed daily	5.7	75	<0.000001	5.2	72	< 0.000001	6	82	< 0.0000023	7	93	< 0.00000004
Specific cleaning and sanitary program to milk pipelines	4.5	45	< 0.000021	4.7	42	< 0.0001	5.4	62	< 0.000023	6.8	73	< 0.000005
Not cleaning inflation and all other rubber plastic like parts and free from cracks	3.5	35	< 0.0021	3.7	32	< 0.001	4.4	62	< 0.0003	5	53	< 0.00002
Summer season	3.21	39	< 0.00012	2.15	19.7	< 0.02	4.24	42.7	< 0.00014	3.27	47.8	< 0.0002
Region variation	2.31	19	< 0.0014	2.46	20.7	< 0.002	3.24	41.4	< 0.0012	2.17	27.8	< 0.0031

OR: Odds Ratio, $\chi 2$: Chi Square, SPC: Standard Plate Count, SCC: Somatic Cell Count, p value: probability value

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Milk residues left on the milking equipment contact surface; support the growth of a variety of microorganisms (Murphy and Boor, 2007: Holm et al., 2004). The main aim of sanitizing is to kill residual microorganisms existing on these surfaces immediately prior to milking. Inadequate or improper cleaning and sanitizing permitted the bacteria to remain on the equipment's surfaces, and to grow and multiply these results in elevated count of *Staphylococcus aureus*, *Salmonella* Typhimurium, SPC and SCC in bulk tank milk. Satisfactory parlor sanitization and disinfection were very important and essential after each and every milking to help and assist to control the bulk tank total bacterial counts, and to limit the risk of cross infection of mastitis pathogens among cows from contaminated milking equipment (Ian ohnstad, 2013). Not being replaced and sanitized the teat cup liners according to manufactures instructions were significantly associated with *Staphylococcus aureus*, *Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 5.79, $\chi 2$: 55 and $P < 0.0006$, OR: 2.9, $\chi 2$: 20.82 and $P < 0.003$, OR: 6.26, $\chi 2$: 86.4 and $P < 0.00037$ and OR: 6.79, $\chi 2$: 76.9 and $P < 0.0000032$, respectively) as shown in table 4.

The teat cup liner is that the only piece of the milking machine that comes into direct contact with the cow. Worn and rough liners are difficult to be cleaned, and will harbor mastitis bacteria, and also, they affect significantly on the bulk tank SPC and SCC level (Roger Blowey and Peter Edmondson, 2010). The milking equipment consists mainly of various rubber parts. Among these rubber parts, the foremost important part could be a teat cup liner which provided direct contact force to teat tissue. Properties of teat cup liners directly affected the technical and technological process of milking, udder health condition and quality of obtained milk, and seriously was that the finding that cracks on the inner surface of teat cup liners were increasing proportionally with exposition time within the primary production, and that they were a source of pathogenic microorganisms (Gálik et al., 2015). The rubbers parts of milking equipment were taken into an account of one of the foremost potential sources of milk contamination (Krzyś et al., 2011). Sanitary condition of the barn, access alleys and cubicle passages as a risk factor was significantly associated with *Staphylococcus aureus*, *Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 2.72, $\chi 2$: 20 and $P < 0.014$, OR: 3.22, $\chi 2$. 30.5 and $P<0.002$, OR. 3.12, $\chi 2$. 32.5 and $P< 0.0014$ and OR. 2.82, $\chi 2$. 27.5 and $P < 0.001$ respectively) as shown in table 4.

Facility hygiene included the cleanliness of the barn, access alleys and milking parlor, thought as an essential and vital part of hygienic milk production and quality control program. Cleaning and renewing the bedding of the cubicles and yards should ideally be performed during milking, so as cows exit from the parlor, they were able to walk back along clean passage ways, pasted fresh food, and then lay down in clean cubicles (Blowey and Edmondson, 2010). This kept the teats as clean as possible during the primary critical 20 to 30 minutes after milking, when the cow was more susceptible to mastitis, because the teat sphincter did not fully close (Blowey and Edmondson, 2010). It also reduced the quantity of feces carried back on to the cubicle beds by soiled feet. Clean farms and houses were strongly related to lower SPC and SCS (Chassagne et al., 2005). Implementing and maintaining few and straight forward hygienic practices in terms of barn cleaning can significantly improve microbiological quality of cow milk, and reduce somatic cell count (Zucali et al., 2011). Surveys were performed to determine relations among measures associated with facility hygiene and microbial counts in bulk tank milk. A correlation between the dirtiness of the access alley to the milking parlor, and therefore the total bacterial count in bulk tank milk were reported by Christiansson et al. (1999). A powerful association was observed among increased bacterial content in bulk tank milk and cleanliness score of the farm facilities and milking cows (Elmoslemany et al. 2009a; Zucali et al. 2011). There was a distinct association amongst cleanliness of housing areas, and therefore the rate of clinical mastitis and high somatic cell counts in bulk tank milk were also established (Barkema et al., 1998). The poor hygienic condition of the farm facilities was a striking feature, which was along with inefficient sanitation of the facilities favored the proliferation and dissemination of *salmonella* among animals and environmental contamination. Proper and regular cleaning and ordinary barn disinfection routines in dairy cattle farms played an important role in controlling salmonellosis in dairy cattle farms. These agreed with Tarazi and Abo-shehada (2015). Sanitation and hygienic measures of dairy cattle farms had a great impact on reduction and lowering *Staphylococcus aureus* levels in dairy cattle farms (Sartori et al., 2017). Not testing and servicing the milking machine every 6 months or annually were considered as risk factor for *Staphylococcus aureus*, *Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR: 6.24, $\chi 2$: 45 and $P < 0.0004$, OR: 4.12, $\chi 2$: 41.2 and $P < 0.0002$, OR. 5.26, $\chi 2$: 68.2 and $P < 0.000001$ and OR: 4.24, $\chi 2$: 48.9 and $P < 0.00002$ respectively) as shown in table 4.

There were a wide variety of milking machine efficiency faults. These usually resulted from gradual changes in milking machine performance due to continued use, wear and age. The efficiency and performance of milking machine components affected mastitis incidence, mainly by their effects on the new mastitis infection rate. It is very important and critical that milking machines should be tested by a qualified technician or adviser on a regular basis. Regular testing, service and maintenance of milking machine is extremely necessary to maintain proper mechanical performance, to boost the speed and completeness of milking, and to improve bulk tank SPC and SCC level. These findings agreed with Rodrigues et al. (2005). Milking machines without proper maintenance in general became the main reason of high bacteriological counts in milk (Reinemann et al., 1997; Murphy and Boor, 2000). The milking parlor should be kept clean, and proper performance of the milking system should be ensured by having the system inspected at the minimum

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annually (Dufour et al., 2011). Not applying pre and post-milking teat dipping was as a risk factor for *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR:2.79, $\chi 2$: 16 and $P < 0.026$, OR: 1.87, $\chi 2$: 17.5 and $P < 0.012$, OR: 3.69, $\chi 2$: 31.4 and $P < 0.00013$ and OR: 2.69, $\chi 2$:21.4 and $P < 0.0023$, respectively) as shown in table 4.

The practice of pre- and post-milking teat dipping was one of the main important and critical components for controlling and preventing of mastitis and a control program in a dairy herd. Teat dipping is now a widely and universally accepted practice for reducing the bacterial population around the teat end, thus for reducing the risk of mastitis. The proper application of an accurate post-milking teat disinfection product to cow teats after milking could be the most significant and important task. After milking, bacteria multiply on the teat skin, and will extend into the teat canal. If the entire surface of every teat is disinfected immediately after milking, this establishment of bacteria would be minimized. Teat disinfection is on was in every of the foremost effective sub-clinical and clinical mastitis control measures available. Failure to cover the entire teat of each cow at every milking was that the most typical error in teat disinfection. Teat dipping was very important in reduction of *Staphylococcus aureus* teat colonization (Da Costa et al., 2014). Number of the advantages of those practices was related to reducing and lowering of the microbial contamination of the teats (Zucali et al., 2011). Post-milking teat sanitization reduced BTSCC, and was clearly and significantly related to individual-cow somatic cell count (ICSCC) (Tadich et al., 2003; Barnouin et al., 2004a; Dufour et al., 2011). Dipping all teats after each milking had the greatest effect on reduction of bulk milk somatic cell counts, and it increased milk yields more than any other milking practice. Pre-milking teat dipping played an important and significant role in reduction of BTSCC. Proper and correct teat-end disinfection before the cluster attached can reduce teat surface bacteria by 75% (Reinemann et al., 2008), and was effective in preventing and controlling mastitis caused by environmental and contagious pathogens (Ruegg and Dohoo, 1997). Not stripping milk regularly into strip cup to detect mastitis as a risk factor was significantly associated with *Staphylococcus aureus*, SPC and SCC in bulk tamk milk (OR: 2.27, $\chi 2$: 19 and $P < 0.015$, OR: 1.37, $\chi 2$: 9.2 and $P < 0.04$ and OR.:2.37, $\chi 2$: 29.5 and $P < 0.001$, OR: 6.2, $\chi 2$:78 and $P < 0.0000001$ respectively) as shown in table 4.

In the herds in which fore-milking was practiced using the fore-stripper, lower SCC was observed. These findings agreed with Skrzypek (2002), Wagner and Ruegg (2002) and Down (2016). Fore-milking and therefore the visual appraisal of fore-milk were widely recommended to be performed before each milking as they eased accurate identification of the clinical mastitis in individual quarters and immediate treatment of affected cows, like wise as their milking using separate equipment. Fore-stripping was extremely useful for checking milk quality, and had a great role in milk let down. Not utilizing separate cleaned and sanitized calving paddocks or pens for cows and heifers was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR: 2.34, $\chi 2$:16 and $P<0.028$, OR: 2.5, $\chi 2$; 17.5 and $P < 0.013$, OR: 4.6, $\chi 2$: 47.5 and $P < 0.00023$ and OR : 3.16, $\chi 2$:37.6 and $P < 0.0013$, respectively) as shown in table 4. The present study provided evidence for the importance of using separate calving pens for cows and heifers, and its disinfection for the successful control of *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk. the current results agreed with Down (2016), Barnouin et al. (2004) and O'Reilly et al. (2006). This effect may could be due to a reduction in pathogen exposure, but additionally may reflect, the negative impact of cross-suckling calves indirectly which was related to mastitis incidence (Green et al., 2007). Cows are susceptible to infection before calving because their natural defense mechanisms are low, new infections occur, and subclinical infections which persisted through the dry period may flare into clinical cases (Down 2016). Housing pregnant heifers with dry cows as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR:4.35, $\chi 2$.:32 and $P < 0.00028$, OR:3.15, $\chi 2$. 27.2 and $P < 0.0018$, OR: 2.2, $\chi 2$: 22.3 and $P < 0.028$ and OR: 2.21, $\chi 2$:20.5 and $P< 0.024$, respectively) as shown in table 4.

These findings agreed with Down (2016). Understanding the mastitis risk factors for dry cows and heifers could also be considered as a critical and vital step to reduce mastitis prevalence. At the time of dry off, cows are not given the protection from post-dip, and their udders are not observed closely for signs of infection. All of those factors contribute and play a key role in elevation the bulk tank level of microbial count and SCC. Herds don't keep SCC records was believed as a risk factor for *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR: 4.27, $\chi 2$. 59.2 and $P < 0.000012$, OR: 2.47, $\chi 2$: 19.4 and $P < 0.0032$, OR: 5.12, $\chi 2$: 79.1 and $P < 0.000002$, OR. 7.23, $\chi 2$. 82 and $P < 0.00000012$, respectively) as shown in table 4. The lowest SCC observed in the herds that was practiced milk recording was possibly because of increased farmer knowledge on individual cows, and its importance as a factor associated with SCC. These results agreed with Hutton et al. (1990) and Down (2016). If dairy producers do not record milk, then it would be difficult to identify cows with constantly elevated SCC, thereby may cause an increasing in the overall bulk SCC. Barkema et al. (1999) found that farmers of herds with a low bulk tank SCC kept better records and were extra acquainted and familiar with every cow in their herds. Milking wet teats was a risk factor for *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR: 2.42, $\chi 2$. 24 and $P < 0.0021$, OR: 2.27, $\chi 2$: 22 and $P < 0.0021$, OR: 5.1, χ: 72 and $P < 0.000002$, OR: 7.1, $\chi 2$. 92 and $P < 0.0000001$, respectively) as shown in table 5.

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Milking wet teats is unacceptable, both for risk of mastitis and elevation of bulk tank SCC and all over total microbial count and subsequent milk quality issues. Water plays an important and vital role in fostering mastitis pathogens on the udder and teats. Water laden with bacteria on udder and teat surfaces can enter teat cup liners, and may increase bacterial contamination of milk, and also increase mastitis incidence, and then subsequently elevate bulk tank SCC (Blowey and Edmondson, 2010). Not using single paper towels or suitable woven cloths for teats' drying was believed as a risk factor for *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR: 2.58, χ2.18 and P<0.013, OR. 2.78, χ2. 15 and P<0.03, OR. 5.58, χ2. 67 and P<0.00004 and OR: 7.4, χ2 .98 and P<0.0000013, respectively) as shown in table 5.

Effective drying had a great impact on the level of SCC in bulk tank milk, and was achieved by using single paper towels or suitable woven cloths. Each cloth must only be used for one cow per milking. Cloths should then be placed in disinfectant solution, washed before the next milking (Blowey and Edmondson, 2010). Not wearing latex gloves was taken as risk factor for *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank milk (OR: 2.92, χ2:20.4 and P < 0.028, OR. 2.32, χ2. 18.9 and P < 0.038, OR: 7.1, χ2. 90 and P < 0.0000003, OR: 7.4, χ2. 93 and P < 0.000000123, respectively) as shown in table 5.

These findings agreed with Bach et al. (2008) and Signorin et al. (2008). The milker can spread contagious mastitis as he handles each cow. The use of gloves was one of the foremost important factors to prevent mastitis caused by *Staphylococcus aureus* agents in dairy cows (Petersson-Wolfe et al., 2010; Arnold and Bewley, 2011), and was included in the National Mastitis Council mastitis control plan (NMC 2001). The operator's hands can be a mean for bacterial dissemination, and gloves have a great role in reducing total bacteria count in bulk tank milk (Nickerson, 2014); Wearing latex gloves decreased *Staphylococcus aureus* prevalence, and incidence in BTM (Dufour et al., 2012). Not washing automatic clusters with hot disinfectant after milking a cow with CM or a high SCC was considered as a risk factor for *Staphylococcus aureus,* Salmonella Typhimurium, SPC and SCC in bulk tank milk (OR: 2.43, χ2:18 and P < 0.024, OR: 2.12, χ2. 15.7 and P < 0.021, OR.:4.23, χ2: 48 and P < 0.00012, OR: 5, χ2: 68.7 and P < 0.00000024, respectively) as shown in table 5.

These findings agreed with Wenz et al. (2007) and Down (2016). At the end of milking, a small quantity of milk is held inside the mouth piece of the line. When the cluster is connected to the next cow, the milk from the previous cow will run down the inner part of the liner, and contaminate the teat of the next cow to which is milking. This represented a risk of infection transferring (Blowey and Edmondson, 2010). Teat contamination occurred via the cluster which was previously contaminated during milking of an infected cow was considered as a great vital point for cross-contamination between cows. Clusters are a usual origin and source of bacteria for dairy cows. Infected cow (clinical case or sub-clinical case with excessive bacterial numbers) had the potential to infect the subsequent 5 to 6 cows milked on that cluster. It was not ideal to wash the cluster (either back-flushing or through-flushing) with cold water. Not applying back-flushing as a risk factor significantly associated with *Staphylococcus aureus, Salmonella* typhimurium, SPC and SCC in bulk tank (OR :5.98, χ2. 59 and P < 0.00001, OR. 3.28, χ2. 39.7 and P < 0.0012, OR. 7.28, χ2. 98.4 and P < 0.0000021, OR. 6.12, χ2. 88.2 and P < 0.000001, respectively) as shown in table 6. Dirty bulk tank surface as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR :7.5, χ2: 85 and P < 0.000002, OR: 5.23, χ2: 68.2 and P < 0.0000001, OR: 6.7, χ2: 88.3 and P < 0.000003 and OR: 7.2, χ2: 95.3 and P <0.0000003, respectively) as shown in table 6.

Claw pieces were not thoroughly brushed after each milking considered as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 3.5, χ2: 41 and P<0.0001, OR. 4.2, χ2: 58 and P<0.000002, OR: 6.7, χ2: 78 and P<0.0000001 and OR. 7, χ2. 93 and P<0.0000002, respectively) as shown in table 6. Gaskets and milk valves were not removed cleaned and brushed daily, it as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 5.7, χ2.:75 and P < 0.000001, OR: 5.2, χ2:72 and P <0.000001, OR: 6, χ2: 82 and P < 0.0000023 and OR. 7, χ2. 93 and P < 0.00000004, respectively) as shown in table 6. Specific cleaning and sanitary program to milk pipelines as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 4.5, χ2: 45 and P < 0.000021, OR: 4.7, χ2: 42 and P < 0.0001, OR: 5.4, χ2: 62 and P < 0.000023 and OR: 6.8, χ2: 73 and P < 0.000005, respectively) as shown in table 6. Not cleaning inflation and all other rubber plastic like parts and free from cracks as a risk factor were significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 3.5, χ2. 35 and P < 0.0021, OR: 3.7, χ2: 32 and P < 0.001, OR. 4.4, χ2: 62 and P < 0.0003 and OR.; 5, χ2:53 and P < 0.00002, respectively) as shown in table 6.

Back-flushing units will help to get rid of both environmental and contagious bacteria, and this may only be of benefit on reducing the bulk tank SCC and SPC level (Blowey and Edmondson, 2010). All internal parts of the tank that can come into contact with milk must be cleaned and disinfected. It was very necessary that the worker checks the internal parts of the tank to be cleaned and disinfected before milking (Blowey and Edmondson, 2010). Dirty bulk tanks played a definite role, and have a great impact on bulk tank SCC and total bacterial level. It is very important to regularly

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check and replace the rubber parts. The rubber plastic like parts was thought to be one of the foremost important sources for bulk tank microbial contamination. Therefore, most standard cleaning program should be beginning with hot water at 35°C to 40°C, flowed by 8 to 10 minutes cleaning with alkaline detergent and disinfectant, and at last cold water rinse (Reinemann et al., 2003). Herd size more than 50 as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 3, $\chi 2$: 39 and $P < 0.0003$, OR: 4.21, $\chi 2$: 49 and $P< 0.00001$, OR: 3.2, $\chi 2$: 37 and $P < 0.0003$, OR: 2.2, $\chi 2$: 18.4 and $P< 0.013$, respectively) as shown in table 5.

These findings agreed with Green et al. (2008). Higher bulk tank SCC was found in larger herds than in the smaller ones (Skrzypek, 2002; Skrzypek et al., 2003; Danko´w et al., 2004; Schewe et al., 2015). The present results disagreed with Norman et al. (2000), Oleggini et al. (2001) and Van Schaik et al. (2002). It would seem that the significant relationship between herd size, and SCC can be described by the fact that the increase in herd size played an important role in elaboration, and magnified the risk of infectious diseases, including mastitis. Another cause may be the fact that in small herds fewer cows were handled by one person, as a result of which animals were treated more individually than in larger herds (Skrzypek et al., 2004). Herd size more than 50 as a risk factor was significantly associated with total bacterial count in bulk tank milk. These results agreed with Goldberg et al. 1992, Gran et al. (2002); Jayarao et al (2004). Herd size (50 cows or more) as a risk factor was significantly associated with Salmonella in bulk tank milk based on the present results which agreed with Ruzante et al. (2010). Large herds may have a greater possibility of buying calves from more than one sources outside with the accompanying the risk of introducing *Salmonella* via subclinical shedders that was stressed by transportation. High cattle density can also additionally be a characteristic of large herds, and may promote *Salmonella* transmission (cummings et al., 2010). Herd size was frequently related to incidence of *Salmonella* in cattle dairy farms (Vaessen et al., 1998; Kabagambe et al., 2000; Warnick et al., 2001; Huston et al., 2002; Blau et al., 2005). Herd size had a great impact on bacterial counts in bulk tank milk (Goldberg et al., 1992; Gran et al., 2002; Jayarao et al., 2004; Elmoslemany et al., 2010). Storage manure indoor as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR :3.27, $\chi 2$: 59 and $P < 0.00123$, OR: 4.17, $\chi 2$: 62.7 and $P < 0.00002$, OR: 6.21, $\chi 2$: 89.7 and $P < 0.000013$, OR: 2.17, $\chi 2$: 29.1 and $P < 0.0021$, respectively) as shown in table 5. Cattle manure was recognized to contain and carry extensive range of microorganisms which can be pathogenic or non-pathogenic to both animals and humans (Godwin, 1997).

It is really worth citing that vectors, e.g., flies and vermin, which might also additionally spread and cause subsequent infections to other animals with pathogens from stored manure and flies, carry a number of mastitis-causing organisms that can colonize teat lesions, and consequently affect the SCC and SPC in bulk tank milk (Holt et al., 2007; Thomson et al., 2017). This requires frequent waste removal, once to several times per day depending on the housing, climate conditions (hot climate leads to drinking, and more frequent urination) and animal density. Animal manure should then be transported to a designated storage or disposal area, out of contact with animal (Manyi-Loh et al., 2016). Muddy bedding materials as a risk factor was significantly related to *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR :2.25, $\chi 2$: 16 and $P< 0.013$, OR: 3.15, $\chi 2$: 36 and $P < 0.00015$, OR: 5, $\chi 2$: 66 and $P < 0.000023$, OR: 6.5, $\chi 2$: 86 and $P < 0.0000003$, respectively) as shown in table 5.

A wide variety of bedding materials are used in cattle barns as straw saw dust, wood and shavings. Bacterial concentrations in fresh and clean bedding were usually much lower than in concentrations in used bedding (Slaghuis et al., 1997). Schreiner and Ruegg (2003) reported that the main and common sources of exposure of environmental mastitis pathogens to the cow were the presence of moisture, mud and manure in the environment of the cow. One of the most vital and important steps affecting bulk tank microbial count and SCC was the transmission of dirt from dirt muddy bedding and soil to milk. Dirt basically was transmitted to milk when it was attached to the exterior of the teats and rinsed off all through milking operation (Murphy and Boor, 2000). The contamination of the teat with muddy soil was regarded one of the foremost important reasons for of elevated concentrations of microbial count in bulk tank milk, and consequently the level of *Salmonella* in bulk tank milk. Practices that expose the teat end to organic bedding sources, moist and muddy pens extended the risk of mastitis prevalence and milk contamination (Ruegg, 2006). Cleanliness of the cows (udder, teats and hocks) as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR: 2.65, $\chi 2$. 19 and $P < 0.023$, OR: 4.25, $\chi 2$: 49 and $P < 0.00023$, OR. 5.15, χ: 69.7 and $P < 0.000001$ and OR: 4.21, $\chi 2$: 45.9 and $P < 0.0012$, respectively) as shown in table 5.

Cleanliness of the cows had a great and vital role in microbial contamination of the bulk tank milk, and had a great impact on the level of *Staphylococcus aureus* SPC and SCC level in bulk tank milk. These results agreed with Ruegg (2003), Schreiner and Ruegg (2003), Elmoslemany et al. (2009b), Piccinini et al. (2009), Zucali et al. (2011) and Cicconi-Hogan et al. (2013). Milking cows should be kept clean, groomed daily and therefore the udders and teats thoroughly should be washed before every milking because the coat and skin were always dirty as this might act as a source of spoilage bacteria. . Cleaning and removal of soil particles, bedding material and manure from the udder and flanks was very essential to prevent the entry of many types of bacteria into the bulk tank milk (Zelalem et al., 2011).

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Summer season as a risk factor was significantly associated with *Staphylococcus aureus*, Salmonella Typhimurium, SPC and SCC in bulk tank (OR: 3.21, $\chi 2$: 39 and P< 0.00012, OR: 2.15, $\chi 2$: 19.7 and P < 0.02, OR: 4.24, $\chi 2$: 42.7 and P < 0.00014 and OR. 3.27, $\chi 2$. 47.8 and P < 0.0002, respectively) as shown in table 6.

The results agreed with Leslie (1996), Sargeant et al. (1998), Norman et al. (2000), Ruegg and Tabone (2000), Zadnik et al. (2001), Berry et al. (2006), Lievaart et al. (2007), Olde Riekerink et al. (2007), Summer et al. (2007), Elmoslemany et al. (2010) and Zucali et al. (2011), Cicconi-Hogan et al. (2013). They reported that bulk tank milk SCC counts were higher in summer, and was perhaps related to the influence and impact of higher temperature and humidity on mastitis risk. Green et al. (2006) suggested that the rise in BMSCC during this period was that the consequences of arise in chronic high individual cow SCC (ICSCC), and disagreed with Berry et al. (2006) who reported that BMSCC was commonly lowest during April, and highest in November. It was additionally recognized that favorable temperature and humidity in the summer played a critical role in favoring growth and quantity of environmental bacteria in bedding material (Harmon 1994). Heat stress may additionally amplify the cow's susceptibility to infection via both lowering host resistance or by increasing host exposure to pathogens (Morse et al., 1988). Variations and fluctuation in temperature and humidity can have a strong impact on bacterial counts in milk. Summer season had a great impact on bulk tank total aerobic count as shown by Izsler (2010). Prevalence of *Staphylococcus aureus* in bulk tank was higher in summer. These results disagreed with findings of Makovec and Ruegg (2003) and Olde Riekerink et al. (2007) who observed a high incidence of *Staphylococcus aureus* in bulk tank milk during December and January. *Salmonella* incidence in bulk tank milk used to be higher in summer season. The present results which agreed with Holley et al. (2006) and Semenov et al. (2007). They mentioned that survival of *Salmonella* in the environment ought to additionally increase in the summer months, although soil composition, moisture and temperature fluctuation appeared to play a role. Region variation as a risk factor was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank (OR.2.31, $\chi 2$: 19 and P < 0.0014, OR: 2.46, $\chi 2$:20.7 and P < 0.002, OR: 3.24, $\chi 2$: 41.4 and P < 0.0012 and OR: 2.17, $\chi 2$: 27.8 and P < 0.0031, respectively) as shown in table 6.

Delta region was significantly associated with *Staphylococcus aureus, Salmonella* Typhimurium, SPC and SCC in bulk tank. These regional variations ought to be variant in climate and agricultural tradition. The climate and geographical aspects and features of region had a great impact on the degree of bacterial contamination and SCC level in bulk tank milk. The results agreed with Raghib et al. (2004). A possible reason for the elevation of TBC in Delta region ought to be due to poor hygienic condition of the collection tanks distributed by milk collectors. This finding agreed with Reinemann et al. (2000). PCR identification of virulence gene (*hlg*) of *Staphylococcus aureus* isolates revealed a product of approximate size 937 bp. The gene (*hlg*) which was found in 13 (54%) *Staphylococcus aureus* isolates (figure 3); these results disagreed with El-baz et al. (2016) (81.1%), Maeda et al. (2016) and Ali (2017) (90%). Hemolysins were produced by *Staphylococcus aureus* isolates, and played a vital role in the pathogenesis of the disease caused by *Staphylococcus aureus* (Lo et al., 2011). γ-hemolysin (*hlg*) gene was found within a 4.5 kb ScaI fragment of *Staphylococcus aureus* chromosome. It was another form of pore forming toxin, unlike α-hemolysin. The *hlg* gene consisted of two transcription units (the first one encodes γ-hemolysin-A (*HlgA*) like protein, a class S (slow) component, and the second one codes γ-hemolysin C (*hlgC*) and γ-hemolysin B (*hlgB*), a category of F(fast) and S(slow) component (Divyakolu et al.2019). Staphylococcal γ-hemolysin (*hlg*) consisted of polypeptides nominated as S (Slow), (*hlgA* or *hlgC*) and F (Fast), (*hlgB*), which coordinated in lysis of target cells, where the S components were proposed to affect cell type susceptibilities to those toxins Meyer et al. (2019). These proteins after determining of their cell targets undertook conformational changes and formed oligomeric complexes. This process resulted in transmembrane-pore formation, and also the cell death (Meyer et al. 2019). This toxin targeted polymorph nuclear cells, monocytes, macrophages and erythrocytes (Vandenesch et al. 2012). γ-hemolysin, belonging to a group of genes that coded for both *hlgA* and *hlgC* as the S (slow) component, or *hlgB* as the F (fast) component which was found within the core genome (Du Mont et al. 2014). On the other hand, molecular identification of *sopB* (*SigD*) virulence gene for *Salmonella* Typhimuriym isolates revealed that the PCR amplification with (*sopB*) gene specific primers was conducted with genomic DNA, which revealed in a product of approximate size 517 bp. *sopB* gene, was found in all *Salmonella* Typhimurium isolates (100%) figures 1 and 2. The *Salmonella* outer protein *(sopB*) gene well was conserved in all *Salmonella* Typhimurium strains. These results agreed with Mirold et al. (2001) who stated that the *sopB* gene was found in the SPI-5 pathogenicity island, and was detected in all sequenced *Salmonella* Typhimurium strains. The outer proteins (Sops) of *Salmonella* were playing a definite role in the manipulation of various stages of polymorphonuclear leukocyte influx, and rearrangement of the cytoskeleton (Boyle et al., 2006). *SopB*/*SigD*, an inositol phosphatase, was needed for fluid and chloride secretion, neutrophil recruitment, and played an important role in causing deformation to epithelial barrier function during the invasion (Bertelsen et al., 2004). It mediated the virulence by inhibiting inositol phosphate signaling pathways (Marcus et al. 2001; Marcus et al., 2002). *SopB*/*SigD* also had a great antiapoptotic activity, and so on they played an important and distinct role in the intracellular replication because of the up keeping and maintain of Akt activation (García-Gil et al., 2018).

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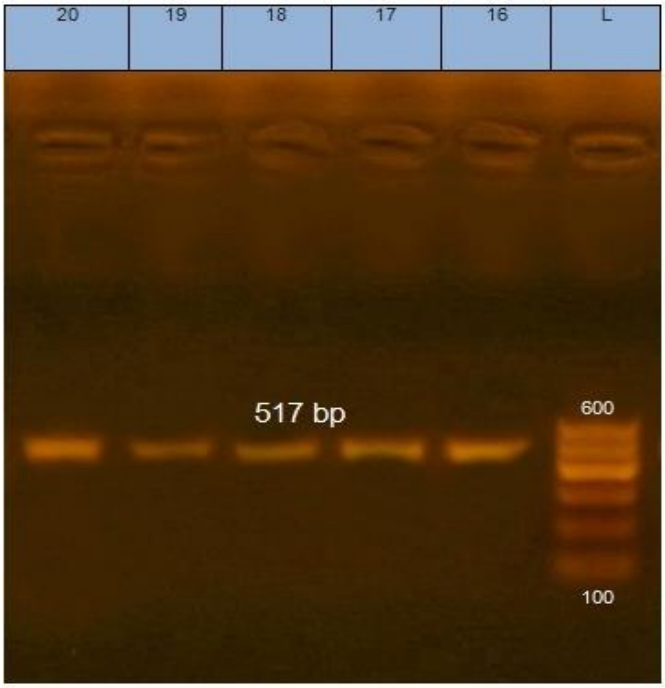

Figure 1. Agarose gel shows a PCR amplified product of 517 bp. of (*sopB*) virulence gene for *Salmonella* Typhimurium, lanes (16) to (20): samples positive for *sopB* gene, Lane (L): MW 100bp ladder (DNA marker)**.**

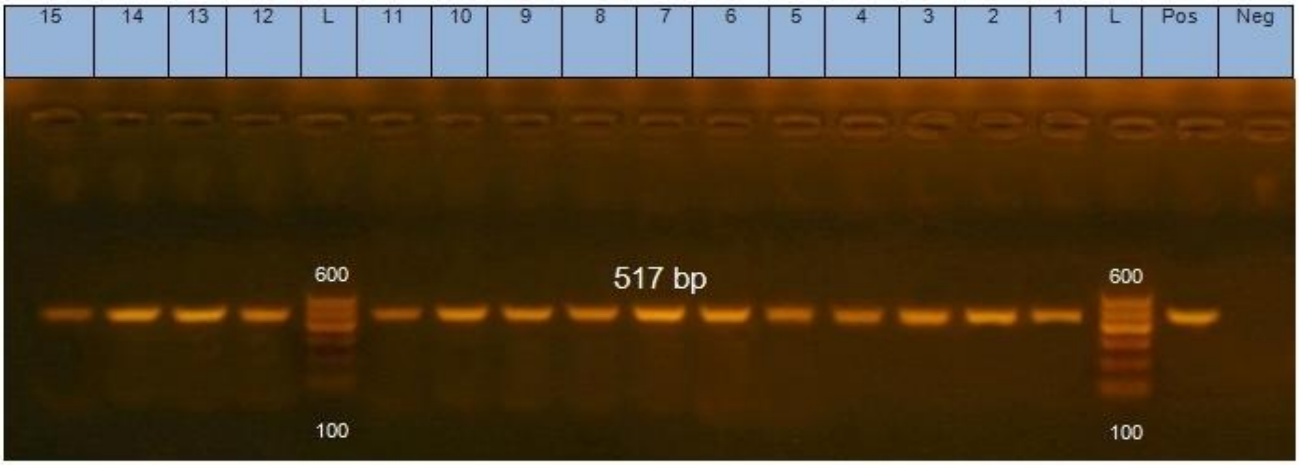

Figure 2. Agarose gel shows a polymerase chain reaction (PCR) amplified product of 517 bp of (*sopB*) virulence gene for *Salmonella* Typhimurium, lanes (1) to (15): samples positive for *sopB* gene, Lane (positive): positive control, Lane (Negative): Negative control, Lane (L): MW 100bp ladder (DNA marker).

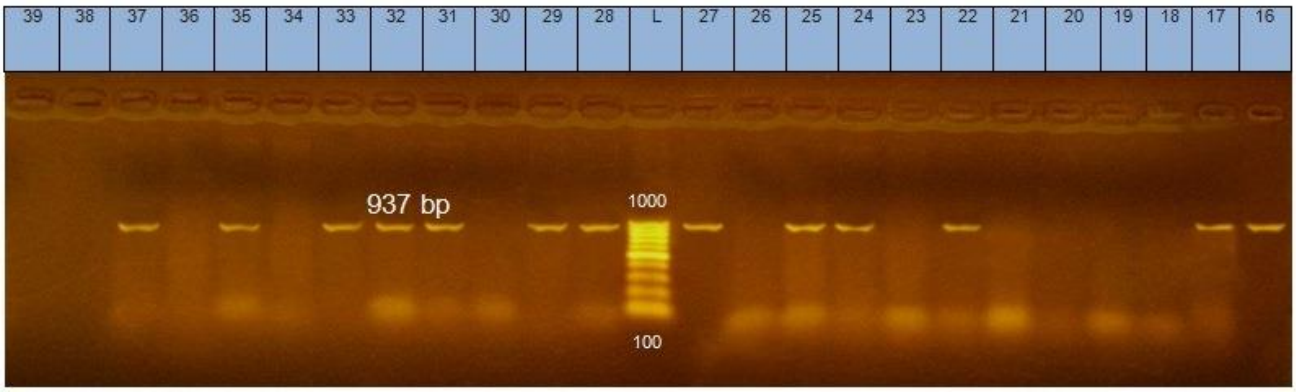

Figure 3. Agarose gel shows a PCR amplified product of 937 bp. of gamma hemolysin (*hlg*) gene for *Staphylococcus aureus*, lanes (16, 17, 22, 24, 25, 27, 28, 29, 31, 32, 33, 35, 37): samples positive for *hlg* gene, Lane (L): MW 100bp. ladder (DNA marker).

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Phylogenetic and partial gene sequence analysis of *hlg* gene of *Staphylococcus aureus* that was generated using neighbor joining in MEGA6 showed a clear clustering of isolated Egyptian *Staphylococcus aureus* strain, and different *Staphylococcus aureus* strains uploaded from gene bank as shown in figures 6 and 7. Sequence distance of *Staphylococcus aureus hlg* virulence gene was created by the MegAlign module of Laser gene DNA Star. Sequence identities between the isolated Egyptian strain and different *Staphylococcus aureus* strains were uploaded from gene bank revealing a 95.8% to 98.9% homology as shown in figure 7. Analyzing of nucleotide sequence of *hlg* virulence gene of the Egyptian isolated strain in the current study showed a 98.9% nucleotide identity with the American *Staphylococcus aureus* strain FDAARGOS-412 (accession no. CP023500) by Minogue et al. (2017), the Belgium *Staphylococcus aureus* strain NMR08 (accession no. CP023560) by Coppens et al. (2017), the Korean *Staphylococcus aureus* strain CN1 (accession no. CP003979) by Chen et al. (2013), the Japanese *Staphylococcus aureus* strains TMUS2126 and TMUS2134 (accession no. AP014652 and AP014563, respectively) by Yamaguchi et al. (2015) and a 98.1% nucleotide identity with the Taiwan *Staphylococcus aureus* strain M013 (accession no. CP003166) by Huang et al. (2012), the Taiwan (MRSA) methicillin resistant *Staphylococcus aureus* strains SA967 and SA40 (accession no. CP003603 and CP003604) by Chen et al. (2013). Amino acids alignment report of the sequenced 934 amino acids of (*hlg*) *Staphylococcus aureus* virulence gene of Egyptian isolated strain (using CLUSTALW) multiple sequence alignment program version 1.83 of MegaAlign module of laser gene DNA star as shown in figure 8 showed a great homology between the *Staphylococcus aureus* Egyptian strain, and the different *Staphylococcus aureus* strains uploaded from gene bank. Phylogenetic and partial gene sequence analysis of *sopB* gene of *Salmonella* that was generated using a neighbor joining in MEGA6 as shown in figures 4 and 5 showed a clear clustering of isolated Egyptian *Staphylococcus aureus* strain and different *S. aureus* strains uploaded from gene bank. Nucleotide sequence distance of *Salmonella* Typhimurium *sopB* virulence gene was created by the MegaAlign module of Laser gene DNA Star. Nucleotide Sequence identities between the isolated Egyptian strain and different *Salmonella* strains uploaded from gene bank revealed a 98.6% to 100% homology as shown in figure 4. Nucleotide sequence analysis of *sopB* virulence gene of the Egyptian isolated strain showed a 100% nucleotide identity with, the Taiwan *Salmonella* Typhimurium strain BL10 (accession no. CP024619) by Hong et al. (2018), the English *Salmonella* Typhimurium strain VNP151-sc-2315230 (accession no. LT795114) by pathogen informatics (2017), the American *Salmonella* Typhimurium RM1160 strain (accession no. CP022658) by Parker et al. (2017), the English *Salmonella* Typhimurium strain DT2 (accession no. HG326213) by Kingsley et al. (2013), the English *Salmonella* Typhimurium strain DT104 (accession no. HF937208) by Mather et al. (2013), *Salmonella* Typhimurium var.5 strain CFSAN001921 (accession no. CP006048) by Hoffman et al. (2013), the English *Salmonella* Typhimurium strain U288 (accession no. CP003836) by Hotton et al. (2012), the American *Salmonella* Typhimurium strain 798 (accession no. CP003386) by Petersson et al. (2012), the American *Salmonella* Typhimurium strain Uk1 (accession no. CP002614) by (Luo et al. (2011), the American *Salmonella* Typhimurium strain ST4/74 (accession no. CP002487) by Richardson et al. (2011), the Japanese *Salmonella* Typhimurium strain T000240 (accession no. AP011957) by Izumiyia et al. (2011), the *Salmonella* Typhimurium strain SL1344 (accession no. FQ312003) by Kroger et al. (2012), the American *Salmonella* Typhimurium strain 14028S (accession no. CP001363) by Jarvik et al. (2010). Amino acids alignment reported of the sequenced 514 amino acids of *Salmonella* Typhimurium (*sopB*) virulence gene of Egyptian isolated strain (using CLUSTALW) multiple sequence alignment program version 1.83 of MegaAlign module of laser gene DNA star as shown in figure 9 showed a great homology between the Egyptian strain *Salmonella* Typhimurium and the different *Salmonella* Typhimurium strains uploaded from gene bank.

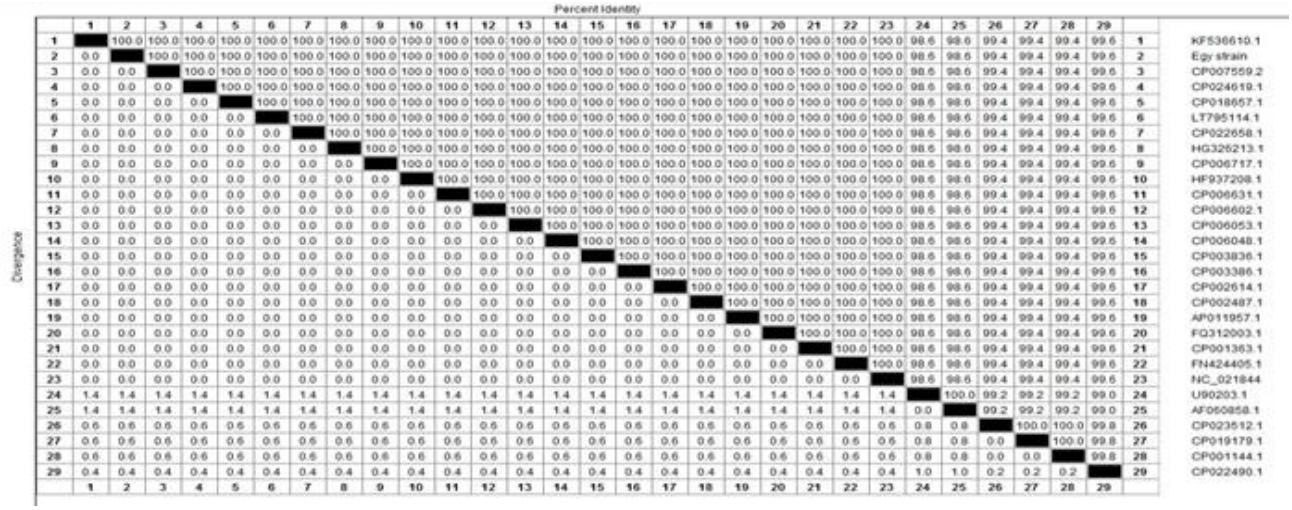

Figure 4. Nucleotide Sequence distance analysis of *Salmonella* Typhimurium (*sopB*) virulence gene between the Egyptian isolated strain and different *Salmonella* Typhimurium strains uploaded from gene bank.

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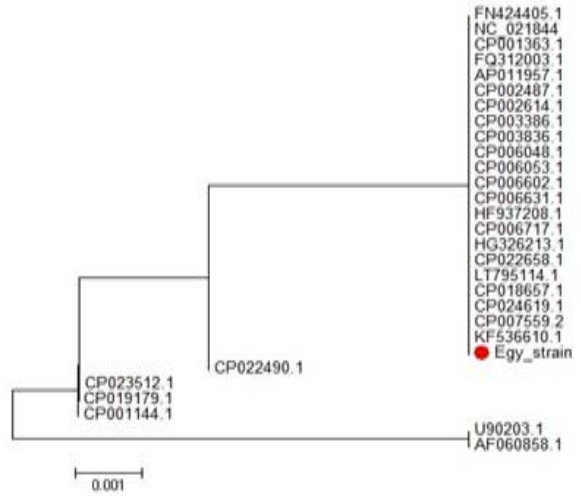

Figure 5. Phylogenetic tree for *Salmonella* Typhimurium (*sopB*) virulence gene partial nucleotide sequences that was generated using a neighbor joining in MEGA6. It shows a clear clustering of the Egyptian isolated strain and different *Salmonella* Typhimurium strains uploaded from gene bank**.**

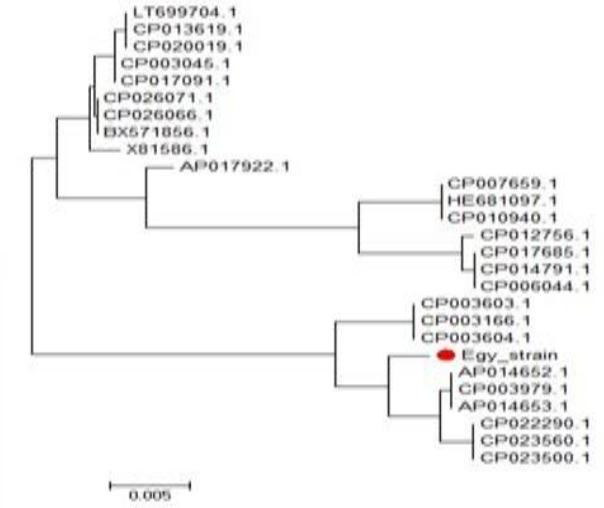

Figure 6. Phylogenetic tree for *Staphylococcus aureus* (*hlg*) virulence gene partial nucleotide sequences that was generated using a neighbor joining in MEGA6. It shows a clear clustering of the Egyptian isolated strain and different *S. aureus* strains uploaded from gene bank**.**

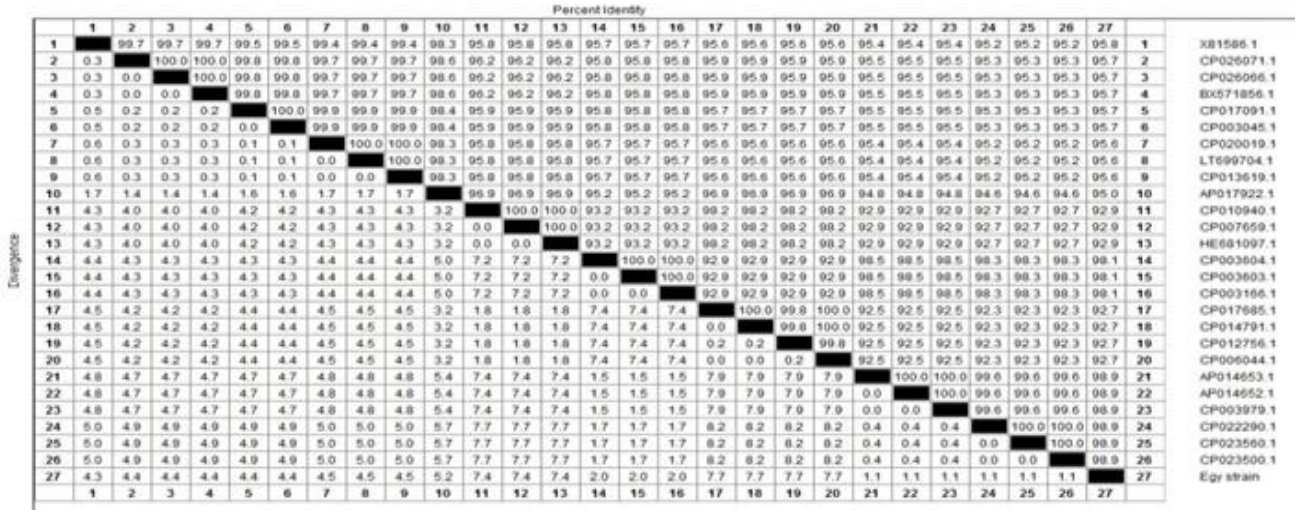

Figure 7. Nucleotide sequence distance analysis of (*hlg*) *Staphylococcus aureus* virulence gene between the Egyptian isolated strain and different *Staphylococcus aureus* strains uploaded from gene bank

```
Majority   ANPLLENAKAANDTEDIGKGNDVEIIKRTEDKTSNKWGVTQNIQFDFVKDKKYNKDALILKMQGFISSRTTYYNYKNTNH
           ---------+---------+---------+---------+---------+---------+---------+---------+
                    10        20        30        40        50        60        70        80
           ---------+---------+---------+---------+---------+---------+---------+---------+
X81586.1   ..........................................G.........V.............................. 238
CP026071.1 ..........................................................................  238
CP026066.1 ..........................................................................  238
BX571856.1 ..........................................................................  238
CP017091.1 ..........................................................................  238
CP003045.1 ..........................................................................  238
CP020019.1 ..........................................................................  238
LT699704.1 ..........................................................................  238
CP013619.1 ..........................................................................  238
AP017922.1 ..........................................................................  238
CP010940.1 ..........................................................................  238
CP007659.1 ..........................................................................  238
HE681097.1 ..........................................................................  238
CP003604.1 ....................S.I.................................................  238
CP003603.1 ....................S.I.................................................  238
CP003166.1 ....................S.I.................................................  238
CP017685.1 ......K...................................................................  238
CP014791.1 ......K...................................................................  238
CP012756.1 ........R.................................................................  238
CP006044.1 ......K...................................................................  238
AP014653.1 ....................S.I.................................................K...  238
AP014652.1 ....................S.I.................................................K...  238
CP003979.1 ....................S.I.................................................K...  238
CP022290.1 ....................S.I.................................................K...  238
CP023560.1 ....................S.I.................................................K...  238
CP023500.1 ....................S.I.................................................K...  238
Egy strain ....................S.I.....................G...........................K...  238
```


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```
Majority   TYGRNMDVTHAIKRSTHYGNSYLDGHRVHNAFKNRNYTVKYEVNWKTHEIKVKGQN-YEHE-ISQIIRCYVY
           ---------+---------+---------+---------+---------+---------+---------+--
                    250       260       270       280       290       300       310
           ---------+---------+---------+---------+---------+---------+---------+--
X81586.1   ..................................................................................  934
CP026071.1 ..................................................................................  934
CP026066.1 ..................................................................................  934
BX571856.1 ..................................................................................  934
CP017091.1 ..................................................................................  934
CP003045.1 ..................................................................................  934
CP020019.1 ..................................................................................  934
LT699704.1 ..................................................................................  934
CP013619.1 ..................................................................................  934
AP017922.1 ..................................................................................  934
CP010940.1 .................................................................................I.  934
CP007659.1 .................................................................................I.  934
HE681097.1 .................................................................................I.  934
CP003604.1 ............................I....V.........................N.........I.  934
CP003603.1 ............................I....V.........................N.........I.  934
CP003166.1 ............................I....V.........................N.........I.  934
CP017685.1 ............................I.....................................  934
CP014791.1 ............................I.....................................  934
CP012756.1 ............................I.....................................  934
CP006044.1 ............................I.....................................  934
AP014653.1 ..............................V.........................N.........I.  934
AP014652.1 ..............................V.........................N.........I.  934
CP003979.1 ..............................V.........................N.........I.  934
CP022290.1 ..............................V.........................N.........I.  934
CP023560.1 ..............................V.........................N.........I.  934
CP023500.1 ..............................V.........................N.........I.  934
Egy strain ..............................V.........................N.............  934
```

Figure 8. Deduced amino acids alignment of (*hlg*) *Staphylococcus aureus* virulence gene of Egyptian isolated strain using (CLUSTALW) multiple sequence alignment program version 1.83 of MegaAlign module of laser gene DNA star and different *Staphylococcus aureus* strains uploaded from gene bank

```
Majority   HPQIIGVMNILGIPTAYALSLISTGKDIFCADFHLCCRELRVGIVMPVMRECIFYGLPGLVIKLLDLSISKRYCRKAMSF
           ---------+---------+---------+---------+---------+---------+---------+---------+
                    10        20        30        40        50        60        70        80
           ---------+---------+---------+---------+---------+---------+---------+---------+
KF536610.1 ..................................................................................  238
Egy strain ..................................................................................  238
CP007559.2 ..................................................................................  238
CP024619.1 ..................................................................................  238
CP018657.1 ..................................................................................  238
LT795114.1 ..................................................................................  238
CP022658.1 ..................................................................................  238
HG326213.1 ..................................................................................  238
CP006717.1 ..................................................................................  238
HF937208.1 ..................................................................................  238
CP006631.1 ..................................................................................  238
CP006602.1 ..................................................................................  238
CP006053.1 ..................................................................................  238
CP006048.1 ..................................................................................  238
CP003836.1 ..................................................................................  238
CP003386.1 ..................................................................................  238
CP002614.1 ..................................................................................  238
CP002487.1 ..................................................................................  238
AP011957.1 ..................................................................................  238
FQ312003.1 ..................................................................................  238
CP001363.1 ..................................................................................  238
FN424405.1 ..................................................................................  238
NC_021844  ..................................................................................  238
U90203.1   ..................................G.........................V..V.........  238
AF060858.1 ..................................G.........................V..V.........  238
CP023512.1 ..................................G............................V.........  238
CP019179.1 ..................................G............................V.........  238
CP001144.1 ..................................G............................V.........  238
CP022490.1 ..................................G.....................................  238
```


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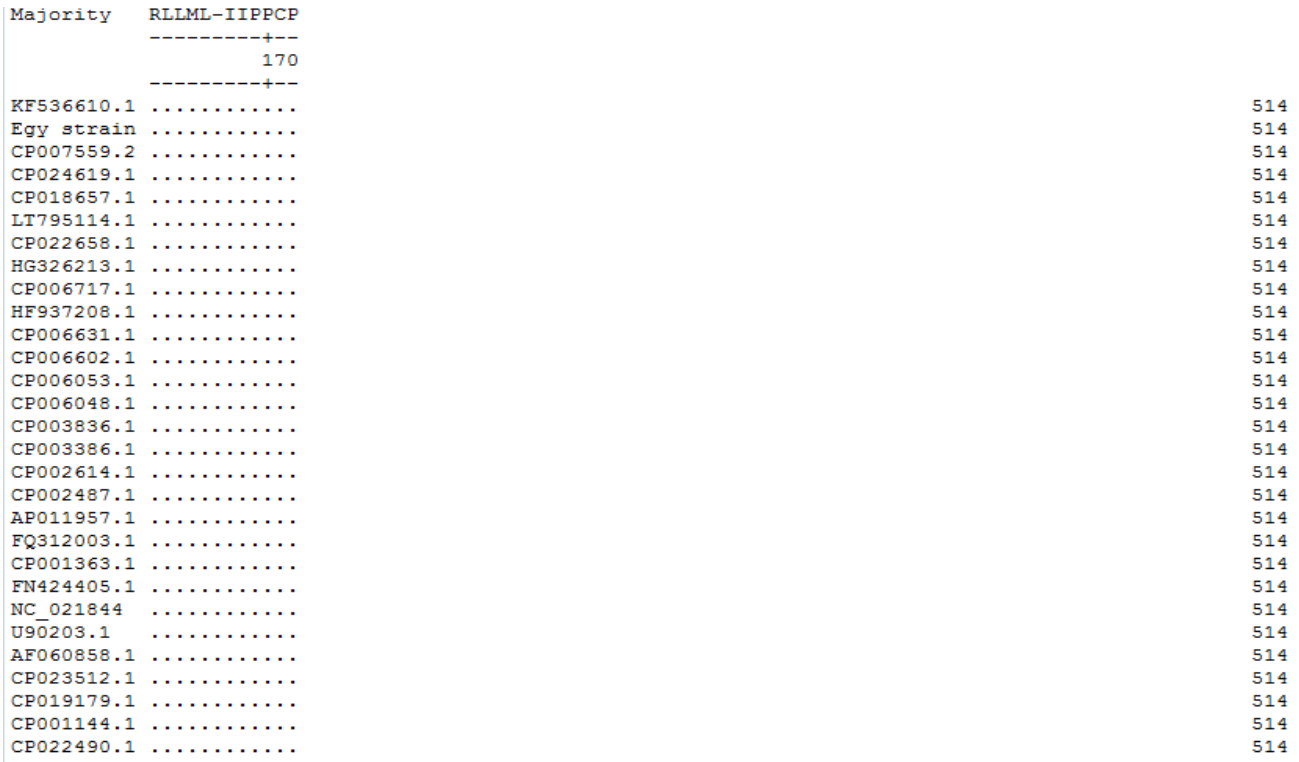

Figure 9. Deduced Amino acids alignment of *Salmonella* Typhimurium (*sopB*) virulence gene of Egyptian isolated strain using (CLUSTALW) multiple sequence alignment program version 1.83 of MegaAlign module of laser gene DNA star and different *Salmonella* Typhimurium strains uploaded from gene bank

CONCLUSION

The results of present study provided an allocation for the elaborateness of more efficacious preventive programs for controlling the mastitis and bacteriological quality of bulk tank milk, enhancement of raw milk quality, decreasing and monitoring the economic losses caused by the disease to dairy industry. Analyzing of nucleotide sequence of *hlg* virulence gene of the *Staphylococcus aureus* Egyptian isolated strain in the current study showed a 95.8% to 98.9% nucleotide identity with different *Staphylococcus aureus* strains uploaded from gene bank. Nucleotide sequence identities between the isolated Egyptian *Salmonella* strain and different *Salmonella* strains uploaded from gene bank revealed a 98.6% to 100% homology.

DECLERATIONS

Author`s contributions

M.F and H.M designed the concept of the review article, and M.F and H.M designed and performed study design and the economic frame work .All authors shared in writing, reviewed and approved the manuscript. All authors read and approved the final version.

Competing interests

The authors declared that they have no competing interests.

Acknowledgments

This work was supported by Department of Udder Health and Neonates, Animal Reproduction Institute, ARC. El-Haram, Giza, for their support.

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To cite this paper: Azooz MF, SEl-Wakeel A, and Yousef HM (2020). Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies. *World Vet. J.*, 10 (3): 338-361. DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

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To cite this paper: Azooz MF, SEl-Wakeel A, and Yousef HM (2020). Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies. *World Vet. J.*, 10 (3): 338-361. DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

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To cite this paper: Azooz MF, SEl-Wakeel A, and Yousef HM (2020). Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies. *World Vet. J.*, 10 (3): 338-361. DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

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To cite this paper: Azooz MF, SEl-Wakeel A, and Yousef HM (2020). Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies. *World Vet. J.*, 10 (3): 338-361. DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

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To cite this paper: Azooz MF, SEl-Wakeel A, and Yousef HM (2020). Risk Factor Analysis of *Salmonella* Typhimurium, *Staphylococcus aureus*, Standard Plate Count and Somatic Cell Count in Bulk Tank Milk in Cattle Dairies. *World Vet. J.*, 10 (3): 338-361. DOI: https://dx.doi.org/10.36380/scil.2020.wvj44

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 362-374, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj45

Effect of Different Dietary Crude Protein Levels and Citric Acid on Broiler Chickens' Performance, Carcass Characteristics, Intestinal Morphology, and Blood Components

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ORIGINAL ARTICLE
pii: S232245682000045-10
Received: 19 Jul 2020
Accepted: 28 Aug 2020

ABSTRACT

The present study was conducted to investigate the effect of dietary protein levels and citric acid on the growth performance, carcass yield, abdominal fat, chemical composition of meat, intestinal morphology, and blood parameters of broiler chickens. A total of 160 Cobb 500 unsexed one-day-old broilers were assigned to 4 dietary treatments, 4 replicates of 10 chickens each. A factorial design arrangement 2×2 was used, including two protein levels, 100% (optimal level) and 90% (low level) of recommended dietary crude protein for cobb 500 broiler chickens, each protein level supplemented with or without 20-gram citric acid /kg. The results showed that chickens fed the diet containing 100% required Crude Protein (CP) supplemented with citric acid which could significantly improve body weight gain, feed conversion ratio, carcass yield, abdominal fat, fat content in meat, intestinal morphology, cecal microbial content, and blood parameters (Albumin, haemoglobin Ac1, fructosamine, and cholesterol). Chickens fed the low CP diet supplemented with citric acid could compensate for the growth performance equivalent to those fed the optimal CP diet. Both required protein level and citric acid were significantly improved blood albumin and reduced haemoglobin Ac1 and fructosamine, which could serve as indicators of the blood protein glycation. In conclusion, citric acid addition could alleviate the negative effect of feeding broiler chickens on low CP diets through its beneficial impact on intestinal morphology, cecal bacterial counts, blood cholesterol reduction, and glycated proteins.

Keywords: Broiler, Citric Acid, Glycation, Performance, Protein level

INTRODUCTION

It is of utmost significance for the livestock industry to benefit from inexpensive, highly efficient, and safe diets. Inclusion of low crude protein content in poultry diets can be considered as one of the effective strategies to reduce ammonia emission from poultry wastes, environmental impact, and diet costs (Belloir et al., 2017 and Barekatain et al., 2019). However, such diets can result in negative consequences, including poor growth performance and carcass yield of broilers (Bregendahl et al., 2002; Liu et al., 2017; Allameh and Toghyani, 2019). The negative effect of a low protein diet on chickens' growth performance might be attributed to the effects of protein deficiency on intestinal morphological changes (Yu et al., 2019). Therefore, nutritionists have developed strategies to lessen the negative effects of the low CP diets on broiler chickens' growth performance (Kermani et al., 2017; Goodarzi Boroojeni et al., 2018 and Sharifuzzaman et al., 2020).

Many endogenous factors limited protein utilization in the chickens' diet, such as gastrointestinal tract physiology, oxidative stress, and glycation. Glycation is one of the non-enzymatic reactions observed in avian species, which might reduce amino acids' nutritional value, therefore low glycation of protein and amino acids could enhance poultry performance (Makino et al., 2015). On the other hand, the low dietary protein could increase the glycated amino acids in chicken plasma (Honma et al., 2017). Therefore, diet adjustments or food additives could be considered as early prevention against glycation (Guilbaud et al., 2016).

As reported, human consumption of several compounds, such as organic acids, minerals, and probiotics induce anti-glycation activity. These compounds, especially organic acids, had been used as alternatives for antibiotics (Polycarpo et al., 2017) and growth promoters in poultry diets (Sabour et al., 2019 and Adhikari et al., 2020). Several studies lend support to the effects of organic acids, especially citric acid, on the enhancement of growth performance (Islam et al., 2011 and Tanzin et al., 2015), carcass yield (Lakshmi et al., 2016), and intestinal histology (Khosravinia et al., 2015). Therefore, the current study aimed to evaluate the impact of adding citric acid to broiler diets with protein levels on growth performance, carcass characteristics, intestinal morphometry, microbiota, and blood biochemical parameters.

To cite this paper: Abd-Elsamee MO, Fathy Abbas Motawe H, Maher Selim M and Ramadan Elsherif HM (2020). Effect of Different Dietary Crude Protein Levels and Citric Acid on Broiler Chickens' Performance, Carcass Characteristics, Intestinal Morphology, and Blood Components. *World Vet. J.,* 10 (3): 362-374. DOI: https://dx.doi.org/10.36380/scil.2020.wvj45

MATERIALS AND METHODS

This six-week study was conducted between March and April 2019 at the Poultry Research Unit, Regional Center for Food and Feed (RCFF), Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. The analyses were carried out at laboratories of RCFF.

Ethical approval

This experiment was performed according to all ethics and animal rights of Agriculture Research Center, Egypt.

Housing and management

The present study consisted of 160 Cobb 500 unsexed one-day-old broiler chicks with 40 gram (g) initial weight. The chickens were randomly assigned into four different groups, four replicates of 10 chickens each, and were housed in a semi-closed house in mesh wire-floored cages equipped with automatic drinkers and pan feeders. The chicks were kept under a similar veterinary control and management system in all treatments. The house temperature was maintained at 32 º C for the first 5 days, and then gradually decreased to 24 º C at the age of 21 days. The chickens were received continuous light for the first 24 hours, and then 23 hours light and one hour of darkness for the remainder of the experiment. All chicks were monitored for general health twice daily. Feed and water were offered *ad-libitum* throughout the experimental period.

The experimental diets and design

The experiment was designed in a 2 (levels of protein) × 2 (levels of Citric Acid, CA) factorial design arrangement during starter (1-14 days old), grower (15-28 days old), and finisher (29-42 days old) periods. The experimental diets included the protein levels of 100% (optimal level) or 90% (low level) of the requirements, and the CA levels of 0 or 20 g/kg diet. The experimental diets were formulated to meet the nutritional recommendations according to Cobb 500 guide with the exception of CP in low protein diets (Table 1).

Broiler performance

The chickens and diets in each pen were weighed weekly, and feed efficiency was adjusted for mortality on a pen basis. The Initial Body Weight (IBW) and Final Body Weight (FBW) of each period were used to calculate Body Weight Gain (BWG). Feed Intake (FI) was recorded at the end of each period during the experiment. Feed Conversion Ratio (FCR) was calculated according to the following equation:

FCR = FI (g) / BWG (g)

BWG, FI, and FCR were calculated cumulatively at the end of the experiment.

The mortality was daily recorded throughout the experiment period.

Carcass measurements

At the end of the experiment, eight chickens from each treatment with approximately similar body weight were chosen for carcass measurements. Prior to slaughter, the selected chicks were deprived of feed for eight hours, individually weighed, and then slaughtered. After complete bleeding, the chicks were plucked by dry-plucking, and their weights were recorded. Head and shanks were removed, then the chicks were eviscerated, and different organs (i.e., intestine, gizzard, lungs, spleen, liver, and heart) were separated. The carcass giblets (i.e., gizzard, liver, and heart), and organs related to the immune system (i.e., spleen, and bursa of Fabricius) were separately weighed.

The proportional weight of giblets or carcass (dressing, %) to live weight was calculated using the following equations:

Giblets weight (%) = [GW / LBW] × 100

Dressing (%) = [DW / LBW] × 100

Where LBW denotes live body weight, GW refers to giblets weight, and DW is dressed weight

Meat composition

The carcass was stored in an airtight polyethylene bag at −18°C for the later evaluation of the whole-body chemical composition (dry matter, CP, fat, and ash).

Intestinal morphology

Small intestines were immediately removed from slaughtered chickens, excised, and flushed with distilled water for content removal. Segments of 2cm in length from the midpoint of the duodenum fixed in a 10% buffered formalin. The samples were evaluated in terms of the Villus Height (VH) and Width (VW), Crypt Depth (CD), Villus Height to Crypt Depth ratio (VH/CD). The surface area of the villus was calculated as the product of the height multiplied by the width (Allameh and Toghyani, 2019).

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Bacteriological analysis of caecal content

At the end of the experimental period, eight birds from each experimental group were selected and slaughtered for bacteriological analysis. The caecum from each chicken was removed aseptically and the contents were transferred into a sterile test tube. The samples of caecum contents were examined for caecum microflora, including *Lactobacillus* bacteria, *E- coil*, and *Clostridium perfringens.*

Blood parameters

At the time of slaughter, eight blood samples were collected from experimental groups. Commercial diagnostic kits (Diamond Diagnostics Company, Egypt) used to measure total protein, albumin, cholesterol, renal function, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT). The globulin concentration was calculated as the difference between total protein and albumin according to Coles (1974). Haemoglobin (Hgb) was measured as mentioned by Van Kampen and Zillstra (1983) and plasma glucose was analyzed based on Trinder's (1969) method. Hemoglobin A1c (HbA1c) values were determined using a Variant II Kit analyzer (Bio-Rad, Hercules, California, USA). Fructosamine was calculated according to the following equation (Cohen et al., 2003):

Fructosamine = (HbA1c–1.61) ×58.82

Statistical analysis

The general linear model was employed for data analysis using SAS software (SAS Institute, USA, 2004). Duncan's multiple range test (Duncan, 1955) was performed to detect significant differences of means. The p-value less than 0.05 was considered statistically significant. The employed model was as following:

$Y_{ij} = \mu + P_i + C_j + (PC)_{ij} + e_{ij}$

Where, Y_{ijis} refers to the observation of the parameter measured, μ is overall mean, P_i denotes the fixed effect of protein level, C_j counts for the fixed effect of CA level, $(PC)_{ij}$ is the fixed effect of interaction between levels of CP and CA, and e_{ij} stands for a random error.

Table 1. Composition and calculated analysis of the experimental diets for broiler during starter (1-14 days old), grower (15-28 days old), and finisher (29-42 days old) periods at the Poultry Research Unit, Giza, Egypt.

Stage / Ingredient	Starter (1-14days old)				Grower (15-28days old)				Finishers (29- 42days old)			
Yellow Corn (8.2% CP)	56.46	53.23	61.30	57.89	59.60	56.00	65.07	61.67	63.92	60.37	69.04	65.34
Soybean Meal (44.5% CP)	31.66	31.557	29.00	29.00	31.85	32.47	27.30	27.30	27.50	27.90	23.40	23.95
Corn Gluten Meal (59.4% CP)	6.00	6.50	4.04	4.50	2.00	2.00	1.65	2.082	1.55	1.62	1.00	1.00
Vegetable Oil	1.82	2.75	1.55	2.50	2.75	3.83	2.05	3.05	3.467	4.60	2.85	4.00
Vitamins & Minerals premix*	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Common Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
L-lysine-HCl	0.17	0.17	0.25	0.25	0.10	0.09	0.205	0.21	0.11	0.11	0.23	0.23
DL-Methionine	0.10	0.1	0.111	0.111	0.125	0.125	0.145	0.145	0.12	0.12	0.15	0.15
Di-calcium Phosphate	2.10	2.05	2.107	2.107	1.96	1.90	1.90	1.91	1.75	1.75	1.70	1.70
Limestone	0.91	0.86	0.86	0.86	0.83	0.80	0.90	0.85	0.80	0.75	0.85	0.85
Choline chloride	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
Citric acid	0.00	2.00	0.00	2.00	0.00	2.00	0.00	2.00	0.00	2.00	0.00	2.00
Total	100	100	100	100	100	100	100	100	100	100	100	100
Composition, calculated**												
Metaboizable E k.cal/kg	3000	3000	3003	3000	3053	3053	3052	3053	3154	3159	3153	3155
Crude protein %	22.3	22.3	20.3	20.3	20.3	20.2	18.5	18.5	18.4	18. 3	16.7	16.6
Calcium %	0.93	0.90	0.90	0.90	0.86	0.84	0.86	0.85	0.79	0.77	0.78	0.79
Available phosphors %	0.46	0.45	0.46	0.46	0.44	0.43	0.42	0.42	0.40	0.39	0.38	0.38
Lysine %	1.23	1.22	1.23	1.22	1.13	1.13	1.12	1.12	1.03	1.03	1.05	1.05
Methionine %	0.49	0.49	0.46	0.46	0.46	0.46	0.45	0.45	0.43	0.42	0.43	0.43
Methionine+ Cysteine %	0.86	0.86	0.81	0.81	0.80	0.80	0.77	0.76	0.74	0.74	0.72	0.72

CP: Crude Protein.*Vitamin and mineral premix each 2Kg contains: Vitamin A 10000000 IU, Vitamin D_3 3000000 IU, Vitamin E 25000 mg, Vitamin K 2000 mg, Vitamin B_1 1500mg; Vitamin B_2 5000mg, Vitamin B_6 1500mg, Vitamin B_{12} 15000mcg, Niacin 30000 mg, folic acid 1000mg, Pantothenic acid 10000mg, Biotin 75000mcg, copper 50000mg, iron 40000mg, manganese 60000mg, zinc (Oxide) 60000mg, iodine 1000mg, selenium 300mg, cobalt 200 mg, BHT (Antioxidant) 250mg and Carrier: Calcium Carbonate up to 2 Kg .**Values were calculated from the data according to NRC (1994). Calculated values of crude protein are based on determined values for corn (8.2% CP), soybean meal (44.5% CP), and corn gluten meal (59.4% CP).

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RESULTS AND DISCUSSION

Growth performance

The effect of adding CA in low protein broiler diet on FI, BWG, and FCR at day 42 of age is presented in table 2. The reduction in dietary CP percentage could significantly increase FI, while BWG and FCR were negatively affected. Although CA addition had no significant effect on FI, it could significantly improve BWG and FCR values ($p \leq 0.05$). The best BWG and FCR values were recorded for the chickens fed 100% CP (optimal level) with the addition of CA. The low protein diet supplemented with CA showed improvements in BWG and FCR of chickens, compared to those fed a low protein diet without CA addition. Regarding citric acid, the BWG and FCR values (2312 g and 1.59, respectively) in chickens receiving a low protein with CA diet were as ameliorated as those fed the required CP (2314 g and 1.57, respectively). Consequently, significant differences were observed in the investigated treatments due to CP and CA interaction.

The obtained results of the present study addressing feed consumption elevation due to low protein levels were in line with the results obtained by Aletor et al. (2000) and Barekatain et al. (2019) indicating increased FI caused by low protein use in the broiler diet. However, Roy et al. (2010) and Park and Kim (2019) stated that feed consumption of broilers was not significantly influenced by dietary protein level. On the other hand, Malomo et al. (2013) and Allameh and Toghyani (2019) concluded that the FI of broiler chickens decreased significantly as the CP level reduced. Several reasons could explain the increase in feed consumption for the low protein diets fed with broilers, including the effect of CP on the appetite of fed broilers (Srilatha et al., 2018), deficiency of some critical amino acids, and the differences in the digestibility of amino acids in the diets (Kamran et al., 2010), and the birds might increase the feed intake to get their CP requirements (Selim, 2015; Belloir et al., 2017 and Mahmoud et al., 2017).

Several studies reported the inferior effects of low dietary protein on broiler growth performance (Hernández et al., 2012; Folorunso et al., 2014 and Liu et al., 2017). In the same vein, Allameh and Toghyani (2019) concluded that the weight of broiler chickens significantly decreased when the dietary CP reduced from 20.4% to 17.9%. Moreover, they concluded that feeding 85% of CP requirements had negative effects on the daily weight gain of broilers. Recently, Hilliar et al. (2020) and Macelline et al. (2020) concluded that birds were fed low protein diets had a low BWG, compared to chicks that were fed high protein diets. However, Tarsewics et al. (2006) found that diets with different protein levels had no significant effect on the BWG of birds.

The FCR results of the current study were in accordance with the findings of a study conducted by Gheorghe (2013) concluding that the highest FCR value in a group fed sub-optimal protein diet might be due to the increase in the feed consumption with low body weight gain. Selim (2015) stated that the different amounts of feed consumed on a 22% CP diet could result in less rapid passage of feed in the intestine, compared to a diet with low CP (20%) with a high rapid passage of consumed feed. This issue could result in a decrease in nutrients utilization, which could have a negative effect on the BWG. The results of the current study suggested that CA had no contributory role in FI which was also supported by Abdel-Fattah et al. (2008) and Dehghani-Tafti and Jahanian (2016). On the contrary, Haque et al. (2010) reported that the CA had a significant effect on the FI of broiler chickens.

More notably, the outcomes of the current study underline the improvement of BW and FCR values due to the use of CA. In the same vein, Panda et al. (2009) asserted that FCR was reduced in treatments with organic acids and had no effect on FI. Moreover, Islam et al. (2011) reported that birds fed CA diets had high body weights of 2.2, 4.68, and 5.03% for the 0.25, 0.75, and 1.25% of CA groups, respectively, compared to the control birds. In addition, Nourmohammadi and Khosravinia (2015) concluded that 3% of CA in the broiler diet led to an increase in BWG around 4.16%. Lakshmi et al. (2016) announced that adding CA (2 %) to the broiler diet improved the broiler performance by 10%. Nourmohammadi and Khosravinia (2015) stated that adding CA (3%) had positive effects on BWG, FI, and FCR of broilers since it could ameliorate nutrients digestibility as a result of proper intestinal conditions. However, Biggs and Parsons (2008) found that using 3% of CA had no significant effect on the broiler's performance.

Carcass measurements

The effects of adding CA to a low protein broiler diet on the whole body chemical composition and abdominal fat percentage are shown in table 3. A significant ($p < 0.05$) increase was found in DM, fat, and abdominal fat percentage due to a low protein level, compared to the optimal CP level. On the other hand, it should be mentioned that the protein level had no significant effect on CP and ash percentage regarding the whole body. Citric acid improved the chemical composition of meat (DM, Fat, and CP %) and abdominal fat percentage compared to non-supplemented groups. The protein level and CA interactions showed significant differences ($p < 0.05$) in DM, CP, fat, and abdominal fat percentage. Citric acid added to a low protein diet significantly ($p < 0.05$) decreased fat and abdominal fat and increased CP percentage in broiler carcass.

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The negative effect of sub-optimal dietary CP on carcass composition in the present study was in agreement with the findings of studies performed by Aletor et al. (2000); Namroud et al. (2008); Sigolo et al. (2017), and Srilatha et al. (2018). Likewise, Javaid et al. (2012) construed the effect of dietary protein on meat DM through the depression of liver lipogenesis leading to a reduction in fat deposition in the muscles and subsequently a reduction in the DM of the meat. Furthermore, the negative correlation between protein levels and fat deposition could initially be due to the increase in feed consumption for birds to meet growth requirements, as a response to any nutrient deficiency (Kamran, 2010) could increase fat deposition (Gous et al., 1990). Second, high Metabolic Energy to CP ratio in a low protein diet promotes lipogenesis (Sigolo et al., 2017) because of the more energy intake per unit of CP intake (Srilatha et al., 2018). Third, the increase of abdominal fat by low CP diets could be due to the reducing in the heat increment which demands the transamination and deamination of superfluous amino acids. Therefore, higher energy was deposited as abdominal fat (Namroud et al., 2008 and Allameh and Toghyani, 2019).

In agreement with our results, Abdel-Fattah et al. (2008) found that 3% of CA dietary supplementation could significantly decrease the abdominal fat. Furthermore, Samanta et al. (2008) stated that the mixture of organic acids induced non-significant increase in meat CP and a decrease in meat fat. However, Dehghani-Tafti and Jahanian (2016) reported that organic acids had no significant effect on the abdominal fat deposition. The effects of CA on the percentage of fat and abdominal fat could be due to the role of dietary organic acids in inhibiting glycolysis and stimulating glycogenesis (Sabour et al., 2019).

Table 4 summarized the effects of adding CA to a low protein broiler diet on the percentage of dressing and the relative weight of the lymphoid organs (Bursa and Spleen). Protein levels had no significant effect on the relative weight of lymphoid organs. However, the low protein level decreased the dressing (from 74.93% for optimal protein levels to 73.86% for low protein levels). The percentage of dressing and relative weight of bursa was significantly higher in groups supplemented with CA. The results showed that adding CA to a low protein diet improved the percentage of dressing percentage and had no significant effect on the relative weight of bursa and spleen. The highest value for the percentage of dressing (75.86%) was recorded by a diet containing 100% of required CP + CA while the lowest value (73%) was for the group fed on 90% of required CP- CA. The addition of CA to a low protein diet had improved the percentage of dressing to be approximately the same as the control group. The negative effects of the low protein diets on dressing percentage in the present study supported by Selim (2015) and Dehghani-Tafti and Jahanian (2016), who found that reducing dietary CP levels decreased the carcass yield of chickens.

The percentage of dressing was significantly higher in CA groups. The findings of the current study are in line with the results of the previous studies (Islam et al., 2011; Dehghani-Tafti and Jahanian, 2016 and Sultan et al., 2018). In the current study, the results showed that the relative weight of bursa of Fabricius and the spleen are not affected by low protein diets. However, CA resulted in the improved relative weight of bursa of Fabricius, there were no differences regarding the spleen percentage. The spleen and bursa of Fabricius are a part of the immunity system (Abdel-Fattah et al., 2008). An immunological response could be expected via the improved in relative lymphoid organs (Katanbaf et al., 1989). The lack of protein levels effect on lymphoid organs was agreed with Golian et al. (2010), while Jahanian (2009) and Barekatain et al. (2019) found that reducing dietary CP had decreased the weights of lymphoid organs.

Intestinal morphology

The effect of adding CA to a low protein broiler diets on VH, VW, CD, VH/CD ratio, and absorption area is presented in table 5. The current study showed that low dietary protein had a significant effect on the intestinal morphology. The decrease in dietary CP content significantly decreased VH, VW, and VW/CD, and absorption area, compared to an optimal protein level. Citric acid addition improved ($p \leq 0.05$) VH, VH/CD, and increased absorption surface. The interaction between protein level and CA showed that the chickens' group fed on CP 100 % + CA had the highest values for VH, VW, VH/CD, and absorption area, while CP 90% - CA recorded the lowest values. The intestinal morphology parameters of the chickens' group fed low dietary protein (CP90) rose to a comparable level to that of the required protein fed (CP100) when CA was included.

Khosravinia et al. (2015) suggested that the increase in digestion and absorption was correlated with the increase in the VH/CD ratio. As a result, the ratio was a marker of the digestive capacity of the small intestine (Allameh and Toghyani, 2019).

The decrease in dietary CP content caused a significant decrease in VH, VW, VW/CD, and absorption area, which lend support to a study conducted by Mahmoud et al. (2017) reporting a slight decrease in the VH of broilers fed with 98% of required CP, compared to 100% of required CP. Moreover, Allameh and Toghyani (2019) stated that the reduction of dietary CP (85% of required CP) resulted in a negative impact on VH in the jejunum. However, Laudadio et al. (2012) found an increase in VH in the duodenum of broilers that consumed low dietary protein (20.5% CP), compared to those consumed high dietary protein (22.5% CP).

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The improvement of VH, VH/ CD, and the enlargement in villus surfaces area of the broilers fed diets supplemented with CA in the present study was also observed by Khosravinia et al. (2015), who found 3% dietary CA increased VH of the broiler. Moreover, Paul et al. (2007) and Samanta et al. (2010) stated that the use of acidifiers in the chickens' diet could increase VH. The improvement in VH resulted in an enlarged surface area for nutrients absorption and thus to higher growth performance (Mohammadagheri et al., 2016).

The amelioration of body gain and FCR by dietary supplementation with CA could be due to the improvement in VH and enlarged absorptive surface area (Dehghani and Jahanian, 2012)**.** The positive influence of organic acids on VH and VH/ CD ratios suggested that CA might improve the rate of nutrient absorption (Nourmohammadi and Afzali, 2013).

Bacteriological analysis of caecal content

The effect of adding CA to a low protein broiler diets on the cecum microbial counts (log^{-1} CFU/g), including *Lactobacillus*, *E. coli*, and *Clostridium perfringens* is shown in table 6. Dietary protein level had no significant effect on cecum microbial counts. However, CA addition had significantly improved *Lactobacillus* counts and reduced *E. coli* and *Clostridium perfringens* counts. The interaction between protein and CA showed significant differences in the bacterial populations (*Lactobacillus*, *Escherichia coli*, and *Clostridium perfringens*). Citric acid caused a significant increase in *Lactobacillus*, compared to non-supplemented groups. Furthermore, smaller numbers of *E- coil* and *Clostridium perfringens* were attributed to CA addition.

The obtained results of the current study addressing the effect of the protein levels on cecum microbial counts were in line with the findings of a study carried out by Ravangard et al. (2017), in which they reported that there were no significant differences in *Lactobacillus* and *Escherichia* bacteria counts in broiler chickens fed on the either low or required dietary protein levels.

Citric acid supplementation resulted in a significant improvement in *Lactobacillus* counts and reduction in *E. coli* and *Clostridium perfringens* counts, which resemble the results of EL-Afifi et al. (2001) and Ghazalah et al. (2011). Furthermore**,** Ahmad et al. (2018) found that CA (40g/kg) increased beneficial (*Lactobacillus*) bacteria, while reduced *E. coli* count, compared to the control group. *Lactobacillaceae* have an important role in host metabolism (Zhu et al., 2015). Similar to the findings of the current study, Cesare et al. (2019) observed a positive relation between *Lactobacillaceae* and amelioration of FCR.

Blood Parameters

The effect of adding CA and dietary protein level on blood components of broilers is presented in table 7. The protein level showed no significant effect on glucose, uric acid (UA), and AST, while the low protein level led to a decrease ($p < 0.05$) in albumin, total protein, and increase in hemoglobin A1c, fructosamine (glycation indicators), and cholesterol. Citric acid addition was increased albumin and decreased hemoglobin A1c, fructosamine, cholesterol, and uric acid. The results showed that the interaction between CP level and CA had no effect on blood glucose, hemoglobin, UA, and AST. The optimal protein level groups recorded the highest values ($p < 0.05$) for total protein, albumin, and globulin, and lowest values for hemoglobin A1C, fructosamine, and cholesterol.

The results of the present study related to the effect of the CP level on the blood parameters were in accordance with the findings of Swennen et al. (2005), Kamran et al. (2010), and Mohamed et al. (2012) concluding that the level of dietary protein did not alter glucose concentration. In addition, Alam et al. (2004) and Mohamed et al. (2012) indicated that the dietary protein level had no significant effect on the haemoglobin concentration of broilers. In addition, Mohamed et al. (2012) stated that the dietary protein caused only the globin section of the haemoglobin to increase with no effect on the haem section.

A low level of albumin is related to the glycation of plasma proteins and HbA1c (Bhonsle et al., 2012). According to Honma et al. (2017), protein levels in chicken diets could modify blood albumin levels and low dietary protein could increase the glycated amino acids in chicken plasma. Haemoglobin A1c reflects non-enzymatic glycosylation and fructosamine examined for glycation, associated primarily with albumin (Anguizola et al., 2013). The *In vivo* experiment conducted by Bhonsle et al. (2012) indicated that blood albumin could control the plasma protein glycation. Moreover, Tiwari et al. (2015) found an inverse correlation between plasma albumin and HbA1c.

The present results for CA were consistent with those obtained by Abdel-Fattah et al. (2008) indicating that dietary acidifiers (e.g. CA, acetic acid, and lactic acid) significantly decreased total lipids, and cholesterol, in the blood.

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Furthermore, Al-Saad et al. (2014) reported that there was no effect of organic acid in the blood hemoglobin and glucose. Ur Rehman et al. (2016) found that organic acids had no significant effect on the hemoglobin of broiler chickens. In the same vein, Nosrati et al. (2017) concluded that organic acid had no effect on the blood total protein and glucose of the broiler.

The positive effects of dietary protein and CA on the glycation reaction may be due to its effects on albumin levels. Moreover, the dietary protein might alter plasma amino compounds (Honma et al., 2017). In the study performed by Ali et al. (2013), it was found that there was a correlation between intestinal microflora and HbA1c, which could explain the effect of CA on HbA1c through its effect on the intestinal microflora. More studies are needed to clarify the correlation between CA, intestinal microflora, and glycation in poultry.

Table 2. The effect of low protein broiler diets supplemented with citric acid on broilers' performance during 1-42 days of age at the Poultry Research Unit, Giza, Egypt.

Treatments		**Parameters**		
CP	**CA**	**Feed intake (g/chicken)**	**BW gain (g/chicken)**	**FCR (g feed: g gain)**
Main effect of CP				
100%	-----	3631^{b}	2376^{a}	1.53^{b}
90%	-----	3682^{a}	2278^{b}	1.62^{a}
Main effect of CA				
-----	0%	3655	2280^{b}	1.60^{a}
-----	2%	3658	2375^{a}	1.54^{b}
CP×CA effect				
100%	0%	3629^{b}	2314^{b}	1.57^{b}
100%	2%	3633^{b}	2438^{a}	1.49^{c}
90%	0%	3681^{a}	2245^{c}	1.64^{a}
90%	2%	3683^{a}	2312^{b}	1.59^{b}
SEM		7.57	16.43	0.01
p-value				
CP		<0.0001	0.0009	<0.0001
CA		0.38	0.001	0.001
CP×CA		<0.0001	<0.0001	<0.0001

Means designated with the same letter within the same column are not significantly different at 0.05 level of probability. The beginning weight was 40g. BWG: Body weight gain, FCR: Feed conversion ratio, CP: Crude protein, CA: Citric acid, SEM: Standard error of the mean.

Table 3. The effect of low protein broiler diets supplemented with citric acid on broilers' whole body composition and abdominal fat percentage at 42 days of age at the Poultry Research Unit, Giza, Egypt.

Treatments		**Parameters**				
CP	**CA**	**Whole-body composition (%)**				**Abdominal fat (%)**
		Dry matter	**Fat**	**Protein**	**Ash**	
Main effect of CP						
100%	---------	27.05^{b}	7.06^{b}	22.55	4.13	1.16^{b}
90%	---------	27.76^{a}	8.26^{a}	22.39	4.05	1.39^{a}
Main effect of CA						
---------	0.00	27.11^{b}	8.57^{a}	21.89^{b}	4.11	1.43^{a}
---------	2.00	27.70^{a}	6.75^{b}	23.05^{a}	4.08	1.13^{b}
CP×CA effect						
100%	0%	26.50^{b}	7.60^{b}	21.83^{c}	4.29	1.32^{b}
100%	2%	27.59^{a}	6.52^{c}	23.27^{a}	3.97	1.00^{d}
90%	0%	27.71^{a}	9.54^{a}	21.95^{c}	3.92	1.53^{a}
90%	2%	27.80^{a}	6.98^{cb}	22.82^{b}	4.18	1.25^{c}
SEM		0.12	0.98	0.43	0.01	0.03
p-value						
CP		0.002	0.02	0.78	0.64	0.0001
CA		0.01	0.0002	0.0011	0.79	<0.0001
CP×CA		<0.0001	<0.0001	<0.0001	0.38	<0.0001

Means designated with the same letter within the same column are not significantly different at 0.05 level of probability. CP: Crude protein, CA: Citric acid, SEM: Standard error of the mean

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Table 4. The effect of low protein broiler diets supplemented with citric acid on the percentage of dressing and relative weight of lymphoid organs at 42 days of age at the Poultry Research Unit, Giza, Egypt.

Treatments		Parameters		
CP	CA	Dressing (%)	Relative weight of lymphoid organs (%)	
			Spleen	Bursa
Main effect of CP				
100%	----------	74.93[a]	0.14	0.22
90%	----------	73.86[b]	0.13	0.21
Main effect of CA				
----------	0.00	73.50[b]	0.12	0.19[b]
----------	2%	75.29[a]	0.14	0.24[a]
CP×CA effect				
100%	0%	74.00[b]	0.13	0.20
100%	2%	75.86[a]	0.14	0.24
90%	0%	73.00[c]	0.11	0.18
90%	2%	74.72[b]	0.14	0.23
SEM		0.22	0.01	0.01
P-value				
CP		0.01	0.10	0.80
CA		<0.0001	0.13	0.007
CP×CA		<0.0001	0.50	0.058

Means designated with the same letter within the same column are not significantly different at 0.05 level of probability. CP: Crude Protein, CA: Citric Acid, SEM: Standard Error of the Mean

Table 5. The effect of low protein broiler diets supplemented with citric acid on intestinal morphology at 42 days of age at the Poultry Research Unit, Giza, Egypt.

Treatments		Parameters				
CP	CA	Villus height (μm)	Villus Width (μm)	Crypt depth (μm)	VH/CD	Absorption area/1000
Main effect of CP						
100%	--------	1905[a]	157.33[a]	152.13	12.57[a]	300.00[a]
90%	--------	1759[b]	132.72[b]	153.46	11.58[b]	233.45[b]
Main effect of CA						
--------	0.00	1770[b]	142.56	154.89	11.53[b]	252.30[b]
--------	2%	1894[a]	147.49	150.70	12.62[a]	279.39[a]
CP×CA effect						
100%	0%	1858[b]	155.31[a]	151.74	12.37[a]	288.56[b]
100%	2%	1952[a]	159.34[a]	152.51	12.77[a]	311.03[a]
90%	0%	1682[c]	129.80[b]	158.04	10.56[b]	218.30[d]
90%	2%	1835[b]	135.64[b]	148.88	12.47[a]	248.90[c]
SEM		14.53	2.09	1.5	0.18	5.48
p-value						
CP		<0.0001	<0.0001	0.67	0.004	0.01
CA		0.002	0.22	0.17	0.001	<0.0001
CP×CA		<0.0001	<0.0001	0.21	<0.0001	<0.0001

Means designated with the same letter within the same column are not significantly different at 0.05 level of probability. VH: Villus height, CD: Crypt depth, CP: Crude protein, CA: Citric acid, SEM: Standard error of the mean.

Table 6. The effect of low protein broiler diets supplemented with citric acid on caecal bacterial counts

Treatments		Parameters		
CP	CA	*Lactobacillus*	*Escherichia coli*	*Clostridium perfringens*
Main effect of CP				
100%	--------	3.49	7.34	11.32
90%	--------	3.36	7.59	10.92
Main effect of CA				
--------	0.00	3.16[b]	7.66[a]	11.54[a]
--------	2%	3.71[a]	7.28[b]	10.71[b]
CP×CA effect				
100%	0%	3.17[c]	7.55[ab]	11.63[a]
100%	2%	3.82[a]	7.14[c]	11.02[c]
90%	0%	3.14[c]	7.77[a]	11.45[b]
90%	2%	3.59[b]	7.42[bc]	10.39[d]
SEM		0.09	0.08	0.14
p-value				
CP		0.46	0.12	0.17
CA		<0.0001	0.009	0.0003
CP×CA		<0.0001	0.01	<0.0001

Means designated with the same letter within the same column are not significantly different at 0.05 level of probability. CP: Crude Protein, CA: Citric Acid, SEM: Standard Error of the Mean

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Table 7. The effect of low protein broiler diets supplemented with citric acid on blood parameters at 42 days of age

Treatments		Parameters										
CP	**CA**	**Glu. (mg/dl)**	**TP (g/dl)**	**Alb. (g/dl)**	**Glob. (g/dl)**	**Hgb (g/dl)**	**HbA1c**	**FA.**	**TC (mg/dl)**	**UA (mg/dl)**	**AST (u/l)**	**ALT (u/l)**
Main effect of CP												
100%	--------	241.98	4.37^{a}	1.55^{a}	2.81^{a}	8.47	3.31^{b}	100.35^{b}	121.01^{b}	3.98	163.33	40.00^{a}
90%	--------	241.23	3.59^{b}	1.35^{b}	2.24^{b}	8.38	3.71^{a}	123.87^{a}	132.66^{a}	3.97	167.50	30.50^{b}
Main effect of CA												
--------	0.00	240.53	3.80	1.35^{b}	2.45	8.45	3.72^{a}	123.87^{a}	134.33^{a}	4.19^{a}	167.50	33.50
--------	2%	242.68	4.16	1.55^{a}	2.60	8.41	3.32^{b}	100.35^{b}	119.35^{b}	3.76^{b}	163.50	37.00
CP$^{\times}$CA effect												
100%	0%	241.53	4.17^{ab}	1.45^{b}	2.72^{ab}	8.63	3.50^{b}	111.13^{b}	129.33^{b}	4.16	167.00	37.00^{ab}
100%	2%	242.43	4.57^{a}	1.65^{a}	2.92^{a}	8.32	3.13^{c}	89.57^{c}	112.70^{c}	3.80	159.67	43.00^{a}
90%	0%	239.53	3.43^{c}	1.25^{c}	2.18^{b}	8.27	3.93^{a}	136.61^{a}	139.33^{a}	4.23	168.00	30.00^{b}
90%	2%	242.93	3.75^{bc}	1.45^{b}	2.30^{b}	8.50	3.50^{b}	111.13^{b}	126.00^{b}	3.73	167.33	31.00^{b}
SEM		2.7	0.13	0.40	0.11	0.07	0.09	5.06	2.53	0.09	7.99	1.88
P-value												
CP		0.80	0.0004	0.01	0.004	0.50	0.01	0.01	0.015	0.69	0.89	0.006
CA		0.67	0.17	0.01	0.40	0.77	0.01	0.01	0.0006	0.006	0.12	0.37
CP×CA		0.17	0.0008	0.001	0.038	0.18	0.001	0.001	0.0001	0.06	0.18	0.03

Means designated with the same letter within the same column are not significantly different at 0.05 level of probability. CP: Crude Protein, CA: Citric Acid, Glu: Glucose, TP: Total Protein, Alb: Albumin, Glob: Globulin, Hgb: Hemoglobin, HbA1c: Hemoglobin A1c, FA: Fructosamine, TC: Total Cholesterol, UA: Uric Acid, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, SEM: Standard error of the mean.

CONCLUSION

Broiler chickens fed a low protein diet supplemented with citric acid addition had the same performance as those with the required protein. The beneficial effect of citric acid on nutrients utilization, growth performance, and carcass may be due to the effect of citric acid on intestinal villus height, absorption area, cecal microbial content, and blood chemistry.

DECLARATIONS

Authors' contribution

All authors have equally contributed to the design of the study, data collection, data analysis writing, and revision of the manuscript. In addition, all the authors approved and agreed to the publication of the manuscript.

Competing interests

The authors have declared no competing interest.

Acknowledgements

The authors would like to appreciate the Deanship of Regional Center for food and feed, Agricultural Research Center, Giza, Egypt, for funding this study.

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To cite this paper: Abd-Elsamee MO, Fathy Abbas Motawe H, Maher Selim M and Ramadan Elsherif HM (2020). Effect of Different Dietary Crude Protein Levels and Citric Acid on Broiler Chickens' Performance, Carcass Characteristics, Intestinal Morphology, and Blood Components. *World Vet. J.*, 10 (3): 362-374. DOI: https://dx.doi.org/10.36380/scil.2020.wvj45

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To cite this paper: Abd-Elsamee MO, Fathy Abbas Motawe H, Maher Selim M and Ramadan Elsherif HM (2020). Effect of Different Dietary Crude Protein Levels and Citric Acid on Broiler Chickens' Performance, Carcass Characteristics, Intestinal Morphology, and Blood Components. *World Vet. J.*, 10 (3): 362-374. DOI: https://dx.doi.org/10.36380/scil.2020.wvj45

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 375-379, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj46

Comparison of Probiotic *Lactobacillus acidophilus* and Oxytetracycline for the Treatment of Early Stage Interdigital Necrobacillosis in Dairy Cows

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ORIGINAL ARTICLE
pii: S232245682000046-10
Received: 20 Jul 2020
Accepted: 30 Aug 2020

ABSTRACT

The objective of this multilocation field trial was to compare the effectiveness of the topical application of a powdered probiotic strain and intramuscular injection of oxytetracycline for the treatment of interdigital necrobacillosis in dairy cows. The current study was conducted from April 2018 to May 2020 on 230 dairy cows with early-stage interdigital necrobacillosis diagnosed by the research veterinarians on 6 farms situated in the Almaty region of Kazakhstan. The animals were recruited randomly to one of the following groups. A trial group in which the affected hoof of a cow was cleaned, coated with powder containing 10^6 colony-forming units of *Lactobacillus acidophilus* per gram, and then bandaged. Cows in the positive control group were subjected to a single intramuscular injection of oxytetracycline at the dosage of 1.0 milligram per kilogram of bodyweight which is considered the routine treatment for interdigital necrobacillosis in the feedlots. Both procedures were executed every 72 hours during a period of two weeks with the subsequent two-week follow-up period. The treatment was discontinued when a cow was assessed as cured. Both groups were monitored daily for their limb condition and the degree of lameness during 28 days. Those cows having no evidence of lameness and lesions attributed to interdigital necrobacillosis with no disease recurrence recorded within the observation period, were considered cured. On day 15, the overall cure rates for *Lactobacillus acidophilus* 015k-1 strain and oxytetracycline were 80.87% and 83.48%, respectively. The overall odds ratio for the cure rate in the probiotic group versus oxytetracycline was 0.837. However, on day 28, all cows in both groups were found to be clinically free from the disease, and cure rates were 100%. No recurrence was recorded in any of the cases. It was concluded that the topical administration of the probiotic powder to dairy cows with early-stage interdigital necrobacillosis can result in cure rates nearly as high as those for intramuscular oxytetracycline within a period of 28 days. This is the first report on the treatment effect of *Lactobacillus acidophilus* locally applied to cattle with early-stage interdigital necrobacillosis.

Keywords: Dairy cows, Foot rot, Interdigital necrobacillosis, *Lactobacillus acidophilus*, Oxytetracycline, Probiotic, Topical administration

INTRODUCTION

Interdigital necrobacillosis (IN) is an infectious podal disease caused mainly by *Fusobacterium necrophorum* and *Dichelobacter nodosus*. These Gram-negative aerotolerant anaerobic bacteria symbiotically produce several toxins and extracellular enzymes involved in the degradation of immunologically competent cells of host, bacterial proliferation, and dermotoxic activity (Nagaraja, 2016; Osová et al., 2018; Carvallo et al., 2020). *D. nodosus* inhabits the epidermis covering the limbs of cattle, whereas *F. necrophorum* is normally present in the digestive tract of healthy cattle, but once penetrated into other tissues, it can turn into a necrosis-inducing opportunistic pathogen (Francis et al., 2019). The common clinical signs of IN include lameness (most frequently unilateral), fever, reduced feed intake, edema, and erythema in the interdigital space and the coronary band (Van Metre, 2017).

Despite the array of steps towards effective treatments for IN the disease is still distributed in many countries (Renault et al., 2018; Dendani-Chadi et al., 2020; Silva et al., 2020). In the past few decades, the efficacy of vaccination against IN has been called into question on account of a number of issues, such as serogroup conversion of the infectious agents or diet-dependent effect (Checkley et al., 2005; Markey et al., 2013). A recent study on sheep with footrot has disclosed that a whole-cell *D. nodosus* vaccine and a recombinant fimbrial vaccine could reduce the disease with approximately twice less efficaciousness as opposed to foot bathing regimens (Allworth and Egerton, 2018).

Regarding probiotics, the experimental evidence demonstrates the capability of different *Lactobacillus acidophilus* strains to suppress *Listeria*, *Shigella*, *Salmonella* Typhimurium, *Escherichia coli*, and other pathogens through a variety

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of mechanisms, such as membrane depolarization and intracellular acidification (Liévin-Le Moal and Servin, 2014). An *in vitro* study has also revealed the antagonistic activity of *L. acidophilus* 015k-1 strain against *F. necrophorum* strains isolated from limbs of cattle (Myktybaeva et al., 2020).

Therefore, the purpose of this multilocation field trial was to compare the effectiveness of the topical application of a powdered *L. acidophilus* 015k-1 strain and intramuscular injection of oxytetracycline for the treatment of IN in dairy cows.

MATERIALS AND METHODS

Ethical approval

All procedures involving the cattle were performed in conformity with Directive 2010/63/EU and were approved by the ethics committee of Kazakh National Agrarian University (Almaty, Kazakhstan).

Study design

This research was conducted on six farms with a total of 1291 cows, located in the Almaty region of Kazakhstan from April 2018 to May 2020. Cattle were housed in open dirt feedlot pens. In each case, research veterinarians performed clinical examination of the cattle. Cows exhibiting early signs of IN represented by lesions graded as 1 or 2 in accordance with a standard scoring system (Jelinek et al., 2001) and a mild degree of lameness according to Jaeger (2016), were allocated randomly to one of two groups. The trial group consisted of cows for which the affected hoof was cleaned, coated with probiotic powder (Lactobacterin-TK2®, Akyntaj LLP, Kazakhstan) containing 10^6 colony-forming units of *L. acidophilus* 015k-1 per gram, and then bandaged (Figure 1). On the other hand, cows in the control group were subjected to a single intramuscular injection of Nitox 200® (oxytetracycline; Nita-farm, Russia) at the dosage of 1.0 milligram per kilogram of bodyweight which is considered the routine treatment for IN in the feedlots. Both procedures were performed every 72 hours during a period of two weeks with the subsequent two-week follow-up period. In case a cow was assessed as cured during this period, its treatment was discontinued. Each cow was ear-tagged. Both groups were monitored daily by licensed veterinarians for the condition of the limb and the degree of lameness during 28 days. Those cows having no evidence of lameness and lesions attributed to IN, with no disease recurrence recorded within the observation period, were considered cured.

Statistical analysis

In order to evaluate the association between variables, two-sided Fisher's exact test was used in the current study. For each of the herds pulled, the odds ratios of clinical cure of cows treated with *L. acidophilus* 015k-1 versus oxytetracycline were computed along with the confidence interval estimated by the Baptista-Pike method. All calculations were carried out in GraphPad Prism version 8.3.0 for Windows (GraphPad Software, San Diego, California, USA).

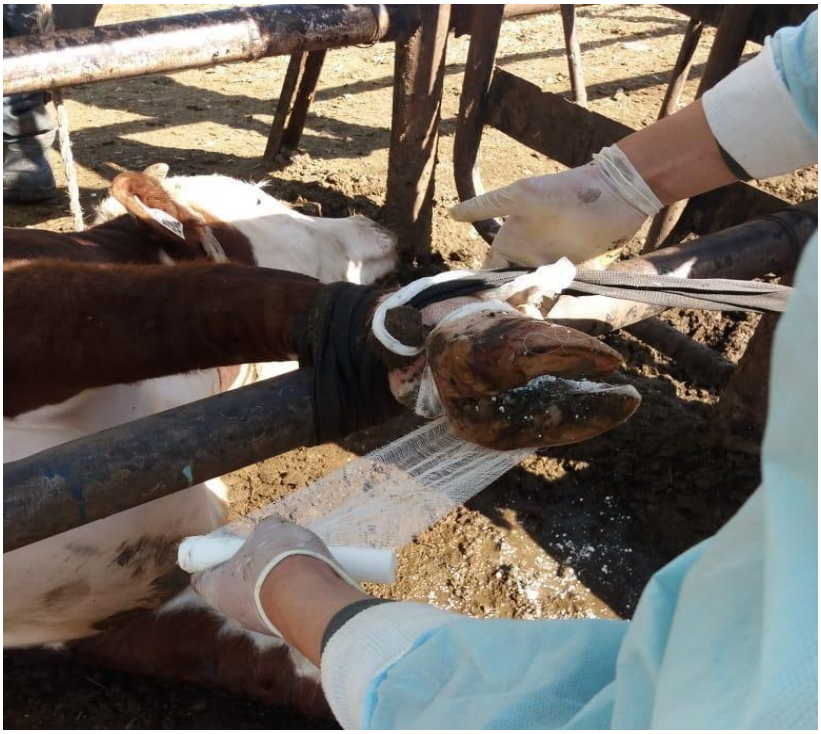

Figure 1. The procedure of applying *Lactobacillus acidophilus* 015k-1 to the foot of a cow with interdigital necrobacillosis

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RESULTS

Among the animals examined, 230 of them were diagnosed with early-stage IN (Table 1).

Cure rates for both treated groups on day 15 of treatment are shown in Table 2.

On day 15, the overall cure rates for *L. acidophilus* 015k-1 and oxytetracycline were 80.87% and 83.48%, respectively. The overall odds ratio for the cure rate in the *L. acidophilus* group versus oxytetracycline was 0.837. This means that the odds of cure after two weeks of the topical application of *L. acidophilus* are 83.7% of the odds of cure after a two-week-long intramuscular injection of oxytetracycline (Ranganathan et al., 2015). However, on day 28, all cows in both groups were found to be clinically free from IN, and consequently, the cure rates were 100%. For this reason, the data are not displayed in a tabular form. There was no recurrence of IN during the observation period.

Table 1. Prevalence of early-stage interdigital necrobacillosis in dairy cows included in the study on livestock farms located in the Almaty region of Kazakhstan

Farm name	Trial period	Cows (total)	Cows with early-stage IN (included in probiotic-treated group[1])	Cows with early-stage IN (included in antibiotic-treated group[2])
Darnis	April – May 2018	262	19	19
Bajserke – Agro	September – October 2018	536	27	27
Almaty	March – April 2019	118	16	16
Anar – K	May 2019	68	13	13
Nurzhan	September – October 2019	75	12	12
Samsybaev M	April – May 2020	232	28	28
Total	April 2018 – May 2020	1291	115	115

IN: interdigital necrobacillosis [1] topical administration of probiotic powder containing 10^6 colony-forming units of *Lactobacillus acidophilus* 015k-1 per gram. [2]single intramuscular injection of oxytetracycline at the dosage of 1 mg/kg of body weight.

Table 2. Cure rates for early-stage interdigital necrobacillosis in dairy cows included in the study on livestock farms located in the Almaty region of Kazakhstan on day 15 of treatment

Farm name	Probiotic-treated group[1]		Antibiotic-treated group[2]		*P*-value (Fisher's exact test)	OR	CI
	cured / treated		cured / treated				
	number	%	number	%			
Darnis	12 / 19	63.16	16 / 19	84.21	0.269	0.321	0.080 – 1.339
Bajserke – Agro	25 / 27	92.59	21 / 27	77.78	0.250	3.571	0.746 – 18.430
Almaty	9/ 16	56.25	14 / 16	87.50	0.113	0.184	0.035 – 1.000
Anar – K	10 / 13	76.92	13 / 13	100.00	0.220	0.000	0.000 – 1.065
Nurzhan	10 / 12	83.33	8 / 12	66.67	0.640	2.500	0.410 – 15.180
Samsybaev M	27 / 28	96.43	24 / 28	85.71	0.352	4.500	0.644 – 56.790
Total	93 / 115	80.87	96 / 115	83.48	0.731	0.837	0.423 – 1.616

OR: Odds ratio, CI: Confidence interval [1] topical administration of probiotic powder containing 10^6 colony-forming units of *Lactobacillus acidophilus* 015k-1 per gram. [2]single intramuscular injection of oxytetracycline at the dosage of 1 mg/kg of body weight. Treatments were repeated every 72 hours for two weeks but when a cow was assessed as cured, treatment was discontinued.

DISCUSSION

Considering that the initial point of foot rot is cutaneous dysbiosis, Ross et al. (2019) hypothesized that a probiotic culture with experimentally established efficacy against skin diseases might be a feasible topical treatment option. In line with the above, *L. acidophilus* has been widely proven to modulate epidermal conditions positively by means of cellular metabolites, antimicrobial peptides, and immune responses (Jeong et al., 2016; Lim et al., 2020). In contrast with antibiotics, the utilization of *L. acidophilus* 015k-1 does not entail the risk of antimicrobial resistance, and it requires no painful injections.

Unfortunately, we have failed to find a published study designed to evaluate the effect of any topical probiotic on foot lesions in livestock. In general, there has been a dearth of research addressing the topical application of a non-antibiotic approach for IN eradication. In a study conducted by Kaler et al. (2012), it was found that at the end of their 28-day-long trial the recovery from the disease was observed in only four out of the ten sheep with at least moderate interdigital phlegmon that had been administered locally with potassium permanganate solution, whilst recovery in a median time of 7 days was recorded for those 52 sheep treated with long-acting parenteral oxytetracycline and enrofloxacin.

Furthermore, Van Metre (2017) in his narrative review, has drawn a skeptical conclusion about the full range of

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transdermal substances utilized as active agents against claw disorders. Nonetheless, Persson et al. (2019) stated that dairy cows with early detected foot rot which were subjected to 100% salicylic acid powder into the interdigital space of the feet showed improvement in their general condition, lameness, body temperature, and coronary circumference after five days since administration. Given the difference in the duration of these trials, it is not possible to compare these findings with the obtained results of the current research.

To the best knowledge of the authors, the current study is the first report on the treatment effect of *L. acidophilus* topically administered to cattle with early-stage IN. The limitation of this research is in the absence of laboratory diagnosis. Meanwhile, it should be noted that the diagnosis of IN on grounds of clinical signs is used in contemporary veterinary practice for a variety of reasons (Osová et al., 2017; Põder, 2018; Kontturi, et al., 2020).

CONCLUSION

In conclusion, it can be stated that the cutaneous administration of *L. acidophilus* 015k-1 to dairy cows with early-stage IN every 72 hours for a period of up to two weeks can result in cure rates nearly as high as those for oxytetracycline intramuscularly injected using the same scheme. In the present case, the full recovery from the disease could be observed in both treatments at the end of the subsequent two-week follow-up period.

DECLARATIONS

Authors' contribution

All authors contributed equally to this work.

Competing interests

The authors declared no conflict of interest.

Acknowledgments

Authors wish to express their thanks to Sergey Sergeevich Kozhevnikov for translating this manuscript into the English language.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 380-390, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj47

Sequencing of *bcfC* Gene of *Salmonella* Typhimurium Isolated from Ducks in Egypt

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ORIGINAL ARTICLE
pii: S232245682000047-10
Received: 22 July 2020
Accepted: 29Aug 2020

ABSTRACT

The main objective of this study was to applying *bcfC* gene sequence of *Salmonella* Typhimurium recently isolated from ducks to give insight into the source and origin, molecular epidemiology, disease pattern of *Salmonella* Typhimurium in Egyptian duck farms. Out of 75 fecal swab samples, 15 (20%) local field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemically and serologically to be *Salmonella* Typhimurium. The PCR amplification with *bcfC* gene-specific primers was conducted with genomic DNA, which revealed a product with the approximate size of 467 bp. The*BcfC* gene was found in 7 (46.6%) isolates of *Salmonella* Typhimurium. Phylogenetic and partial gene sequence analysis of *bcfC* gene of *Salmonella* Typhimurium showed clear clustering of Egyptian isolates of *Salmonella* Typhimurium and different *Salmonella* strains uploaded from GenBank. Sequence identities between the isolated Egyptian strain and different *Salmonella* Typhimurium strains from GenBank revealed 99.8-100% homology. Open reading frame (ORF) analysis of *Salmonella* typhimurium *bcfC* gene using NCBI tool and ORF analysis of *bcfC* gene protein translation using ExPasy (SIB Bioinformatics Resource Portal) indicated all open reading frames of a specified minimum size in a sequence of (453 bp). The 3 conserved domains region in the nucleotide sequence were PapC N-terminal domain (107-394bp), PRK15193 outer membrane usher protein (56-424bp), and FimD Outer membrane usher protein FimD/PapC (cell motility, extracellular structures, 56-424bp). The PapC N-terminal domain was a structural domain found at the N-terminus of *S.* typhimurium PapC protein and had a central role in the pili assembly chaperone usher system (CUP). Amino acids alignment report of the sequenced 415 amino acid of *Salmonella* Typhimurium *bcfC* gene showed great homology between the Egyptian *Salmonella* Typhimurium strain and the different *Salmonella* strains uploaded from GenBank. Nucleotide alignment report of the sequenced *Salmonella* Typhimurium *bcfC* gene at (417bp) demonstrated great homology between the Egyptian *Salmonella* Typhimurium strain and the different *Salmonella* strains uploaded from GenBank. In conclusion, the Egyptian *Salmonella* Typhimurium isolate was related to the common sequence types isolated from humans and bovine-based products across the world especially in the United Kingdom, USA, Ireland, and México. Most of the duck farms from which we isolated the Egyptian *Salmonella* Typhimurium isolates were located in the same geographical area of cattle farms in addition to the duck farms lacked the requirements of biosecurity, which could facilitate the circulatory transmission of salmonella strains between the human beings and other animal farms, including duck farms. Moreover, the PapC N-terminal domain was a central conserved domain encoded by *bcfC* gene of *S.* Typhimurium. A PapC N-terminal conserved domain can be used as a vaccine target for vaccine production against *S.* Typhimurium.

Keywords: *bcfC* gene, Conserved domain, Duck, GenBank, ORF, Phylogenetic tree, *Salmonella* Typhimurium, Sequencing.

INTRODUCTION

Salmonella infections are considered one of among the foremost major problems within the poultry industry. *Salmonella* Typhimurium has been regarded to be frequently related to disease in numerous species, including humans, livestock, domestic fowl, rodents, and birds. Therefore, *Salmonella* Typhimurium is described as the prototypical broad-host-range serotype (Rabsch et al., 2002). *Salmonella typhimurium* has been found in 60% of poultry carcass (Mann and McNabb,1984) and is responsible for 93% of the *Salmonella* infections in ducklings (Badr and Nasef, 2016). *Salmonella* Typhimurium has been isolated from 40% of hatchlings and 1% of older ducklings in Taiwan, although clear host species specific differences have also been detected. 12 Salmonella has been previously isolated from imported day old ducklings in Brazil and also the USA (Ribeiro et al., 2006 and Gaffga et al., 2012). Because the prevalence of Salmonella in duck products poses a risk to human populations, an urgent need exists to research the prevalence, disease risk to human populations, and also the global epidemiology of Salmonella serovars and specific clones. This information could also be wont to address Salmonella risk and promote evidence-based interventions in global public health (Osman et al., 2014).

To cite this paper: El-Maghraby AS, Mwafy A and Ahmed El- Sawy HE-S (2020). Sequencing of *bcfC* Gene of *Salmonella* Typhimurium Isolated from Ducks in Egypt. *World Vet. J.*, 10 (3): 380-390. DOI: https://dx.doi.org/10.36380/scil.2020.wvj47

Pili (fimbriae) play a central role in bacterial colonization and pathogenesis (Li and Thanassi, 2009). Fimbriae are proteinaceous extracellular structures and play a distinct role in adhesion, a major initial step for colonization and entry into host cells. Fimbriae have also referred to as to play a central role in interactions with macrophages, intestinal persistence, biofilm formation and bacterial aggregation in Salmonella serovars (Ledeboer et al., 2006). The fimbrial gene (*bcfC*) is located on a fimbrial structure and play a vital role in attachment and cell invasion of *Salmonella typhimurium* (Huehn et al., 2010). Fimbrial gene *bcfC* is widely distributed among *Salmonella*, these data are according to the essential functions of adhesion factors for the attachment and internalization processes that occur during pathogenesis (Borriello et al., 2012).

bcfC is fimbrial usher protein consists of three functional domains which are PapC N-terminal domain, PRK15193 outer membrane usher protein and FimD outer membrane usher protein FimD/PapC. PapC (pyelonephritis-associated pilus C) is an integral outer membrane usher protein that forms an assembly platform for pilus biogenesis, PapC has five functional domains, all of which are required for pilus biogenesis, It's a 24-stranded β-barrel transmembrane domain that permits translocation of the polymerized pilus fiber across the outer membrane and 4 globular domains: a periplasmic Nterminal domain (NTD), two periplasmic C-terminal domains (CTD1 and CTD2), and a plug domain (Plug) (Henderson et al., 2011 and Phan et al., 2011). The usher PapC N-terminal functional unit represents primary binding site for chaperone-usher formation (Ng et al., 2004; Nishiyama et al., 2005 and Li et al., 2010).

Therefore, the main aim of this study is applying genetic sequencing and phylogenetic analysis of *bcfC* gene by using bioinformatics approach to explore more information about *bcfC* protein and to give insight about the source and origin, molecular epidemiology, disease pattern of *Salmonella* Typhimurium in Egyptian duck farms. Also, identification of highly conserved domains in *Salmonella* Typhimurium *bcfC* gene sequences for vaccine designing production against *Salmonella typhimurium*.

MATERIALS AND METHODS

Ethical approval

No ethical approval was obtained from the Institutional Animal Ethics Committee because no invasive procedure was performed on the animals. However, this study was conducted in accordance to the Institutional Animal Ethics of Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Agricultural Research Center (ARC).

Samples collection

Totally, 75 fecal swabs were collected that contained 30 from apparently healthy ducks and 45 from diseased ducks, these were collected from five duck farms in Qaluobia, Sharkia and Monofia governorates of Egypt.

Isolation of *Salmonella* Typhimurium

It was carried out according to methods described by ISO6579 (2002)

Identification of *Salmonella* Typhimurium

Microscopic examination

Suspected colonies were Gram's stained and microscopically examined according to methods described by Quinn et al. (2002).

Biochemical identification

Biochemical identification was performed on isolated organisms by using the Analytical Profile Index 20E (API 20E) system (Nucera et al., 2006).

Serological identification

Salmonella culture serotyping was carried out according to methods described by Kauffmann-White typing scheme (Popoff, 2001).

Molecular identification

DNA was extracted using the QIA amp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions with modifications. PCR was performed on extracted DNA by using specific primer (Table 1) supplied from Metabion (Germany) to amplify *bcfC* gene according to Huehn et al. (2010**)**. PCR was performed in a 25µl reaction containing 12.5 µl of Emerald Amp GT PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 p/mol concentrations, 4.5 µl of water and 6 µl of DNA template, using an Applied Biosystems 2720 Thermal Cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A100 base pair (bp) DNA Ladder (Qiagen, Germany, GmbH) was utilized to determine the fragment sizes. The gel was photographed by means of a gel documentation system (Alpha Innotech, Biometra).

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Table 1. Primer sequences, target gene and amplicon size

Microorganism	Gene	Primer sequences (5'-3')	Amplified segment product (base pair)	Reference
***Salmonella* Typhimurium**	*bcfC*	F-5'-accagagacattgccttc c-3' R-5'-ttctgatcgccgctattc g-3'	467	Huehn et al., (2010)

Open reading frame analysis

The NCBI tools website was carried out for Open reading frame analysis (ORF) analysis of *bcfC* gene sequence of *Salmonella* Typhimurium (453 bp). ExPASY-Translate Tool-SIB Bioinformatics Resource Portal was used for ORF analysis of *bcfC* gene sequence of *Salmonella* Typhimurium (453 bp) (https://web.expasy.org/translate/).

Conserved domain Search

NCBI Search Tool was performed conserved domain analysis of the *bcfC* protein sequence.

Phylogenetic, amino acids and nucleotide sequence analysis of *bcfC* gene of *Salmonella* Typhimurium

It was performed in Elim biopharmaceuticals, Germany. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis was initially performed according to standard methods described by Altschul et al. (1990). A comparative analysis of nucleotide and deduced amino acids sequences was performed using the CLUSTALW multiple sequence alignment program, version 1.83 of Mega Align module of Lasergene DNA Star software in accordance to methods designed by Thompson et al. (1994) and phylogenetic analysis was performed using neighbor joining in MEGA6 (Tamura et al., 2013).

RESULTS AND DISCUSSION

Isolation and identification of *Salmonella* Typhimurium field isolates

Out of 75 fecal swab samples, 15 isolates were confirmed phenotypically, biochemically and serologically to be *Salmonella* Typhimurium in a prevalence of 20% (15/75). These finding agree with Osman et al. (2014) (18.5%) and disagree with Abd El-Tawab et al. (2015) (9.6%), Lebdah et al. (2017) (14.2%), Hoszowski and Wasyl (2005) (14.3%) and Ismail (2013) (27.02%). The PCR amplification of *Salmonella* Typhimurium *bcfC* gene using specific primer sequences revealed an approximate size of 467 bp (Figure 1). *bcfC* gene was found in (7/15) (46.6%) *Salmonella* Typhimurium isolates. These results disagree with Osman et al. (2014) (100%) and Lebdah et al., (2017) (100%).

In this study, the ORF Finder commonly used on the NCBI tools website was performed (Figure 2). The NCBI tool determines the using of all qualified and alternative genetic codes. The ORF finder locates all ORFs of a specified minimum size during a nucleotide sequence. The sequence when subjected to ORF finder showed that all ORFs found of (453 bp) in (Figures 2 and 3). Also the ORF 2 is the largest one (348) nucleotides (nt) and it begins at complement (2-349 nt). It encodes 115 amino acids (aa). While ORF 1 is found downstream to ORF 2 is about (246 nt) long from (207-452 nt). It encodes 81 aa. ORF finder gives information about the coding and non-coding sequences. Detecting the coding and non-coding regions and final product in the form of its amino acid sequences is essential for understanding the evolutionary processes in various pathogens. By analyzing the ORF we will predict the possible amino acids that are producing during the translation process. The prediction of the proper ORF from a newly sequenced gene is a vital step. ORF is essential to design the primers which are required for PCR and sequencing (Orr et al., 2019).

Nucleotide sequence (453bp) was used to predict the domain region in the sequence (Figure 4). PapC N-terminal domain (107-394bp), PRK15193 outer membrane usher protein (56-424bp) and FimD outer membrane usher protein FimD/PapC (cell motility, extracellular structures); (56-424bp). Three domains were found in the *bcfC* gene sequence of *Salmonella*. The PapC N-terminal domain is a structural domain found at the N-terminus of the Gram-negative bacteria PapC protein. Pili are assembled using the chaperone usher system. In Gram-negative bacteria, this is can be composed of the chaperone PapD and the usher PapC, this domain constituet the N-terminal domain from PapC. N-terminal domain have a central role in substrate binding. The fimbrial usher protein is play a centeral role in assembling of the pilus in Gram-negative bacteria. Pilus known as one of the major fibrous surface organelles, play a great role in attachment to host tissues and given rise to development of a variety of diseases (Nougayrede et al., 2003). The assembly of fimbriae (or pili) need 2 components for assembly and transport system which consist of a periplasmic chaperone and an outer 'usher' membrane protein (Saier and Rosmalen 1993; Hultgren et al., 1994; Schifferli and Alrutz, 1994). The usher protein has a molecular weight of 86-100 kDa and include a membrane-spanning 24-stranded beta barrel domain, reminiscent of porins, and of 4 periplasmic soluble domains: an N-terminal one of about 120 residues (NTD) (*Nishiyama* et al., 2005; Huang et al., 2009), a 'middle' domain (plug domain) about 80 residues *long* (Capitani et al., 2006) and two

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IG-like domains (each about 80 residues long) at the C-terminus (CTD1 and CTD2 (Phan et al., 2011). Interaction between the NTD and Plug domains is essential step for usher gating. A conserved and immunogenic domain considered as a unique target for various vaccine development against *Salmonella* (Jha et al., 2015; Singh et al., 2017).

Phylogenetic and partial gene sequence analysis of *bcfC* gene of *Salmonella* Typhimurium that was generated using neighbor joining in MEGA6 (Figure 6), showed three major clusters or branches, one representing the Egyptian *Salmonella* Typhimurium strain isolate with CPO22491.1, LT795114.1, CPO22497.1, LT571437.1, CPO14975.1, CPO14358.1, CPO22658.1, CPO18657.1, CPO24619.1, LN999997.1, CPO14356.1, CPO11233.1, CPO14977.1, the second cluster for CPO16754.1, CPO22003.1, CPO18659.1, CPO18655.1, CPO18635.1, CPO17232.1, CPO18633.1, CPO19383.1, CPO15526.1, CPO15524.1, CPO18661.1, CPO18651.1, CPO18648.1, and the third one for AF129435.1 and AF130422.1.

Nucleotide sequence distance of *Salmonella* Typhimurium *bcfC* virulence gene (figure 7) was created by the Mega Align module of laser gene DNA star. Sequence identities between the isolated Egyptian strain and different *Salmonella* Typhimurium strains uploaded from GenBank revealed that 99.8% to 100% homology. Nucleotide sequence analysis of *bcfC* virulence gene of the Egyptian isolated strain showed 100% nucleotide identity with the American *Salmonella enterica subsp. enterica serovar* Typhimurium strain CDC 2009K-1640 (accession No.CP014975), the American *Salmonella enterica subsp. enterica serovar* Typhimurium strain USDA-ARS-USMARC-1896 (accession No.CP014977) by Nguyen et al. (2016), the Irish *Salmonella enteric subsp. enterica serovar* Typhimurium strain SL1344RX (accession No.CP011233) by Fitzgerald et al. (2015), the Mexican *Salmonella enterica subsp. enterica serovar* Typhimurium strain YU15 (accession No.CP014358) and *Salmonella enterica subsp. enterica serovar* Typhimurium strain YU15-SO2 (accession No.CP014356) by Silva et al. (2016).

In this study the Egyptian *Salmonella* Typhimurium isolate was distributed into common sequence types isolated from humans and bovine-based products across the world especially in the United Kingdom, USA, Ireland and México. Most of the duck farms from which we isolated the Egyptian *Salmonella* Typhimurium isolates were located in the same geographical area of cattle farms in addition to these farms lacked the requirements of biosecurity, which facilitates the circulatory transmission of *Salmonella* strains between human and animal farms to duck farms, present results agree with Murgia et al. (2015); Ktari et al. (2016) and Yang et al. (2019). Wang et al. (2020) recorded that the relatively high frequency of invasive infection of *Salmonella* in commercial meat-type duck flocks may largely relate to semi-open rearing systems that lack effective biosecurity, the majority of *S.* Typhimurium isolates were grouped into ST19 (63.89%), the most common sequence types isolated from humans and animal-based food products across the world , Perhaps circulatory transmission generated between contaminated poultry meat and human beings.

So, this study concluded that it is possible that the realistic explanation for the existence of similar strains of salmonella typhimurium isolated from duck farms and cows farms and strains isolated from the human host is the absence of disinfection, sterilization operations and the absence of health requirements by farm workers in addition to that the duck farms were located in the same geographical area of bovine farms. Amino acids alignment report of the sequenced 415 amino acid of *Salmonella* Typhimurium *bcfC* gene showed (figure 5) great homology between the Egyptian strain and the different *Salmonellae* strains from GenBank. On the other hand, nucleotide alignment report of the sequenced 417bp of *Salmonella* Typhimurium *bcfC* gene showed (figure 8) high identity between the Egyptian strain and the different *Salmonellae* strains from GenBank**.**

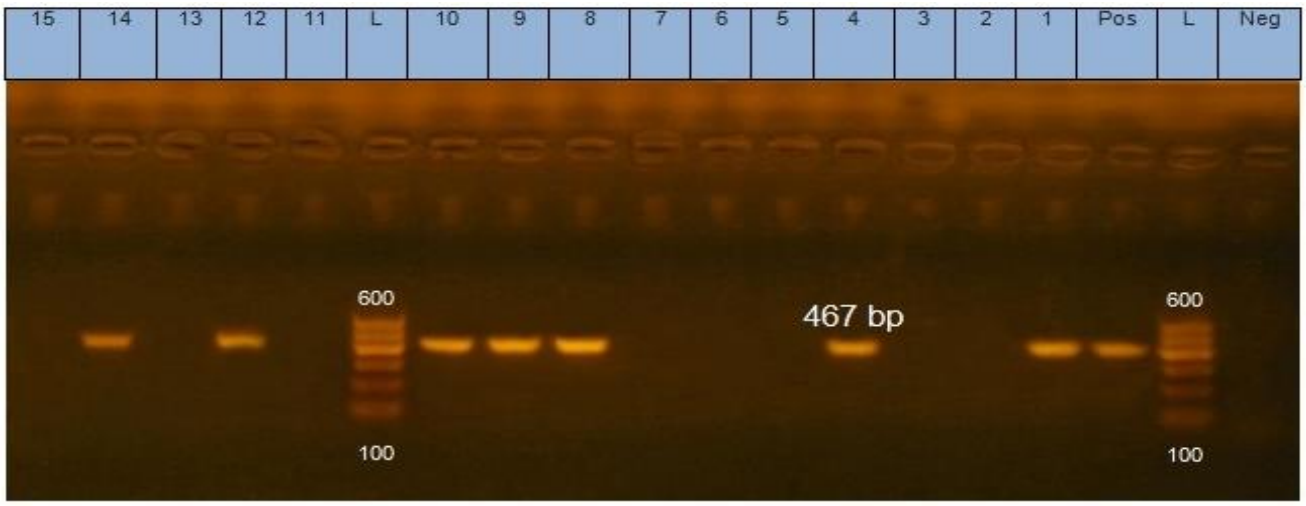

Figure 1. Agarose gel showingPCR-amplified product of *bcfC* virulence gene of *Salmonella* Typhimurium isolated from ducks.Lanes (1, 4, 8, 9, 10, 12, and 14): samples positive for*bcfC*gene (467 bp), Lane (pos.): positive control, Lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).

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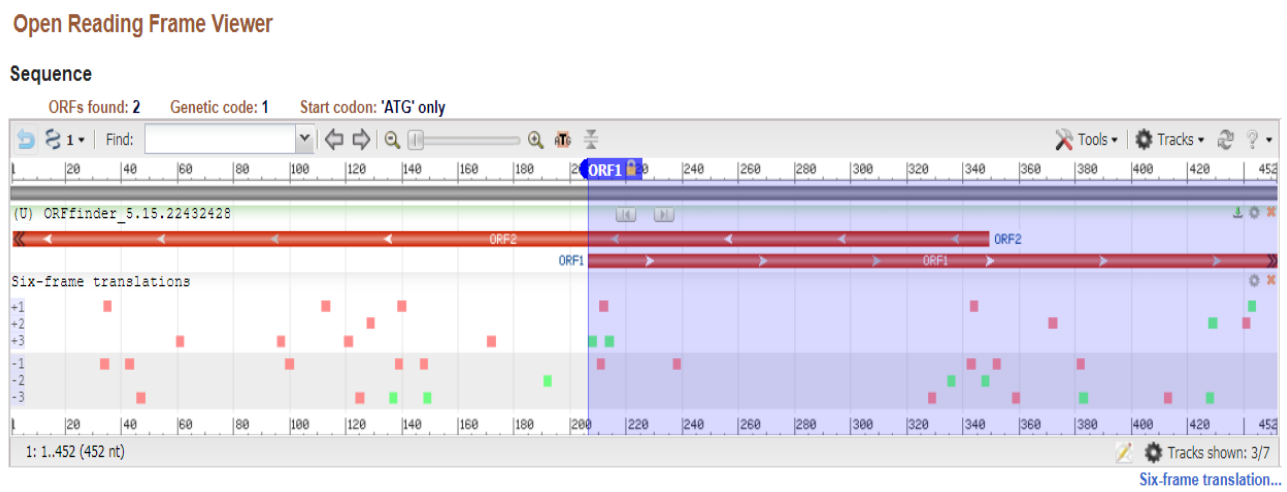

Figure 2. Open reading frame analysis of *bcfC* gene nucleotide sequence

Figure 3. Open reading frame analysis of *bcfC* gene protein translation using ExPasy (SIB Bioinformatics Resource Portal) showed all ORFs. The frame 2 is the longest one.

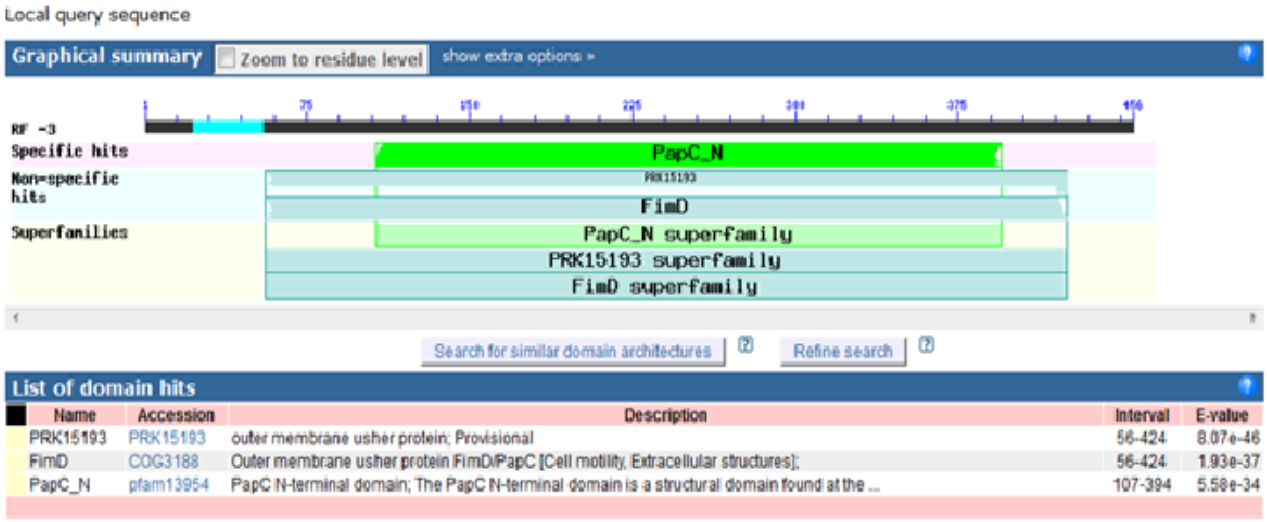

Figure 4. Conserved domains exist within the family region

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```
Majority   SPLFAFHVE-SRNRQAPRLRPLFNPRK-LFSALPPGLRS-LFAPVKV-FNSNALIPSSQYFGGI-PLALATIAACGMLMS
                    10        20        30        40        50        60        70        80
LT795114.1 ..................................................................................  238
Egy_strain ..................................................................................  238
CP022497.1 ..................................................................................  238
CP022491.1 ..................................................................................  238
LT571437.1 ..................................................................................  238
CP014977.1 ..................................................................................  238
CP014975.1 ..................................................................................  238
CP011233.1 ..................................................................................  238
CP014358.1 ..................................................................................  238
CP014356.1 ..................................................................................  238
LN999997.1 ..................................................................................  238
CP019383.1 ..................................................................................  238
CP015526.1 ..................................................................................  238
CP015524.1 ..................................................................................  238
CP018661.1 ..................................................................................  238
CP018651.1 ..................................................................................  238
CP018648.1 ..................................................................................  238
CP016754.1 ..................................................................................  238
CP022003.1 ..................................................................................  238
CP018659.1 ..................................................................................  238
CP018655.1 ..................................................................................  238
CP018635.1 ..................................................................................  238
CP018633.1 ..................................................................................  238
CP017232.1 ..................................................................................  238
AF129435.1 ..........G.....................................................................  238
AF130422.1 ..........G.....................................................................  238
CP024619.1 ..................................................................................  238
CP018657.1 ..................................................................................  238
CP022658.1 ..................................................................................  238

Majority   RRCCAKSKRVRAAGIALARSGQLPAAAFANSGNAPVFTPIFLSISGVRQARNPLSSVGA
                    90        100       110       120       130
LT795114.1 ..................................................................  415
Egy_strain ..................................................................  415
CP022497.1 ..................................................................  415
CP022491.1 ..................................................................  415
LT571437.1 ..................................................................  415
CP014977.1 ..................................................................  415
CP014975.1 ..................................................................  415
CP011233.1 ..................................................................  415
CP014358.1 ..................................................................  415
CP014356.1 ..................................................................  415
LN999997.1 ..................................................................  415
CP019383.1 ..................................................................  415
CP015526.1 ..................................................................  415
CP015524.1 ..................................................................  415
CP018661.1 ..................................................................  415
CP018651.1 ..................................................................  415
CP018648.1 ..................................................................  415
CP016754.1 ..................................................................  415
CP022003.1 ..................................................................  415
CP018659.1 ..................................................................  415
CP018655.1 ..................................................................  415
CP018635.1 ..................................................................  415
CP018633.1 ..................................................................  415
CP017232.1 ..................................................................  415
AF129435.1 ..................................................................  415
AF130422.1 ..................................................................  415
CP024619.1 ..................................................................  415
CP018657.1 ..................................................................  415
CP022658.1 ..................................................................  415
```

Figure 5. Amino acids alignment of *bcfC* virulence geneof Egyptian isolated strain *Salmonella* Typhimurium and different *Salmonella* Typhimurium strains retrieved from GenBank using CLUSTALW multiple sequence alignment program version 1.83 of MegAlign module of Lasergene DNASTAR.

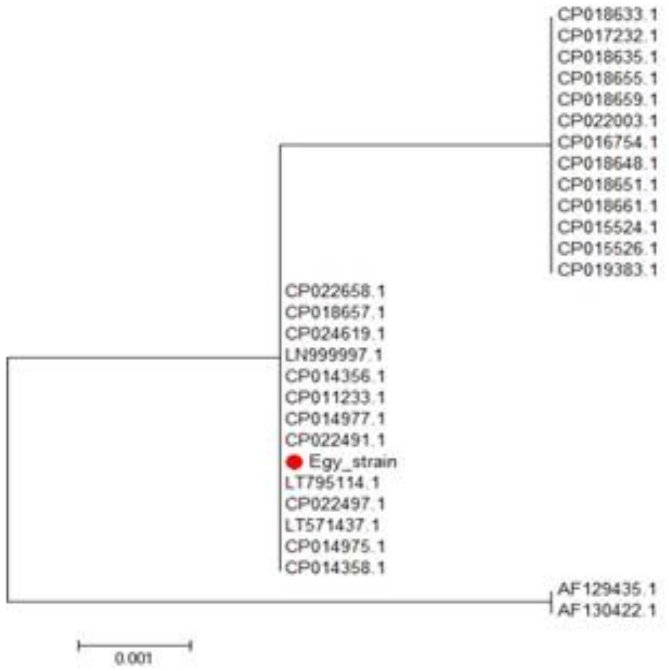

Figure 6. Phylogenetic tree for *Salmonella* Typhimurium *bcfC* virulence gene partial nucleotide sequences that was generated using neighbor joining in MEGA6, showing clear clustering of the Egyptian isolated strain (marked with red color) and different *Salmonella* Typhimurium strains uploaded from GenBank**.**

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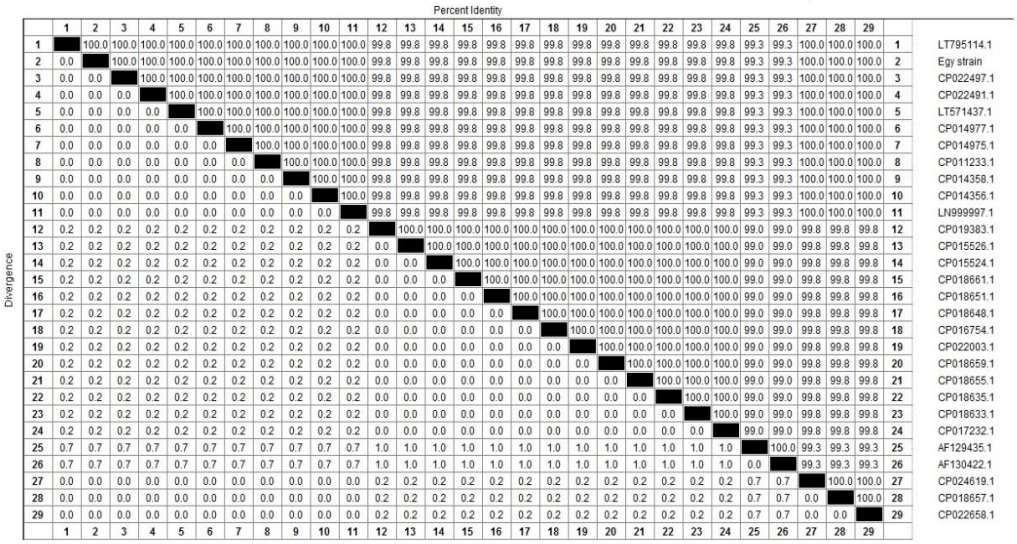

Figure 7. Nucleotide sequence distance analysis of *bcfC* virulence gene of Egyptian isolated strain and other *Salmonella* Typhimurium strains from GenBank.

```
Majority   TCGCCGCTATTCGCGTTCCATGTGGAGTAGTCGCGTAACCGCCAGGCCCCCAGATTAAGGCCGCTATTCAATCCAAGAAA
                    10        20        30        40        50        60        70        80
LT795114.1 ..............................................................................  80
Egy strain ..............................................................................  80
CP022497.1 ..............................................................................  80
CP022491.1 ..............................................................................  80
LT571437.1 ..............................................................................  80
CP014977.1 ..............................................................................  80
CP014975.1 ..............................................................................  80
CP011233.1 ..............................................................................  80
CP014358.1 ..............................................................................  80
CP014356.1 ..............................................................................  80
LN999997.1 ..............................................................................  80
CP019383.1 ..............................................................................  80
CP015526.1 ..............................................................................  80
CP015524.1 ..............................................................................  80
CP018661.1 ..............................................................................  80
CP018651.1 ..............................................................................  80
CP018648.1 ..............................................................................  80
CP016754.1 ..............................................................................  80
CP022003.1 ..............................................................................  80
CP018659.1 ..............................................................................  80
CP018655.1 ..............................................................................  80
CP018635.1 ..............................................................................  80
CP018633.1 ..............................................................................  80
CP017232.1 ..............................................................................  80
AF129435.1 ..............................G...............................T.............  80
AF130422.1 ..............................G...............................T.............  80
CP024619.1 ..............................................................................  80
CP018657.1 ..............................................................................  80
CP022658.1 ..............................................................................  80

Majority   ATAGCTGTTCTCCGCACTGCCGCCTGGGCTCCGATCCTGACTATTCGCGCCGGTAAAGGTGTAATTCAATAGCAACGCAT
                    90        100       110       120       130       140       150       160
LT795114.1 ..............................................................................  160
Egy strain ..............................................................................  160
CP022497.1 ..............................................................................  160
CP022491.1 ..............................................................................  160
LT571437.1 ..............................................................................  160
CP014977.1 ..............................................................................  160
CP014975.1 ..............................................................................  160
CP011233.1 ..............................................................................  160
CP014358.1 ..............................................................................  160
CP014356.1 ..............................................................................  160
LN999997.1 ..............................................................................  160
CP019383.1 ..............................................................................  160
CP015526.1 ..............................................................................  160
CP015524.1 ..............................................................................  160
CP018661.1 ..............................................................................  160
CP018651.1 ..............................................................................  160
CP018648.1 ..............................................................................  160
CP016754.1 ..............................................................................  160
CP022003.1 ..............................................................................  160
CP018659.1 ..............................................................................  160
CP018655.1 ..............................................................................  160
CP018635.1 ..............................................................................  160
CP018633.1 ..............................................................................  160
CP017232.1 ..............................................................................  160
AF129435.1 .........T....................................................................  160
AF130422.1 .........T....................................................................  160
CP024619.1 ..............................................................................  160
CP018657.1 ..............................................................................  160
CP022658.1 ..............................................................................  160
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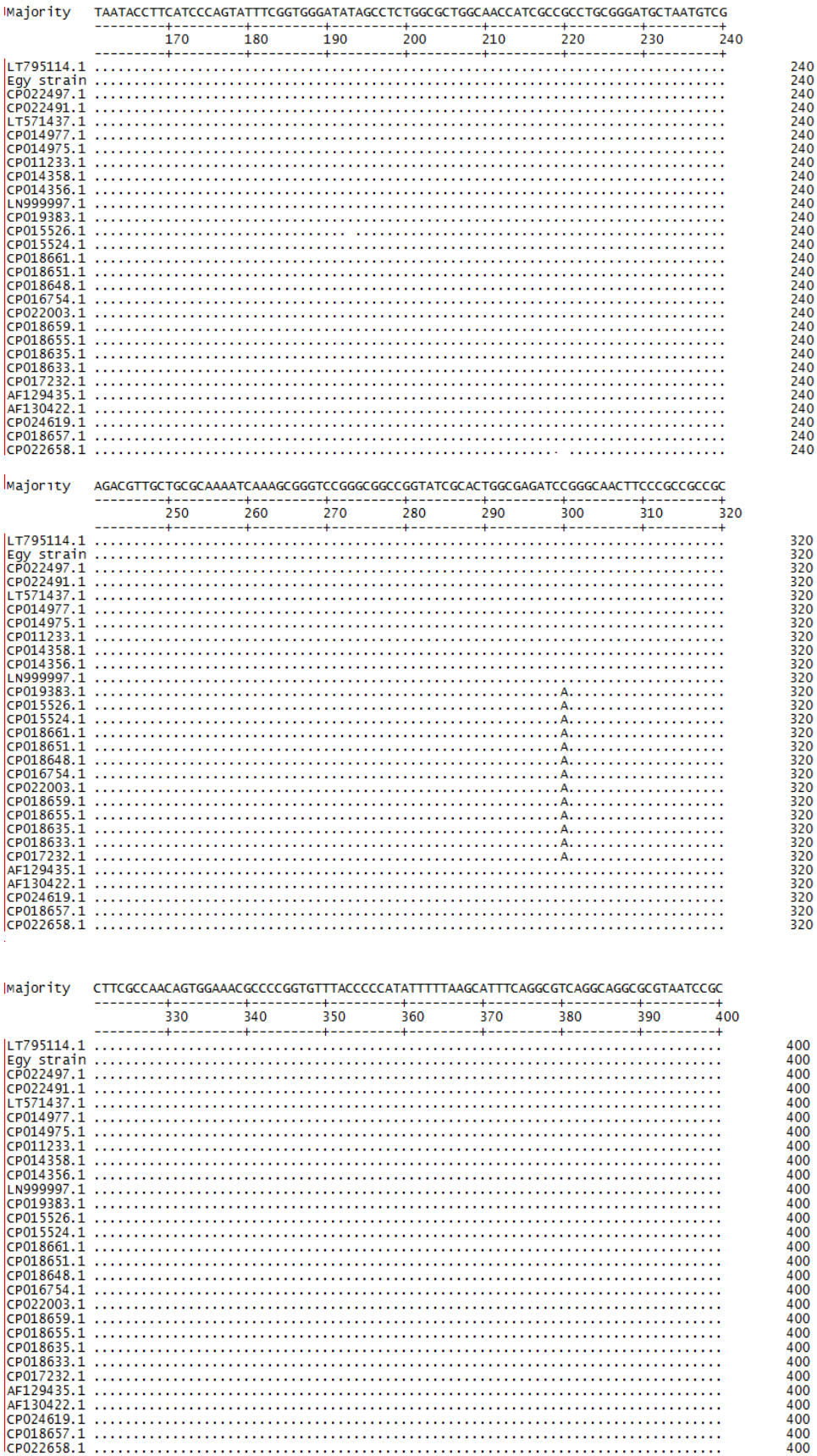

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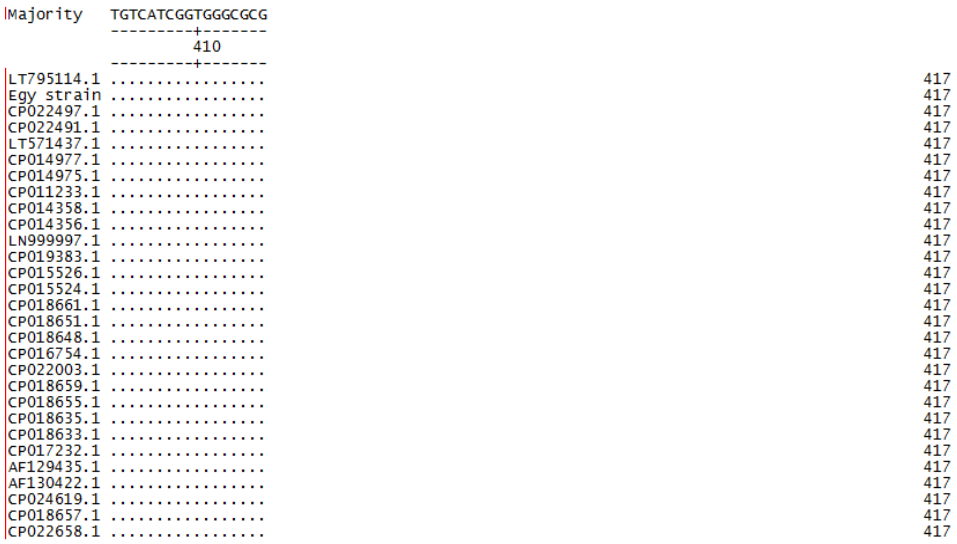

Figure 8. Nucleotides alignment of *bcfC* virulence gene sequence of Egyptian strain of *Salmonella* Typhimurium and different *Salmonella* Typhimurium strains retrieved from GenBank using CLUSTALW multiple sequence alignment program version 1.83 of MegAlign module of Lasergene DNASTAR.

DECLARATIONS

Author's contributions

Abeer Saad El-Maghraby designed the idea and concept of the review article, planned the study and Abeer Saad El-Maghraby, AbeerMwafy and Hala Ahmed Al-Sawy designed and performed study design. All authors shared in writing, and approved the final version of manuscript.

Acknowledgements

This study was supported by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo and Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

Competing interests

The authors declared that no competing interests exist.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 391-397, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj48

Effects of Dietary Inclusion of *Pentadiplandra brazzeana* Powder on Growth Performances, Gut Microbiota, and Haemato-Biochemical Indices of Broiler Chickens

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ORIGINAL ARTICLE
pii: S232245682000048-10
Received: 18 Apr. 2020
Accepted: 25 May 2020

ABSTRACT

This study was designed to assess the rearing performances of broiler chickens under two *Pentadiplandra brazzeana* powder feeding diets. A total of 256 day-old Cobb 500 broiler chicks were randomly assigned to 4 treatment groups with 4 replicates of 16 birds each in a completely randomized design. This trial involved administrating *Pentadiplandra brazzeana* powder to broiler chickens via drinking water (2 g/l) or dry feed (2 g/kg) and comparing the result to those obtained from chickens fed on antibiotic (1 g/kg) and chickens fed without additive. The results revealed that the incorporation of *Pentadiplandra brazzeana* powder in chickens' diet and drinking water could decrease feed intake, compared to the negative control diet. The inclusion of *Pentadiplandra brazzeana* powder in water and antibiotic in diet recorded the high live weight and weight gain, compared to the birds fed with the powder in feed and the negative control diet. The low feed conversion ratio was reported with the water supplemented with *P. brazzeana* powder. The carcass yield was significantly higher with the inclusion of the powder via drinking water, compared to the inclusion via feed and with the control diet. The feeding method did not affect the haemato-biochemical parameters, compared to the control diet. Chickens fed with a diet containing *Pentadiplandra brazzeana* powder in water recorded the highest ($p<0.05$) lactic acid bacteria count, compared to *E. coli* and *Salmonella*. In conclusion, dietary supplementing with *Pentadiplandra brazzeana* powder through drinking water at a rate of 2 g/l can be used as an alternative to antibiotics to improve the growth performances of broiler chickens.

Keywords: Broiler chicken, Growth performances, Gut microflora, Haemato-biochemical profile, *Pentadiplandra brazzeana*

INTRODUCTION

The use of Antibiotic Growth Promoters (AGPs) in animal diet has led not only to the development of resistance by pathogenic bacteria (Toghyani et al., 2011), but also residues found in livestock products could be harmful for consumer health (Vicente et al., 2007). It has been resulted to the systematic interdiction and removal of AGPs in animal nutrition (Cervantes, 2006, Kana et al., 2017a). Therefore, the searches for alternative feed supplements have been increased extensively and considerable attention has been given to phytogenic as replacements for antibiotics growth promoters, which should have the same beneficial effect as AGPs. Phytobiotic or phytogenic are compounds of plant origin which are incorporated in animal feed to improve growth performances through their anti-oxidative and antimicrobial action (Oko and Agiang, 2009; Gakunga et al., 2013; Alonge et al., 2017). Example of some phytobiotics includes herbs, spices as well as extracts of plants (Windisch et al., 2008). The beneficial properties of phytobiotic is due to the presence of actives substances such as phenol, tannins, alkaloids and terpenoïds (Alloui et al., 2011). These actives substances in animal industry, have the ability to activate feed intake and digestive secretion, anti-inflammatory activity, antimicrobial and antioxydant properties. Previous study revealed that *Zingiber officinale* and *Allium sativum* (Karangiya et al., 2016), *Tetrapleura tetraptera*, *Afrostyrax lepidophyllus* and *Dichrostachys glomerata* improve growth performance of broiler chickens (Kana et al., 2017abc).

Pentadiplandra brazzeana is a spontaneous arborescent shrub or climber growing from the West coast of Africa, Central Africa to the basin of the Zambezi (Kouka, 2001). This species is widely used in traditional medicine. The roots of these plants are often harvested by the local people to treat many types of ailments such as kidney pains and hemorrhoids, constipation, cough and post-partum hemorragy (Cimanga et al., 2018; Lautenschläger et al., 2018). These supplements are commonly used for their culinary properties (Abdou et al., 2012). Phytochemical test of the roots has revealed the presence of alkaloids, flavonoids, tannins, phenols and triterpernes (Ngbolua et al., 2011; Lautenschläger et al., 2018). Also, *in vitro* studies have revealed antimicrobial (Nyegue et al., 2009), antifungic (Dzoyem et al., 2014),

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antioxidant (Abdou et al., 2012) and cytotoxic (Kuete et al., 2011) properties of the roots of this plant. Based on these properties, it's proposed that roots of this plant, commonly use as spice could balance the gut microbiata, ensure good health, thus improving growth performances of broiler chickens. However, the active compounds present in this spice are easily damageable, which can prevent adequate levels from reaching target sites and lead to a decrease of the potential bioactivity of these compounds, a qualitative loss of properties (Mahmood et al., 2018), thus reducing the effectiveness of the powder. Hence, the need to find a way of administrating of the spice powder through the diet, making it possible to better conserve and limit the alteration of its active compounds in order to make them more available and accessible along the digestive tract of the animal. So, the aim of this study was to evaluate the effects of *Pentadiplandra brazzeana* powder either in water or in feed supplement on growth performances in order to determine the best way of incorporating spices powder in poultry diet.

MATERIALS AND METHODS

Ethical approval

The present study has been performed in agreement with the guidelines of the ethical standards from the 1964 Helsinki declaration and latterly amendment under approval code # CU/II/F/34/19#.

Conditions of experiment

The study was carried out at the Teaching and Research Farm of the Faculty of Agronomy and Agricultural Sciences, University of Dschang, Cameroon. Annual temperatures vary between 10°C and 25°C.

Feed additives

Dried sample of *Pentadiplandra brazzeana* roots was ground into powder in a harmed mill, sieved and incorporated in the experimental diets. Sample of the ground spices was put into polyethylene plastics and stored at 4°C in a refrigerator until analysis. Phytochemical screening of *Pentadiplandra brazzeana* was done according to the common phytochemical methods described by Harborne (1973). The screening revealed that saponins and sterols were absent. Phenols, tannins, flavonoids alcaloids, tripernoids, anthocyanins and anthraquinons tests were positive. Antibiotic (Doxycyclin ®) used in the positive control diet was bought from a local veterinary pharmacy.

Animal and experimental diets

A total of 256 day-old Cobb 500 broiler chicks were randomly assigned to four treatments groups in a completely randomized design with 64 birds per treatment. Each group was sub divided into 4 replicates of 16 birds each. Two experimental diets were formulated from the negative control diet (T0$^-$) formulated to meet their requirements (table 1) by incorporating 1 g of antibiotic/kg of feed and considered as positive control diet (T0+) and 2 g of *P. brazzeana* powder/kg of feed (T1). The last treatment consisted of incorporating 2 g of *P. brazzeana* powder in drinking water (T2). Throughout the experiment, vaccination and other routine poultry management practices were maintained. Chicks were weighed at the beginning of the experiment and on a weekly basis thereafter. Data on feed intake (FI) and body weight gain (BWG) were estimated and use to calculate feed conversion ratio (FCR). Feed and water were offered *ad libitum*.

Sample collection and analysis

Growth performances, hematological and serum biochemical parameters

Feed intake, weight gain and feed conversion ratio were evaluated on a weekly basis in both starter and finisher phases of the study. At the end of the feeding trial (49 days of age), 10 birds (5 males and 5 females) from each treatment group were randomly selected, fasted for 24 hours and slaughtered for carcass evaluation as preceded by Kana et al. (2017). From each slaughtered bird, blood was collected in 2 test tubes, one of which contained as anticoagulant. Blood with anticoagulant was used for hematological analysis using a full automatic blood cell counter (model PCE-210N Hong kong, china). Hematological parameters included White blood cell (WBC), Red blood cell (RBC), Hemoglobin (Hgb), Mean cell hemoglobin concentration (MCHC), Mean cell volume (MCV), Mean cell hemoglobin (MCH) and Packed cell volume (PCV). Meanwhile, after centrifugation of blood free from anticoagulant, serum was collected and preserved at -20°C for the evaluation of total protein, albumin, globulin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), total cholesterol, high density lipoproteins (HDL), low density lipoprotein (LDL), triglyceride, urea and creatinine, using the colorimetric method as prescribed by the commercial kits (Spinreact ® kits).

Microbial count

A sterile spatula was used to collect freshly bird feces through cloacal swab. The identification and quantification of bacteria were assessed on appropriate specific culture media (MRS Agar for lactic acid bacteria, Mac conkey Agar for *E. coli*, SS Agar for *Salmonella*). Samples were incubated at 37°C for 24 h.

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Statistical analysis

Data recorded on growth, hematological, biochemical and microbial parameters were submitted to one-way analysis of variance test by the general linear model procedure of statistical package for Social Science (SPSS ver. 20.0) software. The differences were tested using Duncan test and probability values less than 0.05 was considered as significant (Steel and Torrie, 1980).

Table 1. Composition and chemical analysis of basal diet

Ingredients (%)	Starter (0-21d)	Finisher (22-49d)
Maize	60	68
Wheat bran	3	2
Soybean meal	21	14
Cotton seed meal	5	5
Fish meal	5	5
Oeister shell	1	1
Premix 5%*	5	5
Total	100	100
Chemical analysis		
Metabolizable energy (Kcal/kg)	2895.04	2998.50
Crude protein (%)	21.58	19.29
Energy/protein	134.15	155.68
Calcium (%)	1.17	1.15
Phosphorous (%)	0.48	0.44
Calcium/phosphorous	2.37	2.60
Lysin (%)	1.37	1.15
Methionin (%)	0.48	0.44
Lysin/methionin	2.85	2.63
Crude fibre (%)	5.00	5.19

*Premix 5%: crude proteins 400 mg, Lysin 33 mg, Methionin 24 mg, Calcium 80 mg, Phosphorous 20.5 mg, metabolizable energy 2078 kcal/kg, Vitamins: Retinol 10 000 000 IU, Cholecalciferol 3 000 000 UI, Tocopherol 2500 IU, Phylloquinon 4000 mg, Thiamin 5000 mg, Riboflavin 500 mg, Pyridoxin 2500 mg, Cyanocobalamin 5 mg, Folic acid 10 000 mg and Niacin 2000 mg.

RESULTS AND DISCUSSION

Growth performances

Table 2 indicated the effect of the feeding mode of *P. brazzeana* powder on growth performances. During the growing phase and throughout the production period, the administration of *P. brazzeana* powder through feed and water induced a significant ($p<0.05$) decrease in feed intake compared to the negative control diet. This decrease in feed intake could be explained by the strong smell of the spice and the presence of alkaloids which may have induced a bitter taste and have not been well appreciated by the birds inducing the drop of the consumption. These results are in agreement with the finding of Kana et al. (2017c) who reported that administration of *Tetrapleura tetraptera* powder in broiler chickens feed significantly decreased feed intake. The present result is in contradiction with the finding of Nweze et al. (2011) who reported that feeding broilers with *Tetrapleura tetraptera* through feed, fresh and boiled water has no effect on feed intake. Similarly, Kana et al. (2017b) recorded no significant effect on feed intake with the incorporation of 2g of *Afrostyrax lepidophyllus* fruit and bark powder in broilers feed. Likewise, Ebile et al. (2018a) reported that feeding quails with 0.5% of *Dichostachys glomerata* powder through feed or water had no significant effects ($p<0.05$) on feed intake. The difference between all these results can be attributed to the variability of composition in actives compounds in those spices.

As shown in table 2, birds fed on antibiotic and *P. brazzeana* powder in drinking water recorded the highest live weight and weight gain ($p<0.05$) compared to the negative control and birds receiving *P. brazzeana* powder through feed. These decreased due to the administration through feed of the spice powder could be explained by a loss of some active ingredients in the feed due to their volatility. This reduction of growth is similar to the finding of Kana et al. (2017c) who reported that during the brooding phase diet supplemented with *Tetrapleura tetraptera* powder decreased live body weight. In contrary, the highest body weight obtained with water administration could be explained by the effectiveness of water in extracting secondary metabolites from the spice and made it more disponible for the animal. These active metabolites have antibacterial and antioxidant properties known to reduce the risk of developing microbiota pathogens, by destroying the bacterial membranes, making unavailable certain substrates for the bacteria and inactivate bacterial enzyme (Windisch et al., 2008), resulting in greater availability of certain nutrients, thus improving in live weight. This result is similar to those of Vivian et al. (2015) who reported that the aqueous extract of ginger significantly improved growth performances of chickens.

Data presented in table 2 showed that, birds receiving antibiotic and *P. brazzeana* powder through water recorded the lowest ($p<0.05$) FCR of all the treatments. This decrease in FCR can be understood by a greater availability of active ingredients in water than in feed, due to their antibacterial, anti-inflammatory and anti-oxidant properties improved the availability and increased the absorption and better utilization of nutrients.

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The effects of *P. brazzeana* feeding diets on carcass yield and relative weight of organs are presented in table 3. Regardless of *P. brazzeana* feeding diets, there were no significant (p>0.05) effects on relative weight of the legs, heart, liver, gizzard and abdominal fat compared to the control diets. Bird fed with antibiotic and *P. brazzeana* powder in drinking water recorded the highest (p<0.05) carcass yield compared to the negative control and the administration of the powder through feed. This improvement of carcass yield may result in the positive effect on live weight. These results are in contradiction with those of Kana et al. (2017c) who reported no significant effect on carcass yield when birds were fed with *Tetrapleura tetraptera* powder.

Table 2. The effects of *P. brazzeana* diets in growth performance of broilers

Characteristics	Study period (days)	Treatments				
		T0$^-$	T0$^+$	T1	T2	*P*
Feed intake (g)	01- 21	1152.50±29.41^a	1014.94±76.05^b	1089.03±64.40^{ab}	1135.55±35.69^a	0.01
	22 – 49	4152.40±63.88^a	4024.47±74.58^b	4026.84±70.96^b	4005.97±25.92^b	0.02
	1 – 49	5304.90±46.68^a	5039.40±37.54^c	5115.37±75.78^b	5141.53±21.56^b	0.00
Live weight (g)	01 – 21	636.73±24.00^a	583.31±26.74^b	574.78±35.08^b	635.90±21.26^a	0,01
	22 -49	2146.26±17.02^b	2281.52±24.60^a	2114.38±27.21^b	2270.30±25.51^a	0,00
Body weight gain (g)	01 – 21	596.73±24.00^a	543.31±26.74^b	552.28±25.20^b	595.64±17.37^a	0.01
	22 – 49	1518.39±23.66^b	1698.27±24.70^a	1580.43±45.40^b	1643.51±27.75^a	0.00
	01 – 49	2115.12±21.11^b	2241.58±20.15^a	2082.54±54.60^b	2239.15±42.52^a	0.00
Feed conversion ratio	01 - 21	1.93±0.05	1.87±0.18	1.96±0.17	1.91±0.09	0.11
	22 - 49	2.69±0.04^a	2.37±0.06^c	2.54±0.11^b	2.40±0.05^c	0.00
	01 - 49	2.48±0.02^a	2.22±0.02^c	2.37±0.09^b	2.27±0.05^c	0.00

$^{a, b}$: Means with the same superscript on the same row are not significantly different (p>0,05). P= probability**.** T0$^-$ =control diet; T0$^+$ = T0+ 0.1% Doxycycline; T1 = T0+ 2 g of *P. brazzeana* /kg of feed; T2 = T0 + 2 g of *P. brazzeana* /liter of water.

Table 3. The effects of *P. brazzeana* diets in carcass characteristics of broiler chickens

Parameters (%BW)	Treatments				
	T0$^-$	T0+	T1	T2	P
Carcass yield	72.18±1.84^b	74.54±1.84^a	71.59±1.67^b	74.12±1.70^a	0.00
Head	2.50±0.14^a	2.27±0.16^b	2.12±0.23^b	2.27±0.24^b	0.00
Leg	3.55±0.33	3.88±0.47	3.59±0.52	3.83±0.34	0.12
Liver	1.79±0.12^b	2.35±0.45^a	1.94±0.28^b	1.68±0.42^b	0.00
pancreas	0.17±0.03	0.16±0.02	0.18±0.04	0.16±0.03	0.61
Gizzard	1.48±0.18	1.52±0.25	1.58±0.21	1.45±0.13	0.72
Heart	0.46±0.07	0.48±0.08	0.42±0.06	0.45±0.10	1.05
Abdominal fat	2.48±0.50	2.44±0.54	2.79±0.69	2.30±0.41	1.46

$^{a, b}$: Means with the same superscript on the same row are not significantly different (p>0.05). P= probability**.** T0$^-$ =control diet; T0$^+$ = T0+ 0.1% Doxycycline; T1 = T0+ 2 g of *P. brazzeana* /kg of feed; T2 = T0 + 2 g of *P. brazzeana* /liter of water.

Gut microbiata

As shown in table 4, the analysis of variance revealed that, the number of *Escherichia coli* and salmonella was significantly lower with the administration of antibiotic and *P. brazzeana* powder through feed and water compared to the negative control diet. Thus, as non-significant, the number of these pathogenic bacteria was less in birds receiving the spice through water compared to feed. This could be explained by the presence of active substances like phenols, flavonoid and alkaloids in the spice which promoted the development of lactic acid bacteria by reducing the development of pathogeneous bacteria. Antimicrobial property of the spice is considered to arise from phenols (Muanda et al., 2011), and hydrophobic compounds present in spices that intrude into the bacterial cell membrane inducing the disintegration of the membrane structure and cause leakage thus making microbes less virulent (Windisch et al., 2008). This result are in agreement with the findings of Ngouana et al. (2017) who observed an increased in lactic acid bacteria population in the intestine of healthy chickens. The lactic acid bacteria multiply and eliminate pathogenic bacteria by acidifying the intestinal gut and producing antibacterial substances like organic acids (Elarousi et al., 2008).

Table 4. Fecal microbial load of broiler chickens as affected *P. brazzeana* diets

Bacteria load log10 (UFC)	Treatments				
	T0$^-$	T0+	T1	T2	P
Lactobacillus spp	8.65±0.14^{ab}	8.42±0.17^b	8.51±0.16^{ab}	8.60±0.16^a	0.05
Escherichia. coli	8.91±0.09^a	8.47±0.08^b	8.64±0.20^b	8.42±0.11^b	0.00
Salmonella spp	8.69±0.12^a	8.35±0.07^b	8.50±0.10^b	8.42±0.09^b	0.00

$^{a, b}$: Means with the same superscript on the same row are not significantly different (p>0.05). P= probability**.** T0$^-$ =control diet; T0$^+$ = T0+ 0.1% Doxycycline; T1 = T0+ 2 g of *Pentadiplandra brazzeana*/kg of feed; T2 = T0 + 2 g of *Pentadiplandra brazzeana*/liter of water.

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Biochemical parameters

Table 5 summarizes the effects of feeding diets of *P. brazzeana* powder on biochemical parameters. With the exception of ASAT and LDL cholesterol which were markedly affected ($p<0.05$), experimental diet did not significantly affect the other studied biochemical parameters. Diet receiving antibiotic induced a significant increase in serum level of ASAT ($p<0.05$). There were no significant effects between *P. brazzeana* feeding diets and the negative control diet on serum content of ASAT, ALAT, creatinine, urea, total cholesterol, triglycerides and HDL cholesterol. These findings indicated that supplementation of *P. brazzeana* powder in feed or water had no negative effect on the liver and kidney. This can be due to the presence of hepato-protectory effect of substances present in the studied spice like saponins (Barbosa, 2014). It can be suggested that active compounds have improved the secretion of bile salt and led to a better digestion of lipids present in the feed (Frankic et al., 2009). This finding is in agreement with the finding of Ebile et al. (2018a), who recorded no significant effects on serum biochemical parameters of quails. The present results contradict the findings of Kana et al. (2017a, b) who respectively reported a marked ($p<0.05$) increased in creatinine content with 0.4% *Dischrotachys glomerata* and *Afrostyrax lepidophyllus* in broiler chickens. Diet receiving antibiotic induced a significant increase in serum content of ASAT. These results are in contradiction with the result of Ali et al. (2007) who reported that the addition of thyme in broiler chickens diet induced a significant decrease in serum total cholesterol.

Table 5. Effects of *P. brazzeana* feeding diets on serum parameters of broilers chickens

Parameters	Treatments					
	T0$^-$	T0+	T1	T2	SEM	P
ASAT (U/I)	157.60^b	221.08^a	124.35^b	106.89^b	12.35	0.00
ALAT (U/I)	39.20	46.25	42.65	38.93	5.96	0.97
Urea (mg/dl)	0.51	0.57	0.71	0.65	0.19	0.51
Creatinine (mg/dl)	1.50	1.51	1.33	1.25	0.25	0.24
Total cholesterol (mg/dl)	122.74	114.69	119.11	116.16	6.29	0.82
Triglycerid (mg/dl)	85.83	86.86	89.91	89.17	5.03	0.99
HDL cholesterol (mg/dl)	119.66	125.64	113.28	133.81	5.42	0.64
LDL cholesterol (mg/dl)	26.67^b	53.32^a	33.65^{ab}	21.91^b	4.55	0.04

$^{a, b}$: Means with the same superscript on the same row are not significantly different ($p>0.05$). p: probability**.** T0: control diet; T0$^+$: T0+ 0.1% Doxycycline; T1: T0+ 2 g of *Pentadiplandra brazzeana*/kg of feed; T2: T0 + 2 g of *Pentadiplandra brazzeana*/liter of water.

Hematological parameters

Table 6 indicates the effects of *P. brazzeana* feeding diets on hematological parameters of broiler chickens at 49 days of age. Feeding broilers with *Pentadiplandra brazzeana* powder had no significant effects on blood hematological parameters irrespective of the feeding regime. This result is in agreement with the finding of Zomrawi et al. (2012) who stated no significant effect on Hgb, MCV, MCH and MCHC in blood of broiler chicken supplemented with different levels of ginger root powder. The present results are in contradiction with the findings of Al-Kassie et al. (2011), who recorded a significant decreased in WBC, RBC, Hgb and PCV when 0.25%, 0.5%, 0.75% and 1% hot red pepper was incorporated through broiler diet compared to the control diet. Furthermore, Vivian et al. (2015) reported a significant increase in WBC, RBC and Hgb compared to the control treatment when 50 ml ginger, garlic and the combination of the two were incorporated in broiler drinking water.

Table 6. Effects of *P. brazzeana* feeding diets on hematological parameters of broiler chickens

Parameters	Treatments				
	T0$^-$	T0+	T1	T2	P
WBC (10^3/µl)	83.83±7.36	86.57±5.97	82.98±5.45	82.27±4.65	0.85
RBC (106/µl)	2.43±0.24	2.88±0.40	2.60±0.28	2.56±0.15	0.09
Hgb (g/dl)	11.70±1.14	13.17±1.75	12.60±1.14	11.87±0.72	0.35
PCV (%)	33.23±2.18	37.37±4.97	33.88±3.39	33.80±2.38	0.15
MCV (fL)	137.43±4.96	130.17±6.43	131.10±5.25	132.47±5.99	0.35
MCH (pg)	48.07±1.64	45.67±1.79	48.70±4.40	46.40±1.86	0.84
PLT (10^3/µl)	47.17±21.37	51.00±22.14	46.40±18.67	37.00±11.71	0.67

CONCLUSION

It's concluded that dietary administration of *P. brazzeana* powder to through drinking water at level of 2g/L improved growth performances of broiler chickens. Hence, *P. brazzeana* powder could be used through water as alternative to antibiotics growth promoters in poultry production.

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DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Author's contribution

Necdem T. Boris, Donfack Mikael, Deffo T. Gilchrist and Kengni N. Josiane went to the field to carry out the research and collect the samples. Kana J. Raphaël and Ngouana T. Ruben supervised the overall research work. Necdem T. Boris wrote the first draft before being revised by Kana J. Raphaël and Ebile D. Agwah. The final draft of article is approved by all the authors.

Consent to publish

All persons gave their informed consent prior to their inclusion in the study.

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To cite this paper: Necdem TB, Kana JR, Ngouana TR, Ebile DA, Donfack M, Tchouan DG and Kengni NJ (2020). Effects of Dietary Inclusion of *Pentadiplandra brazzeana* Powder on Growth Performances, Gut Microbiota and Haemato-Biochemical Indices of Broiler Chickens. *World Vet. J.*, 10 (3): 391-397. DOI: https://dx.doi.org/10.36380/scil.2020.wvj48

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 398-404, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj49

Cross Protectivity of Yolk Immunoglobulin Anti-Hemagglutinin Protein of High Pathogenic Avian Influenza A subtypes H5N1 Administered on Chicken Infected by High Pathogenic Avian Influenza A subtypes H5N1

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ORGINAL ARTICLE
pii: S232245682000049-10
Received: 17 Jun. 2020
Accepted: 09 Aug 2020

ABSTRACT

Yolk Immunoglobulin (IgY) against Avian Influenza (AI) is commonly used as immunotherapy and immunodiagnostic techniques. Application of IgY mixed in drinking water is known effective to inhibit AI replication. The effectivity of IgY anti-Hemagglutinin Protein (anti-HA) of High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) was tested against infection of High Pathogenic Avian Influenza clade 2.3.2 (A/Duck/Sidoarjo/2012). The inhibiting activity was observed through Immunohistochemistry. Sixty chickens were infected with 10^5 EID_{50}/ml of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012). Yolk Immunoglobulin with different amounts (0 µg, 100 µg, 200 µg and 400 µg) were administered at three different times which were 24 hours before infection, at the time of infection, and 24 hours after infection. The observation was conducted for 7 days. During post infection observation, death chickens were managed for immunohistochemistry assay to observe the present of virion and IgY sialic acid 2,3-alfa galactosa (SA α 2,3 gal) blocking activity in septa alveoli. By the end of observation all chickens were euthanized for immunohistochemistry assay. The result showed that anti-HA IgY obtained from HPAI clade 2.1 could protecting infection of HPAI clade 2.3.2. According to immunohistochemistry assay, the administration of IgY can neutralize the infecting virus marked by the number of virions observed in septa alveoli of the lungs. Regarding the assay, the dose of 200 µg and 400 µg of IgY applied 24 hours before the infection, can reduce clinical signs and mortality of infected chicken (80-100%). The best dose of the IgY to protect them from infection of clade 2.3.2 (A/Duck/Sidoarjo/2012) was 400 µg administered 24 hours before infection. It could be concluded that administration of IgY anti-Haemaglutinin Protein (anti-HA) of High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) could protect chickens against the infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012), even though they belong different clades. The protection rate was 80-100%. Further research should be done to discover the cross-protectivity of IgY as preventive method against HPAI outbreak.

Key words: Avian influenza virus, IgY anti-HA, Immunotherapy, Productivity.

INTRODUCTION

Avian Influenza (AI) is commonly known as fowl plaque which is a disease caused by infection of Influenza A virus which belongs to the family Orthomyxoviridae. This disease is susceptible for many species of birds (Bouma et al., 2009; Webby and Webster, 2003; De Jong et al., 1997). According to the genotype, It is classified into 16 Haemagglutinin and 9 Neuraminidase subtypes (Bergervoet et al., 2019). According to virulence, It is classified into two groups which are Low Pathogenic Avian Influenza (LPAI) and High Pathogenic Avian Influenza (HPAI) (OIE, 2016). Both LPAI and HPAI are originated from H5 and H7 subtypes (Bouma et al., 2009; Webby and Webster, 2003; De Jong et al., 1997), and it has become attention-getting to international trade community since HPAI causing a great loss by the outbreak, and the LPAI causing annual problem and has potency to mutate into HPAI (MacLachlan et al., 2016). It has become endemic in many countries such as Indonesia (Daniel et al., 2012).

Prevention has been already conducted such as routine vaccination and biosecurity management but annual outbreak remained ongoing. Poultry farm companies in Indonesia have conducting vaccination more than 400 million doses since 2004 (Bouma et al., 2009). AI is an enveloped segmented single-stranded negative sense RNA virus. Under electronic microscopes it is seen on pleomorphic, spherical, or velamentous forms. Its virion consists of 10-14.6 kb genome divided into eight segments arranged on helical-symmetrically order. It has seven structural proteins such as Haemagglutinin protein (HA), Neuraminidase protein (N), two Matrix proteins (M1 and M2), and three Polymerase proteins (PB1, PB2, and PA). HA and N are enveloped protein lining on the membrane form spikes that has important roles on pathogenicity, classification and neutralization of the virus (MacLachlan et al., 2016; Knipe and Howley, 2013). Specific antibody is usually used as a diagnostic rule or as a prevention for specific diseases. The antibody obtains from animals needs a good production procedure regarding to animal welfare instructions (Hau and Hendriksen, 2005). Antibody obtained from Yolk Immunoglobulin (IgY) is homolog to Immunoglobulin G (IgG) obtained from mammals.

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Recently the application of IgY obtained from eggs as immunotherapy is rising because the concentration of immunoglobulin is higher compared to IgG obtained from mammals. One of the privileges of obtaining immunoglobulin from chickens is that chickens have high sensitivity of antigen exposure, thus immune response and IgY production are persistent (Hau and Hendriksen, 2005).

AI virus transfers through the airway or orally, then Haemagglutinin protein of the virus bounds to the receptors of sialic acid alfa 2 and 3- galactosa (SA α 2,3 gal) proteins. This binding triggers the fusion of the virus into cells (Knipe and Howley, 2013). This binding could be failed if specific antibody against HA protein block the process. HA antibody obtained from Yolk Immunoglobulin might have the potency to block this process to prevent AI infection in chickens. This research was conducted to know the effectivity of anti-HA from HPAI clade 2.1 (A/Chicken/Blitar/2003) against infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012).

MATERIALS AND METHODS

Sixty chickens (21 days-old) were used in this experiment. They were divided into three groups randomly. Each group consisted of four subgroups of treatments which were consisted of five chickens respectively. IgY was obtained by infecting HA protein of HPAI clade 2.1 (A/Chicken/Blitar/2003) into Specific Pathogen Free (SPF) layer chicken. IgY was extracted from the eggs, and stored at -20°C (Narat, 2003). Yolk Immunoglobulin was given to each major group at three different times. IgY was administered 24 hours before infection, 24 hours after infection, and at the time of infection on Group I, II, and III respectively. The amounts of IgY given on each sub groups were 0 µg, 100 µg, 200 µg, and 400 µg respectively. Each of dose was diluted on distilled water till the total volume was one ml. The research was conducted at Biosecurity Level-2 (BSL-2) of Faculty of Veterinary Medicine, Airlangga University Indonesia. The temperature was set at 16 ^{o}C.

The chickens were infected with 10^5 EID_{50}/ml dose of the antigen (A/Duck/Sidoarjo/2012). The observation has been started at the time of infection continuing for 7 days. During this period, all death chickens were recorded, then prepared for immunohistochemistry assay for determining the degree of cell destruction and sialic acid 2,3-alfa galactosa (SA α 2,3 gal) blocking activity from anti-HA on cell tropism according to the antigen (virion detection) and antibody detection (anti-anti HA). By the end of observation, the rest of living chickens were euthanized through cervical dislocation method. Samples for immunohistochemistry were collected from the lung. The lung was dipped in 10% formalin buffer, then processed to make slides (Damayanti et al., 2004). Before the immunohistochemistry procedures applied to the slides, they were prepared for deparaffinization to wear the wax off. After cleaning up the slide, 250 µl of primer antibody (anti H5N1 and anti-anti HA) that had been diluted (1:1600) was added to the slides, then it was incubated for 60 minutes. Then activity of peroxidase was blocked by adding three drops of hydrogen peroxidase (H_2O_2), then it was incubated for 20 minutes. The slides were then rinsed using PBS for three times. Moreover, anti-rabbit conjugate labelled with Biotin-Streptavidin was added followed by DAB substrate. After that the slides were rinsed, they were dipped on Haematoxilyn for two minutes. They were transferred into Scott solution, then incubated for 2 minutes. They were rinsed, and then covered by cover glass. Positive result marked by the present of brown color on the slide (Damayanti et al., 2004). Obtained data was analyzed using ANOVA (Analysis of Variance) on Statistical Programs for Social Scientific (SPSS) program. The possible results were analyzed according to the Least Significance Different (LSD) analysis (Kusriningrum, 2012).

Ethical approval

The arrangement of this research had been approved by the ethics commission of experimental animals of Faculty of Veterinary Medicine Airlangga University, Indonesia.

RESULTS AND DISCUSSION

Observation has been conducted for seven days after the infection. It revealed that each treatment showed different effects according to mortality rate; the presence of virion captured on septa alveoli, and the presence of IgY in septa alveoli of chickens. In group I, chickens which were not treated by anti-HA were death on day 2 until day 3 after infection of (A/Duck/Sidoarjo/2012) (Diagram 1). In contrary, chickens treated with anti-HA (with amounts of 100µg, 200µg, and 400µg) showed healthy condition, and no clinical signs were present. Even though clinical signs were absence, one of the chickens administered with 100µg of antibody died on the second day after the infection while others remained intact until the end of the observation period (Diagram 1). It could be concluded that administration of anti-HA 24 hours before the infection could give 80-100% of protectivity (Table 1).

Group II which were treated with anti-HA at the same time of infection showed different results. Administration of anti-HA has protected the chickens from mortality only on day 1 after being infected. On the second day of infection, the

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mortality of chickens could be seen on each subgroup; primely on the subgroup not treated by anti-HA. The subgroup which were not treated with anti-AH (0 µg) has started the mortality prior to the subgroup treated with 100 µg of anti-HA. Mortality still could be seen even on the subgroup treated by 400 µg anti-HA (Diagram 2). According to the protection rate, administration of anti-HA at the same time of infection could gave 40-80% of protection (Table 1).

While administration of anti-HA on Group III has completely protected the chickens from mortality only on day 1 after the infection, Mortality occurred on all subgroups even on the one administrated with 400 µg of anti-HA. The mortality rate was significantly around 60% of the group population (Diagram 3). This rate is the largest among other treated groups. By the end of the observation, only subgroups of chickens treated with 200 µg and 400 µg of anti-HA have survived, while all chickens in other subgroups were death. It could be concluded that anti-HA given 24 hours after being infected gave a protection of 40% (Table 1). Regarding the dose of anti-HA, administrations of 200 µg and 400 µg of anti-HA are more protective than 100 µg of anti-HA. They could give protection around 40-100%. Administration of both doses 24 hours before the infection indicated a protection lasting longer than other times of administration (Diagrams 1-3). All data were collected, then processed into ANOVA analysis. According to the ANOVA analyses, the results showed a significant difference ($p<0.05$) (Table 2). Thus, it was processed into LSD analysis. The results of analyses revealed that the administration of anti-HA is influenced by the time of administration and the doses. Administration of anti-HA 24 hours before infection could give aa appropriate protection more and last longer than the other administration times. This discovery was supported by the result of immune-histochemistry (IHC) assay. According to IHC results, there was an absence of AI virus in septa intra-alveola from chickens treated with 400µg anti-HA on Group I. It was marked by the absence of dark-brown colour like formation observed on IHC slides (Figure 1). In contrast, the presences of AI virus observed on the chickens treated with anti-HA with 200µg and 100µg of anti-HA on Group I. The presence of AI virus also has been observed on the chickens which were not treated with anti-HA antibody (Figure 1). Administration of anti-HA in Group II and Group III seemed that they could not neutralize the virus as good as Group I, thus the number of virions have increased on both groups (Figure 1). The presents of virion inside the septa-alveoli of lungs could disturb respiration of infected chickens (OIE, 2016). Regarding the effective dose of protection, 400 µg of anti-HA gave best protection among others. In poultry, AI virus enters the host body through respiratory system and orally, then attaches to receptor sialic acid alfa 2,3- galactosa (SA α 2,3 gal) protein which laid on mucosal epithelium located on both respiratory and gastrointestinal tracts (Costahurtado et al., 2014; Webby and Webster, 2003). The transmission commonly occurs through contaminated water source, ingestion of contaminated feed and nasal discharge (Achenbach and Bowen, 2011).

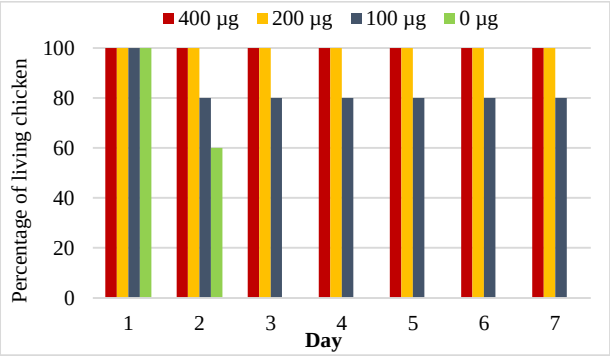

Diagram 1. Percentage of living chicken after administration of anti-HA 24 hours before infection

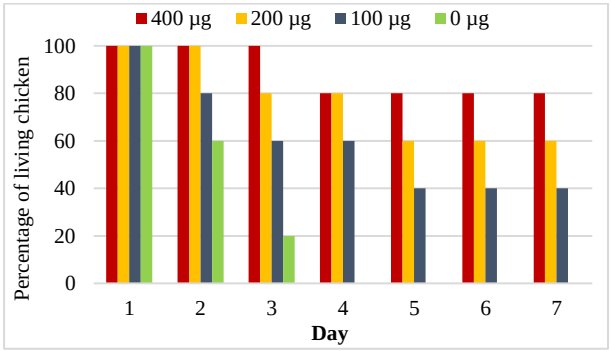

Diagram 2. Percentage of living chicken after administration of anti-HA at infection time

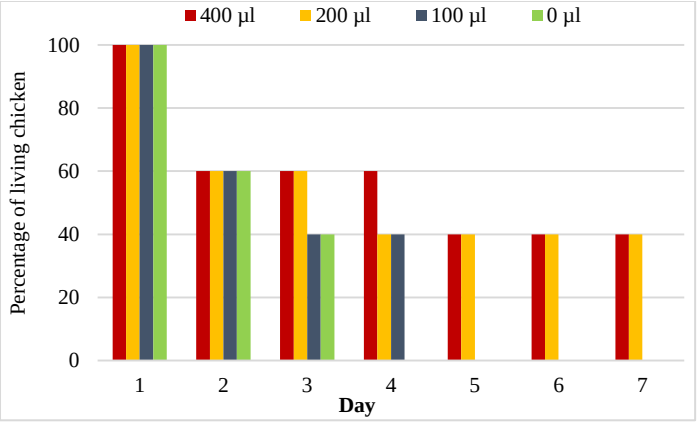

Diagram 3. Percentage of living chicken after administration of anti-HA 24 hours after infection

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Table 1. Protectivity rate of anti-HA antibody obtained from egg yolk (IgY).

	Application of Antibody Anti-HA (IgY)		
Dose	**24 hours before infection (%)**	**0 hours before infection (%)**	**24 hours after infection (%)**
0 µg/head	0	0	0
100 µg/head	80	40	0
200 µg/head	100	60	40
400 µg/head	100	80	40

%: means protectivity rate

Table 2. The amount of IgY and the time administration influence the protectivity of chickens

Amount	**Time of apllicatipn of anti-HA**	**(Mean ± SD)**
400	D-1	$4,8^{a} \pm 0,83$
	D-0	$10^{b} \pm 1,41$
	D+1	$23,2^{c} \pm 1,09$
200	D-1	$12,8^{d} \pm 1,09$
	D-0	$18,8^{e} \pm 1,09$
	D+1	$35,2^{f} \pm 1,09$
100	D-1	$30,8^{g} \pm 1,09$
	D-0	$35,6^{f} \pm 0,89$
	D+1	$41,2^{h} \pm 1,09$
0	D-1	$47,6^{i} \pm 0,89$
	D-0	$47,2^{i} \pm 1,09$
	D+1	$47,6^{i} \pm 0,89$

Different superscript on the same column showing significant different ($p < 0.05$). D-1: 24 h before infection, D-0: at the time of infection, D+1: 24 hours after infection.

In this research anti-HA obtained from egg yolk called IgY was used which is equivalent to mammalian Immunoglobulin G (IgG), since it is distinguished as the ancestor. As the IgY is equivalent to mammalian IgG, it has similar functions as the main humoral immune-system to eradicate antigens (Agrawal et al., 2016). IgY is frequently used as substitution from mammalian antibody because the production process is more respecting animal welfare. Moreover, it is easier to be done and the amount of immunoglobulin obtained is larger among small-sized animals (Narat, 2003; Ko and Ahn, 2007; Wen et al., 2012; Agrawal et al., 2016). Its capability to binding and target specifity is higher than mammalian Immunoglobulin G (IgG) which makes it has potential as therapeutic therapy for respiratory infections (Abbas et.al., 2018). Application of IgY is noticed capable to prevent bacterial and viral infections (Narat, 2003; Ko and Ahn, 2007; Wen et al., 2012; Agrawal et al., 2016). It could be applied in human too which gives many advantages (Pereira et.al., 2019; Constantin et. al., 2020). According to the IHC of anti-HA detection, anti-HA administered orally could be seen on septa alveoli of lungs as brown color (Figure 2). Immunotherapy given orally is capable to block receptors on the epithelium of mucosa on digestion system. It is directly transported through all over the body absorbed by intestine to capillaries, then transported to portal vein in liver and then vena cava in heart (Rahimi et al., 2007). Administration of anti-HA 24 hours before being infected suggested that it could compete binding of the virus to receptor SA α 2,3 gal protein. Administration of anti-HA obtained from horses given to the mouse intraperitoneal before infection can give 100% protection (Lu et al., 2006).

This research used anti-AH obtained HPAI clade 2.1 (A/Chicken/Blitar/2003) against the infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012). Both viruses belong to different clades, different hosts and different time isolations. According to the results, even both viruses are different; the cross-protection was observed. This finding extents the fact that cross-reactivity among clades of H5 subtypes occurred (Dharmayanti et al., 2017; Ducatez et al., 2011). This evidence is not only occurred among H5 subtypes. Cross-reactivity also occurred among H7 subtypes to H3 and H4 subtypes. It is noticed that cross-reactivity between H7 and H3 is stronger than H7 and H4 (Guo et al., 2016). Cross-reactivity among subspecies in same family not only occurs in AI. It also occurs in Newcastle Disease (Aldous et al., 2016). The distinct point is that cross-reactivity in Newcastle Disease seems stronger than AI. Both viruses are single stranded negative sense RNA virus. RNA viruses are easy to mutate because their polymerase enzymes lack of proof-reading. Among them, the mutation rate of AI is higher because its genome arranged on some segments leading to antigenic shift and antigenic drift (MacLachlan et al., 2016).

This finding reveals the possibility of anti-HA hyper-immune serum application on AI prevention. Routine vaccination as one of the main prevention methods could be possibly optimized by application of anti-HA serum orally. Further researches need to be done since this research is conducted in controllable and variables environment.

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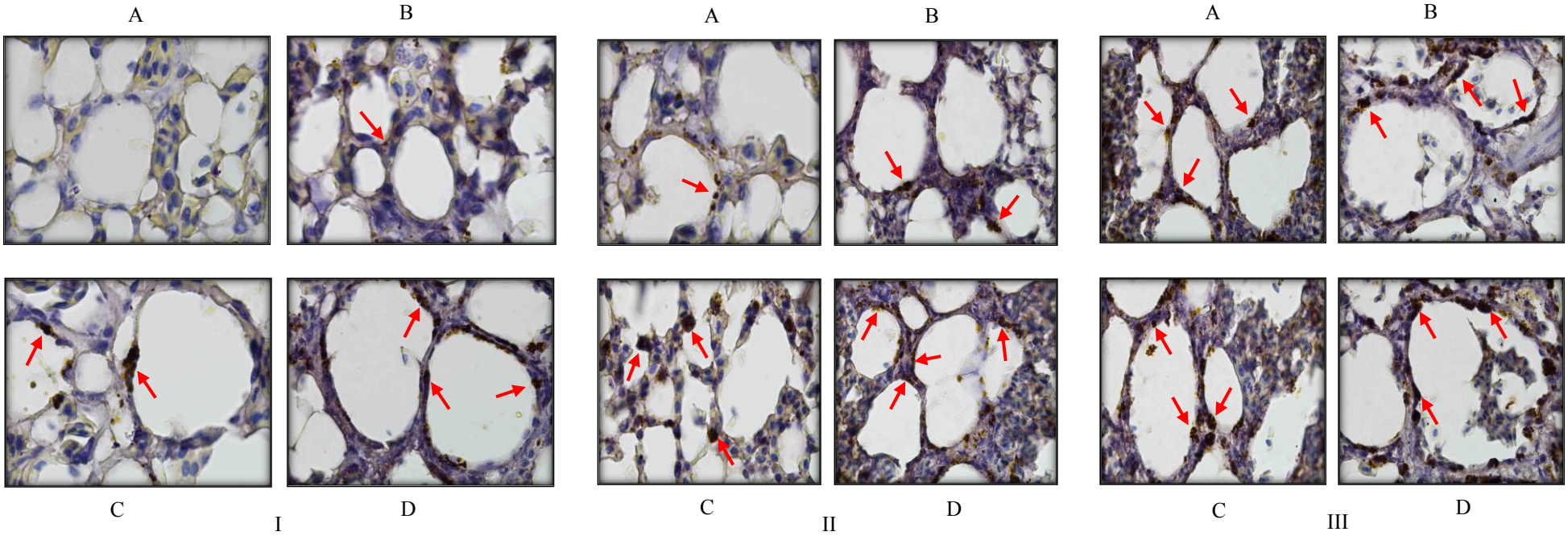

Figure 1. Immunohisochemistry of chicken lungs. Arrows indicate the presence of Avian Influenza Virus on septa alveoli. I; chicken administered with anti-HA IgY 24 hours before infection. II; chicken administered with anti-HA IgY 24 hours after infection. III; chicken administered with anti-HA IgY 24 at time of infection. A; Amount of administered IgY is 400 µg. B; Amount of administered IgY is 200 µg. C; Amount of administered IgY is 100 µg. D; Amount of administered IgY is 0µg.

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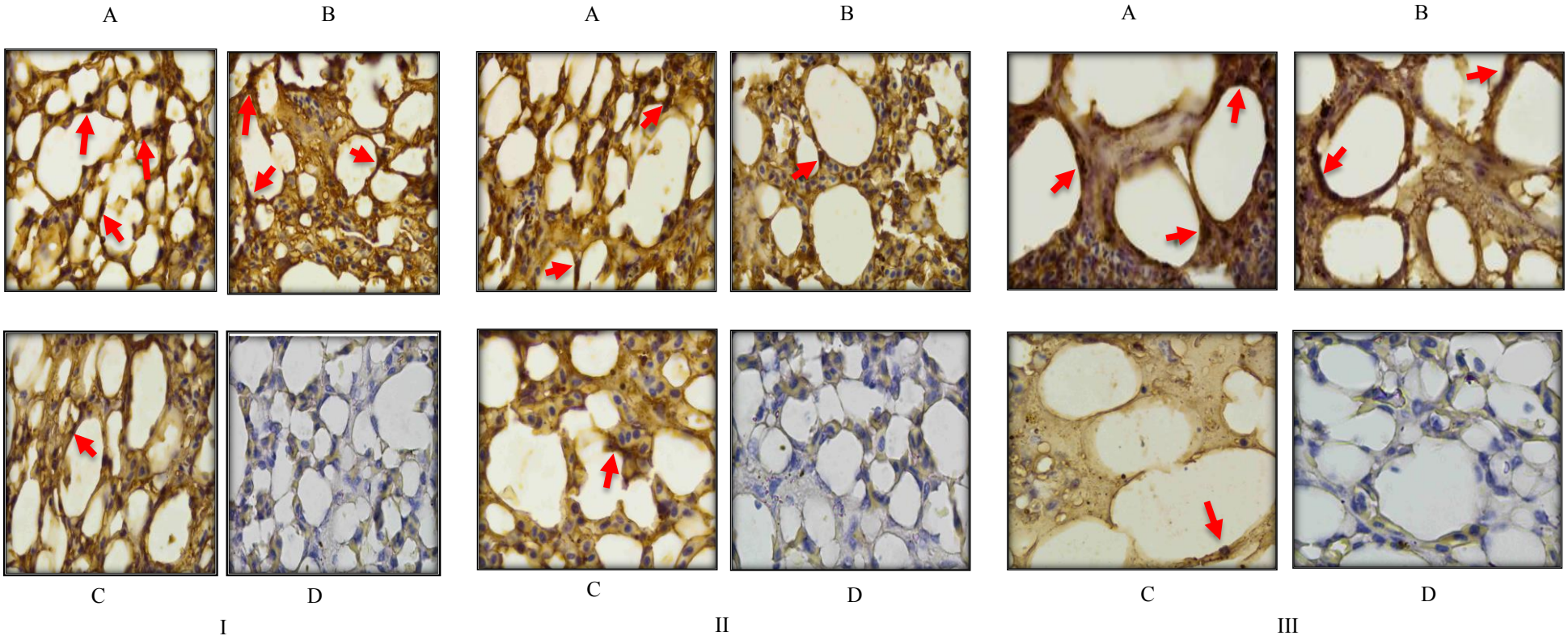

Figure 2. Immunohisochemistry of chicken lungs. Arrows indicate the blocking activity of anti-HA IgY on Avian Influenza Virus on septa alveoli. I; chicken administered with anti-HA IgY 24 hours before infection. II; chicken administered with anti-HA IgY 24 hours after infection. III; chicken administered with anti-HA IgY 24 at time of infection. A; Amount of administered IgY is 400 µg. B; Amount of administered IgY is 0µg.

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CONCLUSION

It can be concluded that application of anti-HA obtained High Pathogenic Avian Influenza (HPAI) clade 2.1 (A/Chicken/Blitar/2003) could give a protection from infection of HPAI clade 2.3.2 (A/Duck/Sidoarjo/2012), although they were originated from different clades. The protection rate was 80-100% applied 24 hours before infection.

DECLARATIONS

Authors' contribution

Suwarno contributed on data analysis and the write up of the manuscript. I also contribute on the production of IgY and formulating the dose of administered IgY and processing the sample on Immunohistochemistry assay. Rahaju Ernawati and Nanik Sianita Widjaya contributed on data analysis and the write up of the manuscript and calculating the dose of EID_{50}/ml and conducting the challenge test. All authors read and approved the final draft of manuscript.

Competing interests

The authors have not declared any conflict of interests.

Consent of publish

All the authors agree to publish this manuscript in World`s Veterinary Journal.

Acknowledgements

This research was fund by Director of Research and Community Development Ministry of Technology and Higher Education on 2016.

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To cite this paper: Suwarno, Ernawati R and Widjaja NS (2020). Cross Protectivity of Yolk Immunoglobulin Anti-Hemagglutinin Protein of High Pathogenic Avian Influenza A subtypes H5N1 Administered on Chicken Infected by High Pathogenic Avian Influenza A subtypes H5N1. *World Vet. J.*, 10 (3): 398-404. DOI: https://dx.doi.org/10.36380/scil.2020.wvj49

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 405-413, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj50

Assessment of Genetic Capability for Post-Weaning Growth Traits of Reciprocal Cross between Gabali and V-Line Rabbits Using an Animal Model

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ORIGINAL ARTICLE
pii: S232245682000050-10
Received: 05 Aug 2020
Accepted: 23 Sept 2020

ABSTRACT

This study aimed to assess the conceivable impact of hereditary factors on the crossbreeding rabbit groups set up by proportional going between Sinai Gabali and V-Line. Reciprocal cross on the post-weaning performances was performed by estimating the genetic capability for their crosses. The study samples included two pure rabbit breeds (a male Saini Gabali (G) and a female V-Line (V)), and reciprocal crosses to compromise 10 groups. Records of 448 kits delivered by 45 does and 16 bucks were utilized to estimate Heritability (h^2), genetic and phenotypic correlations, and breeding values of litter weight traits. First generation was created from the consequences of four parities (½G ½V, and ½ V ½G; sire breed was demonstrated first). Weaning was implemented on the 28th day of the kits' age. Post-weaning litter traits were measured Body weight (BW) at 4, 5, 6, 8, 10, and 12 weeks of their age; and average daily gain was measured during 4-8 weeks (ADG_{4-8}, ADG_{4-12}, ADG_{8-10}, and ADG_{8-12}). Data were examined by animal model, which was performed utilizing derivate free limited maximum likelihood. The results revealed that h^2 was moderate for both breeds, and its reciprocal cross ranged from 0.2 to 0.25, and BW at weaning was 0.22 ± 0.07. Meanwhile, there was a positive genetic correlation between BW and ADG at different age ranges (ranged 0.02 to 0.77, 0.04 to 0.76, respectively). Assessments of environmental correlation between BW at different age ranges were negative, except of those between BW_8 and BW_{12} which were positive, but not significant. Additionally, the progeny had higher predicting breeding values for BW at 4, 8,10, and 12 weeks for both breeds, but that was obtained from G×V exceeding those from their reciprocal cross. In conclusion, direct additive variance was considerably effective, and consequently body weight at weaning and post-weaning growth traits could be improved by utilizing bucks of Sinai Gabali with doesof V-line based on the performance of their progenies, and selection of sires and dams.

Keywords: Genetic correlation, Heritability, Post-weaning, Sinai gabali, V-line, Weaning weight

INTRODUCTION

The expected global needs for the meat at 2050 are about 73% compared to present mass production. The developing countries will contribute for most of this expansion due to fast population growth and ascending of capital income (FAO, 2011; OECD, 2017). Obike and Ibe (2010) conveyed that rabbits are considered as a high-quality source of protein with cheap price, which can be used to improve the animal production of protein in low revenue populations (Makkar et al., 2014). Furthermore, the rabbits had many compensations above other animals for low cost meat production, for instance, high growth rate, high feed conversion, and their ability to utilize fibrous feeds, both of sexual maturity and short gestation length resulted in a short generation interval, also, the rabbits' meat was classified as a high-quality meat (Herbert, 2011). In addition, growth rate in rabbits, particularly their post-weaning was intensely affected by genotype and environmental condition including the availability of both quality and quantity of feed. Furthermore, the diversity of the rabbit breeds gave more opportunities to improve the meat production by divergent breeds crossing (Piles et al., 2004).

Heritability, which is an element of variance components, gave data about the hereditary idea of a characteristic and is required for genetic assessment and selection procedures (El-Raffa, 2005). Phenotypic variance assumed a vital part for post-weaning traits in rabbits was generally depicted to be overwhelmed by ecological impacts inferable from the does or potentially litter, which might owe the short interval from the weaning to marketing. Moreover, low heritability and direct proportions of genetic responses to select for post-weaning growth traits were conveyed (Lukefahr

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et al., 1996). The rabbits' genetic improvements were mainly conditional on the heritability of the defined traits, and its association with different other traits of economic importance were measured. A moderate to high heritability (0.7-0.9) for litter weight at 7-8 weeks old, while the repeatability generally indicating low estimates (0.003-0.008) were obtained by Okoro et al. (2012). In oppose with Iraqi, 2008 who found that estimating of heritability were 0.05 to 0.38 body weight (BW) at 4, 8, and 12 weeks old, while these estimations were 0.23 and 0.19 for daily gain from 4 to 8, and 8 to 12 weeks of age, correspondingly. Though, additive genetic variability was far from being considered negligible. To facilitate, and to make rapid improvements in rabbits' performance, it could be inaugurated from selection and use of divergent breeds in the crossbreeding (Chineke and Raheem, 2009). In addition, crossbreeding is efficient for improving the post-weaning growth potential (Piles et al., 2004). Besides, selection in maternal lines in rabbit was somewhat considering determination inside limited population which amassed in mating grouping impacts (Ragab et al., 2015), extending the hereditary variety amongst lines and, verifiably, changing the gene frequencies between population. Furthermore, to employ genetic resources effectively, the genetic and environmental causes of phenotypic alteration in economic attributes need to be differentiated (Gorbani and Salamatdoust, 2011). The intention of rabbits' breeding was to improve execution characteristics of rabbits' population through both mating and selection. Accordingly, the targets of the existing research were to assess conceivable impact of hereditary factors on crossbreeding rabbit groups set up by proportional going between Sinai Gabali and V-Line (as a unique lines) by reciprocal cross on post-weaning performances by estimate the genetic capability for their crosses.

MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethics Committee, Egypt.

Breeding plan

Two pure rabbit breeds were used in current study (Saini Gabali (G), and V-Line (V) as a standard exotic line). Does and bucks of the V were acclimatized descendants of the Spanish synthetic line. Crossbreeding system was applied in ten mating groups which contained 4 to 5 does per group. The first five groups consisted of V does which were mated with five G bucks (G♂ X V♀), and reciprocal crosses (V♂ X G♀) for the further five groups. Each buck was represented as a sire to all litters in each group to produce F_1 (½ G ½ V and ½ V½ G; sire breed was indicated first). Weaning was implemented at 28 days of kits' age.

Rabbitry, housing and management

Animals were raised in a semi closed rabbitry, depending on the natural ventilation. Does were housed individually in pens where there were settle boxes, feeders, and automatic drinkers. All rabbits were fed on a commercial lactating-pelleted-diet containing approximately 2600 Kcal/kg ration as digestible energy; 16.3% crude protein; 13.2% crude fiber and 2.5% fat. Both of feed and water were provided *ad libitum*. Does mated their same respective group assigned bucks 10 days post-kindling. Pregnancy was ascertained by palpation 10 days of succeeding mating. Females that neglected to conceive were come back to the same assigned buck to be re-reproduced. Inside 12 hours once encouraging, litters were checked and recorded. In this way, weaned a month kits were sexed and exchanged for additional study to standard descendants prepared pens.

Source of data

Post-weaning litter traits were body weight (BW_4) at , 5, 6, 8, 10, and 12 weeks old, average daily gain between 4 to 8 weeks (ADG_{4-8}), 4 to 12 weeks (ADG_{4-12}), 8 to 10 weeks (ADG_{8-10}), and 8 to 12 weeks (ADG_{8-12}).

Statistical analysis

Linear model

Data were preliminary analyzed using general linear model (GLM) and VARCOMP procedures of Statistical Analysis System (SAS, 2001, version 8.1). In addition, single and multi-traits animal model analyses (AM), were performed using derivate free restricted maximum likelihood as recommended by Boldman et al. (1995). For the derivative- free model, convergence (co) variance components estimation was when the global maximum of the log likelihood function was found**.**

Estimation of heritability (h^2)

The following formula was used by the animal model software to estimate h^2

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$$h^2 = \frac{\sigma^2 a}{\sigma^2 a + \sigma^2 P_e + \sigma^2 e}$$

Where,

$\sigma^2 a$: was additive genetic variance $\sigma^2 P_e$: was permanent environmental variance, and $\sigma^2 e$: were random residual associated with each observation.

Estimation of correlations

The following formula was used as recommended by Franzese and Iuliano (2019).

$$r_{XY} = \frac{cov(xy)_{ij}}{\sqrt{\sigma^2 xij}\sqrt{\sigma^2 y_{ij}}}$$

Where,

$cov(xy)_{ij}$ = The additive genetic covariance, $\sigma^2 xij$ = The additive genetic variance of the trait (or permanent environmental, environmental and phenotypic). $\sigma^2 y_{ij}$ = ?????

Estimation of Predicted breeding value

The mixed model equations (MME) for the best linear unbiased estimator (BLUE) of Estimable function of *b*, and for the BLUP of *a* and pe, in matrix notation was as follows:

$$\begin{bmatrix} X`X & X`Z & X`w \\ Z`X & Z`Z + A^{-1}\alpha_1 & Z`Z \\ W`X & w`Z & w`w + I\,\alpha_2 \end{bmatrix} \begin{bmatrix} b\hat{} \\ a\hat{} \\ p\hat{} \end{bmatrix} = \begin{bmatrix} X\,y` \\ Z\,y` \\ wy` \end{bmatrix}$$

Where, A^{-1} is the inverse of the numerator relationship matrix,

$\alpha_1 = \sigma^2_e / \sigma^2_a$ and $\alpha_2 = \sigma^2_e / \sigma^2_{pe}$

Estimation of Accuracy of Predicted breeding value

The accuracy of predicted breeding values for each animal was assessed as follows:

$R\ A`A = (1- d_j\ a)^{2,}$

Where,

R A`A = the accuracy of prediction of the individual breeding values, d_j = the $_j$th diagonal elements of inverse of the appropriate block coefficient matrix standard error, (Se) was estimated by the following relation: $Se = d_j\ \sigma^2_e$. In addition, simple correlations between breeding values for litter traits and progeny weight were estimated.

RESULTS AND DISCUSSION

Heritability estimates for body weight and average daily gain

Heritability estimates h^2 for BW at 4, 6, 8, and 12 weeks were 0.65 ± 0.169, 0.09 ± 0.224, 0.01 ± 0.118 and 0.29 ± 0.143, respectively which were estimated from MTAM, and using all data. h^2 are moderate for both breeds and ranged from 0.22 to 0.25 and 0.20 to 0.25 for G♂ X V♀, and V♂ X G♀ crosses, respectively (Table 1). In addition, the moderate h^2 for BW at weaning 0.23 and 0.22 for G♂ X V♀, and V♂ X G♀ respectively, which suggested that the selection for weaning weight will give greater improvement for BW at marketing, thus, this could be situated as the endorsement goal of animal breeder. Furthermore, a little higher h^2 of BW for G♂ X V♀ than its reciprocal indicated the importance of using bucks of Saini Gabali to improve BW at different ages.

The extant results indicated that h^2 estimates were higher for BW at weaning and decreased gradually, and then increased at older ages. The equivalent results were confirmed by Khalil (1986). Also, the moderate h^2 for BW_4, and BW_{12} suggested that the selection for weaning weight will give a greater improvement in BW at marketing. Similarly, according to El-Kelany (2005) who concluded that the additive genetic variances at younger age (4 weeks) for the five breeds (New Zealand White, California, Basucat, Flander and Black and Baladi) were beneath those at older age (8 weeks). This could be a direct result of non-added substance of genetic impacts and changeless environmental impacts at 4 weeks than at 8 weeks. In addition, Anous (2000) found that h^2 estimates for BW at 4, 6, 8 and 10 weeks old were 0.67, 0.81, 0.98 and 0.67, respectively, and concluded that the selection for BW at earlier ages may be convenient procedure for improving early rabbit growth. The present estimate of h^2 for weaning weight were exceeding those reported before (0.224-0.26) (Argente et al., 1999; García and Baselga, 2002). These differences among results may be attributed to differences in sampling errors, available number of observations, methods of analysis and statistical model used. Contritely, low h^2 for BW at various ages of California were 0.17 and 0.18 for 8 and 12 weeks old, while h^2 were

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0.16 and 0.19 for daily gain during the period of 4 to 8 and 8 to 12 weeks old, respectively (Gharib, 2008). At the present study, the favorable estimates of h^2 for post-weaning growth traits were underneath those obtained by other scientists using the AM ranged from 0.30 to 0.72 (Iraqi et al., 2002). The acquired outcomes were correspondingly to the small sample' size of progeny per generation, second the inadequacy of progeny per sire, and third to the non-randomness in the allocation of progeny within sire group and sampling errors (Khalil et al., 2000). Intestinally, it will be an encouraging factor to enrich growth performance of these standard breeds raised in hot climates through the selection of sires in future. Regarding to gain weight, the present estimates of ADG among different ages were lower than those reported by many authors working on various rabbits' breeds and ranged from 0.018 to 0.61. These results implied that the post-weaning ADG tends to be low to moderate heritable. Alike body weights, the h^2 for post-weaning ADG estimated from sire components were inferior to those estimated from dam components, which it was due to the maternal effect on BW from birth to weaning. These results corresponded to the previous studies where maternal genetic effects were realized significantly positive for the favors of V-Line dams, BW_8 and BW_{12} (Abou Khadiga et al., 2008). The V-line had an unfavorable maternal genetic effect on the BW at 32 days old (ranging from 54.3 to 86.0 gram). In addition, Orengo et al. (2009) reported that maternal genetic effects were significant for BW at 60 days. Furthermore, the estimate of h^2 was higher for BW_8 weeks than other ages, and the selection of the animals would be more effective at 8 weeks of age to improve post-weaning growth traits in Gabali rabbits (Iraqi, 2008).

Table 1. Estimates of heritability for body weights, and average daily gains from weaning to marketing weight for Gabali X V-line, and its reciprocal cross by animal model

Traits	Heritability (h^2 ± SE)		
	All data	Gabali x V- line (G♂ X V♀)	V- line x Gabali (V♂ X G♀)
BW4	0.65 ± 0.16	0.23 ± 0.07	0.23 ± 0.07
BW_6	0.09 ± 0.22	0.25 ± 0.10	0.25 ± 0.10
BW_8	0.01 ± 0.11	0.25 ± 0.05	0.25 ± 0.05
BW_{12}	0.29 ± 0.14	0.22 ± 0.02	0.22 ± 0.02
ADG_{4-8}	0.01 ± 0.13	0.36 ± 0.135	0.15 ± 0.135
ADG_{4-12}	0.12 ± 0.06	0.38 ± 0.069	0.61 ± 0.069
ADG_{8-12}	0.08 ± 0.05	0.08 ± 0.055	0.04 ± 0.055

BW: Body weight at 4, 6, 8, and 12 weeks of age (BW4, BW6, BW8, and BW12, respectively). ADG_{4-8}: Average daily gain between 4-8 weeks interval, 4-12 weeks (ADG_{4-12}), and 8-12 weeks (ADG_{8-12})

Genetics and environmental correlations

The MTAM analysis of variance and covariance were performed on the data of V-Line and Saini Gabali breed to derive estimates of direct additive genetic (r_g) and environmental correlations (r_e) among different traits studied. Genetic correlation for both BW and ADG at distinctive ages were positive, and ranged from 0.02 to 0.77, and 0.04 to 0.76, respectively. The highest genetic correlation was 0.76 between ADG_{4-12} and ADG_{8-10} (Tables 2 and 3). These results possibly explained by that the selection for higher BW at weaning would cause an associated increase in marketing body weight. Assessments of environmental correlation among BW at different ages were negative, except the r_e between BW_8, and BW_{12} were positive (0.08, 0.124), but not significant (Table 2). Also, r_e among ADG in different intervals were lower in most incidents than the genetic correlation (Table 3). The present findings advocated that using early information for higher BW selection at weaning would cause a correlated increase in marketing BW. Comparable results were acquired where r_g ranged from 0.31 to 1.0 (El-Kelany, 2005; Khalil et al., 2000). Moreover, Shebl et al. (1997) stated that r_g amongst BW at 8, 12 and 16 weeks of age, and the daily gain (8 and 12 weeks) for New Zealand White (NZW) and Gabali line (G) were positive. The current estimates of r_g indicated that BW_8 was more correlated than BW_4, and BW_6 compared to r_g for BW_4 with that at BW_6, and BW_{12}, indicating that high weaning weight was associated with heavier body weight as BW_4, and BW_{12}. Enab et al. (2000) found that r_g were positive and generally high in Cal and NZW breeds at all ages (4, 8, and 12 weeks). Also, it was noted that r_g in general tended to diminish the value as the intervals between two ages increased in both breeds (Enab et al., 2000). Prospectively, r_g between ADG in different ages were positive; the highest r_g between ADG_{4-12} and ADG_{8-10} indicated that the selection of animals that have heavy BW at 4 weeks of age, would increase BW at marketing in the next generation. Analogously, Shebl et al. (1997) found that the r_g between BW_8, and ADG_{8-12} weeks was lower than the corresponding r_g between BW_{12} and ADG in the same period. Regarding to estimates of environmental correlation (r_e) concerning BW at different ages were negative, except for r_e between BW_8 and BW_{12} which was positive, but not significant. In addition, r_e between average daily gains in different ages were lower than the genetic correlation in most incidents (Table 3). In other incidents, both r_g and r_e had the same indication, and they were not distinct in magnitude. Accordingly, this tendency might possibly be owing to several physiological mechanisms.

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Table 2. Estimates of genetic (above diagonal) and environmental (below diagonal) correlations among weekly body weights from weaning to marketing weight in rabbits as estimated by Multi-trait animal model

Correlated traits[a]	BW_4	BW_6	BW_8	BW_{12}
BW_4	-	0.14 (0.683)	0.51 (2.257)	0.20 (0.346)
BW_6	-0.17 (0.231)	-	0.77 (1.472)	0.02 (0.579)
BW_8	-0.29 (0.196)	-0.38 (0.114)	-	0.02 (1.207)
BW_{12}	-0.75 (0.122)	0.00 (0.139)	0.08 (0.124)	-

[a] Body weight at 4, 6, 8, and 12 weeks of age (BW4, BW6, BW8, and BW12, respectively);

Table 3. Estimates of genetic (above diagonal) and environmental (below diagonal) correlations between post weaning average daily gains in rabbits as estimated by Multi-trait animal model

Correlated traits[a]	ADG_{4-8}	ADG_{4-12}	ADG_{8-10}	ADG_{8-12}
ADG_{4-8}	-	0.13 (2.298)	0.53 (5.410)	0.08 (2.637)
ADG_{4-12}	-0.18 (0.081)	-	0.76 (0.338)	0.04 (0.385)
ADG_{8-10}	0.01 (0.076)	0.35 (0.071)	-	0.02 (0.360)
ADG_{8-12}	-0.09 (0.119)	1.00 (0.026)	0.34 (0.084)	-

[a] Average daily gain between 4-8 weeks interval (ADG_{4-8}), 4-12 weeks (ADG_{4-12}), 8-10 weeks (ADG_{8-10}), and 8-12 weeks ($_{ADG8-12}$).

Breeding values

Body weight

Estimates of minimum and maximum predicted breeding values (PBV), and their accuracies (r) for BW at various ages estimated from does breeding values (DBVs), bucks breeding values (BBVs) and progeny breeding values (PBVs) were presented in table 4. The range of DBV for BW at four, five, six, eight, 10, and 12 weeks of age were 360, 584, 541, 561, 600, and 232 gram, respectively, and that of BBV were 219, 247, 250, 213, 270, and 290 gram, respectively. Whereas the range of PBV was 517, 575, 550, 505, 535 and 548 gram for BW at 4, 5, 6, 8, 10, and 12 weeks of age, respectively. Table 5 shows the higher numbers of PPVs which were obtained from crossing between bucks of G♂ X V♀, and V♂ X G♀, respectively. The present results indicated that for both breeds, progeny had higher breeding values for BW at different ages (4, 8, 10, and 12 week). Also, the predicted breeding values achieved from G♂ X V♀ cross are superior to those attained from reciprocal cross. Then, it is important to recognize that there was a potentially moderate improvement in BW at weaning and marketing (12 weeks) in G♂ X V♀ raised in adverse environmental through sire selection. According to high ranges in breeding values for most body weights obtained Does and progeny, indicated the importance of selection of doe and progeny in the second line to improve BW for different ages. The similar results were also obtained by Khalil et al. (2000) and Sabra et al. (2001). Moreover, Khalil et al. (2000) concluded that among all sires in NZW and California breeds, percentage of sires with positive estimates of sire transmitting ability (46.20 to 61.10%) for BW (At 5, 6, 8, 10 and 12 weeks). From the genetic point of view, there is a potentially for a moderate improvement in post-weaning growth traits in NZW and Cal rabbits raised in adverse environmental through sire selection. In addition, Sabra et al. (2001) noticed that the PBV for BW at 8 weeks and 12 weeks of age ranged from 269 to 303 grams, and from -315 to 324 grams in NZW, and Z-line. Also, wide variations in PBV of BW between the two strains was found to be in favor of Z-Line rabbits (Iraqi et al., 2002). The exactness of lowest and greatest evaluations of PBV were for the most part higher in NZW than those in Z-Line.

Average daily gain

Estimates of minimum and maximum PBV with standard errors (SE) and their accuracies (r) for ADG between 4 to 8 weeks (ADG_{4-8}), between 4-12 weeks (ADG_{4-12}), between 8 to 10 weeks (ADG_{8-10}), and between 8 to 12 weeks (ADG_{8-12}) estimated from DBVs, and PBVs are presented in table 6. The range of DBVs were 6.29, 1.845, 8.31, and 11.38 gram for ADG_{4-8}, ADG_{4-12}, ADG_{8-10}, and ADG_{8-12}, respectively. Also, the BBVs for the above-mentioned traits were 3.08, 1.32, 6.11, and 15.29 gram, respectively. Whereas, the range of PBVs were 6.95, 1.86, 6.16, and 15.36 gram, respectively. The present results indicated that the range of predicted breeding values of does for most traits studied were higher than those of progeny and sires. The obtained results revealed the importance of dams, since it gives the higher range of breeding values and selection of dams for the next generation in maternal line would place emphasis on good genetic maternal effects. Similar results have found where the genetic trend of post-weaning daily gain in lines A and V rabbits, had positive trend, small for line V, and considerably higher for line A (Baselga and García, 2002).

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Table 4. The ranges of predicted breeding values for does, bucks and progeny in weekly body weights from weaning to marketing for Gabali, V-line rabbits and their crosses as estimated by multi-trait animal model

Traits[a)]	Predicted breeding values[b]																	
	DBVs						BBVs						PBVs					
	Minimum			Maximum			Minimum			Maximum			Minimum			Maximum		
	BV	SE	R	BV	SE	r	BV	SE	r	BV	SE	R	BV	SE	r	BV	SE	r
BW_4	-0.207	0.10	0.88	0.153	0.05	0.37	-0.143	0.04	0.73	0.076	0.07	0.92	-0.201	0.06	0.68	0.316	0.08	0.85
BW_5	-0.241	0.05	0.37	0.343	0.11	0.88	-0.174	0.05	0.73	0.073	0.08	0.91	-0.223	0.07	0.00	0.352	0.11	0.85
BW_6	-0.214	0.06	0.35	0.327	0.11	0.87	-0.184	0.05	0.71	0.066	0.08	0.79	-0.217	0.07	0.00	0.333	0.11	0.83
BW_8	-0.228	0.06	0.00	0.333	0.83	0.81	-0.116	0.05	0.00	0.097	0.08	0.89	-0.197	0.07	0.00	0.314	0.11	0.82
BW_{10}	-0.254	0.07	0.00	0.346	0.13	0.80	-0.141	0.06	0.39	0.129	0.12	0.89	-0.229	0.08	0.00	0.323	0.13	0.84
BW_{12}	-0.113	0.08	0.00	0.119	0.14	0.83	-0.100	0.06	0.38	0.198	0.13	0.88	-0.212	0.09	0.00	0.336	0.14	0.83

[a)] Body weight at 4, 5, 6, 8, 10, and 12 weeks of age (BW_4, BW_5, BW_6, BW_8, BW_{10}, and BW_{12}, respectively), [b)] DBVs: Doe predicted breeding value, BBVs: buck predicted breeding value, PBVs: progeny predicted breeding value, BV: Breeding value, SE: Standard error, R/r: Accuracies of breeding value.

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Table 5. Predicted breeding values for higher number of progenies of body weight (g) at different ages (4, 8, 10 and 12 weeks of age) for Gabali x V-line and its reciprocal cross

Gabali x V- line (G♂ X V♀)					V- line x Gabali (V♂ X G♀)				
No.	**BW_4[a)]**	**BW_8**	**BW_{10}**	**BW_{12}**	**No.**	**BW_4**	**BW_8**	**BW_{10}**	**BW_{12}**
100	-293	-272	-197	-185	351	-185	-177	-223	214
101	426	292	601	350	357	120	210	153	112
216	212	164	253	282	354	136	035	150	164
274	326	279	285	113	356	040	101	080	122
277	503	395	552	301	369	013	078	039	059
279	328	270	320	133	370	104	162	151	173
282	117	111	067	060	384	024	088	051	069
301	303	270	237	169	385	-113	-106	-166	240
387	673	550	676	454	401	071	095	-110	-152
910	478	404	440	318	406	220	240	304	374
915	353	259	445	406	410	036	026	007	053
960	190	170	145	065	441	097	092	128	145
967	134	117	107	041	487	087	039	082	090
4006	209	155	263	203	489	418	384	573	739
4008	678	599	544	378	498	133	192	175	168
4009	-071	-060	-068	-393	4055	472	525	654	801
4016	130	020	423	257	4056	354	480	446	390
4098	270	220	270	068	5001	044	104	060	038
					5002	188	207	268	347

[a)] Body weight at 4, 8, 10, and 12 weeks of age (BW_4, BW_8, BW_{10}, BW_{12}, respectively)

Table 6. The ranges of predicted breeding values for does, bucks and progeny in post-weaning average daily gain for Gabali, V-line rabbits and their crosses as estimated by multi-trait animal model

Traits[a)]	Predicted breeding values[b]																	
	DBVs						BBVs						PBVs					
	Minimum			Maximum			Minimum			Maximum			Minimum			Maximum		
	BV	**SE**	**R**	**BV**	**SE**	**r**	**BV**	**SE**	**r**	**BV**	**SE**	**R**	**BV**	**SE**	**r**	**BV**	**SE**	**r**
ADG_{4-8}	-2.850	3.58	0.16	3.444	4.460	0.61	-1.421	3.12	0.40	1.656	4.15	0.72	-4.542	3.77	0.32	2.404	4.30	0.63
ADG_{4-12}	-1.004	0.67	0.17	0.841	0.92	0.69	-0.702	0.66	0.41	0.612	0.85	0.74	-0.840	0.34	0.33	1.024	0.88	0.63
ADG_{8-10}	-3.735	2.73	0.14	4.577	3.49	0.63	-2.862	2.60	0.36	3.245	3.30	0.68	-2.886	0.303	0.28	3.273	3.82	0.59
ADG_{8-12}	-4.590	4.31	0.23	6.786	6.58	0.77	-10.25	3.88	0.51	5.032	5.81	0.82	-8.327	5.15	0.43	7.033	6.090	0.72

[a] Average daily gain between 4-8 weeks interval (ADG_{4-8}), 4-12 weeks (ADG_{4-12}), 8-10 weeks (ADG_{8-10}), and 8-12 weeks ($_{ADG8-12}$); [b] DBVs: Doe predicted breeding value, BBVs: buck predicted breeding value, PBVs: progeny predicted breeding value

To cite this paper: Rabie T, Nowier A, Abou-Zeid A and Khattab A (2020). Assessment of Genetic Capability for Post- Weaning Growth Traits of Reciprocal Cross between Gabali and V-Line Rabbits Using an Animal Model. *World Vet. J.*, 10 (3): 405-413. DOI: https://dx.doi.org/10.36380/scil.2020.wvj50

DECLARATIONS

Competing interests

There is no conflict of interest.

Authors' contributions

Rabie T., Khattab A., and Abou-Zeid A. designed research. Rabie T. wrote the paper. Khattab A. and Nowier A. analyzed the data. All authors read and approved the final manuscript.

Acknowledgments

The support of the Department of Animal Production and Fish Resources, Faculty of Agriculture, Suez Canal University, Ismailia, and the Department of Animal Production, Faculty of Agriculture, Tanta University are gratefully acknowledged.

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To cite this paper: Rabie T, Nowier A, Abou-Zeid A and Khattab A (2020). Assessment of Genetic Capability for Post- Weaning Growth Traits of Reciprocal Cross between Gabali and V-Line Rabbits Using an Animal Model. *World Vet. J.*, 10 (3): 405-413. DOI: https://dx.doi.org/10.36380/scil.2020.wvj50

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 414-420, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj51

Effects of *Tribulus terrestris* Fruits on Renal and Lung Tissues in Female Mice Administered with Cadmium

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ORIGINAL ARTICLE
pii: S232245682000051-10
Received: 08 Aug 2020
Accepted: 20 Sept 2020

ABSTRACT

The current study was designed to evaluate the protective effects of *Tribulus Terrestris* on kidney and lung tissues against cadmium toxicity in female mice. In this regard, 20 female albino mice were randomly assigned into three groups; the first group served as the control group, the second group was given the toxic substance (cadmium 6 mg/kg) only, and the third group was given cadmium (6 mg/kg) plus the alcoholic extract of the *Tribulus terrestris* fruit (200 mg/kg). The substances were administered orally by stomach tube daily for 10 days. On the last day of the study, the animals were euthanized, and their kidney and lung were sampled for histological study. The kidney tissue in mice exposed to cadmium showed cellular inflammation, necrosis, hyperplasia, and large urinary space in Bowman's capsule in comparison to the normal appearance of tissues in the mice in the other two groups. The large aggregations of lymphocytes around the bronchus and edema in the lungs exposed to cadmium were observed. The lungs of some mice exposed to cadmium and treated with *Tribulus terrestris* fruit indicated normal tissue appearance, while others showed large aggregations of lymphocytes between alveolar sacs and thick interalveolar septa. The *Tribulus terrestris* protected the kidneys against the toxicity of the cadmium while this plant had fewer protective effects against cadmium in the lung tissue. These results demonstrated that *Tribulus terrestris* ameliorated cadmium toxicity.

Keywords: Cadmium, Kidney, Lung, Mice, *Tribulus terrestris*

INTRODUCTION

Tribulus terrestris, a flowering plant in the family Zygophyllaceae, has many medicinal and therapeutic benefits, and it is used directly to treat many diseases in different countries (Zhu et al., 2017). Many diseases treated by this plant include heart diseases, urinary tract diseases, hepatic disorders, immune diseases, and sexual dysfunctions. Also, this plant is used as dietary supplements and tonic for muscles in athletes (Hashim et al., 2014). The fruit of *Tribulus terrestris* contains many compounds such as alkaloids, flavonoids, glycosides, saponins, and sapogenins, having important therapeutic roles including anti-inflammatory, antibacterial, antitumor, metal chelating, antidiabetic, and analgesic activities (Miraj, 2016; Semerdjieva and Zheljazkov, 2019).

A number of herbal drugs have potent nephroprotective effects due to their diuretic, antispasmodic and anti-inflammatory properties (Maharana and Dadhich, 2016). It has been shown that *Tribulus terrestris* is effective in lowering blood pressure and preventing kidney failure (Mohd et al., 2012). In addition, *Tribulus terrestris* exert hypolipidemic, cardiotonic, and hepatoprotective effects (Sivapalan, 2016). Also, *Tribulus terrestris* has a very important property that increases the effectiveness of antioxidant enzymes (Junior, et al., 2017; Dakshayini and Basha, 2018).

Cadmium, a heavy metal, is not a major component of the body and has no beneficial effects in the body. When entering an organ, it is very poisonous. Cadmium mainly affects kidneys and causes renal failure (Yang and Shu, 2015). Cadmium is known for its effects on proximal convoluted tubules in the kidneys, but the mechanism is uncertain. Some researchers believe that cadmium works to break down cells through programmed cell death (apoptosis) (Shagirtha and Miltonprabu, 2016). It has strong toxicity for kidneys as it breaks down DNA and increases oxidative stress (Karimi et al., 2012).

Cadmium dust inhalation affects the lung, leading to lung damage and lung cancer in smoky humans (Ganguly et al., 2018). Exposure to cadmium dust causes inflammation and changes the morphology of the lung cells, in addition, it has a negative effect on the immune system (Blum et al., 2014). The cadmium injection in the trachea of rats led to the accumulation of fluid in the alveolar spaces, then the death of type I alveolar cells, and finally lung fibrosis (Kwon et al., 2003). A study assessed the extent of DNA breakdown in animals exposed to cadmium and revealed that cadmium

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produces genotoxic effects such as DNA damage and chromosomal aberration (Skipper et al., 2016). A study showed that increasing the concentration of cadmium leads to DNA damage in the lung cells (Yang et al., 2012).

The present study aimed to evaluate the activity of *Tribulus terrestris* against the toxicity of cadmium. For this purpose, this study evaluated the histopathological changes of liver and kidney in mice exposed to cadmium and treated with the plant extract.

MATERIALS AND METHODS

Ethical approval

The research carried out in accordance with the ethic roles of Anbar University (Ethic committee Anbar University)

Animals and study design

Twenty two-month-old female albino mice weighing 25 to 30 g were obtained from the animal house of the Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Iraq. They were housed in plastic cages containing sawdust. Water and diet (pellet) were available free for the mice. They were kept for six weeks for adaptation. The animals were distributed randomly to three groups: six mice in the control group, seven mice in the cadmium-exposed group (6 mg/kg), and seven mice in the cadmium-exposed and *Tribulus terrestris* extract (TTE)-treated group (cadmium: 6 mg/kg and TTE: 200 mg/kg). Stomach tube was used to give cadmium and TTE to the mice daily for 10 days.

Alcoholic extract preparation

Fruits of *Tribulus terrestris* were collected, air dried, and then finely powdered using an electric grinder. Next, 100 g of powder was mixed with 1 L of 95% ethanol in round bottom flasks and refluxed for 6 hours. The liquid extract obtained was separated from the solid residue by vacuum filtration, concentrated using an incubator at 60 $^{\circ}$C for 3-5 days, and then stored at 4 $^{\circ}$C until use (Abdel-Kader et al., 2016).

Histopathological examination

Finally, all mice were euthanized, and their kidneys and lungs were isolated. The organs were fixed in 10% formaldehyde at least 24 hours, and paraffin-formatted tissue blocks were then prepared. Tissue sections were cut into 4 μm sections by microtome and stained with hematoxylin and eosin. All stained sections were examined microscopically to observe histological changes.

RESULTS

Kidney

Histopathological examination of kidney tissues of the mice exposed to cadmium illustrated some changes. It showed the presence of cellular inflammation and necrosis (Figure 1A and B). Also, hyperplasia in convoluted tubule (Figure 1C) and large urinary space in Bowman's capsule (figure 1D) were observed.

All control animals had a normal histological appearance as shown in Figure 2. Each mouse in the cadmium and TTE-treated group had a normal appearance in both cortex and medulla of their kidney (Figure 3).

Lung

The results showed that cadmium affected the mice lung tissue. A large pool of lymphocytes around the bronchus and edema was observed in mice exposed to cadmium Figure 4.

Figure 5 shows the lungs of control animals whose alveolar cells and alveolar sacs appeared in normal consistency. In the cadmium and TTE-treated group, some cases had normal tissues as shown in Figure 6, while large aggregations of lymphocytes among alveolar sacs in some cases (Figure 7) and thick interalveolar septa in others were observed (Figure 8).

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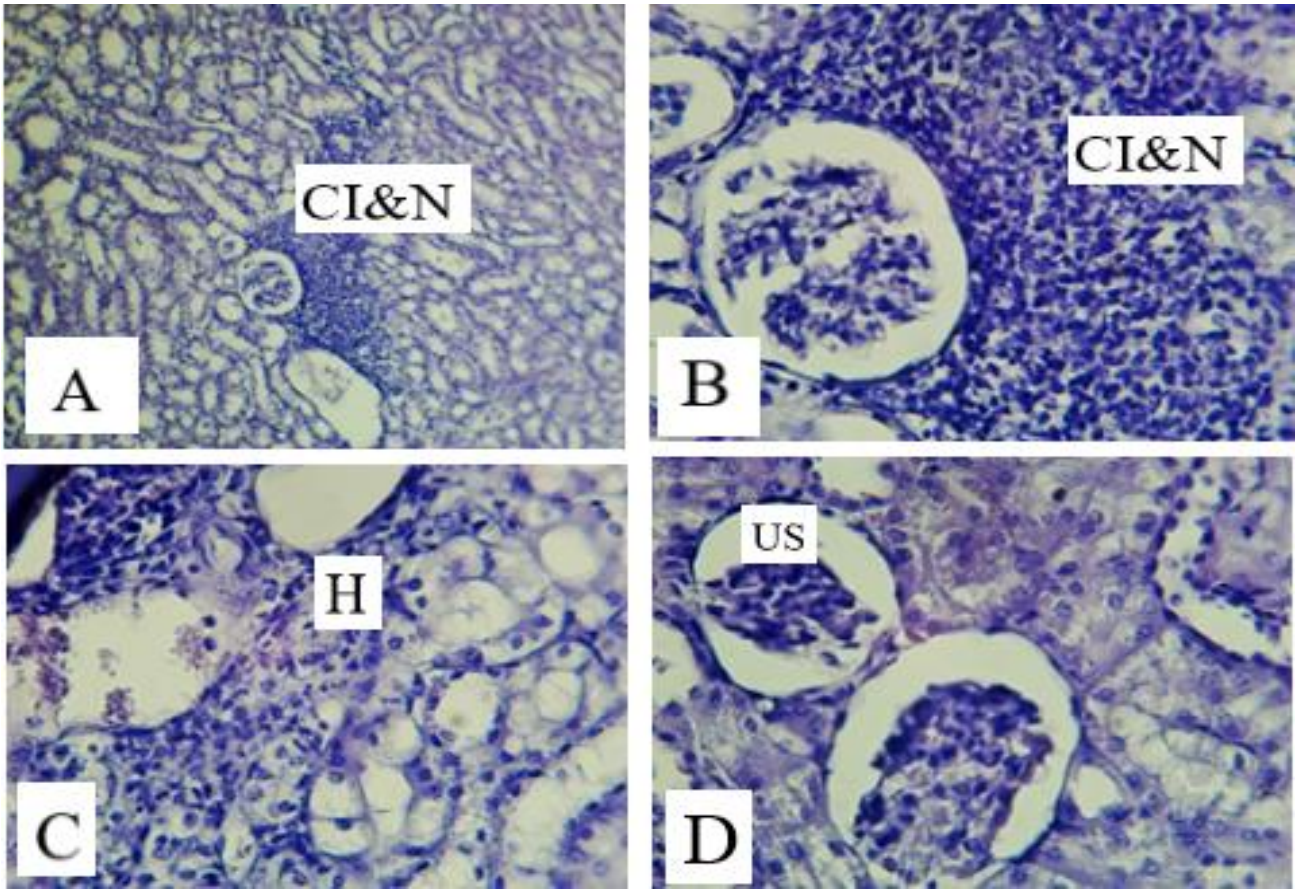

Figure 1. Cross-sections of kidneys of mice exposed to cadmium. CI & N: Cellular inflammation and necrosis; H: hyperplasia; US: urinary space in Bowman's capsule. A and C: 10X; B and D: 40X. (H&E).

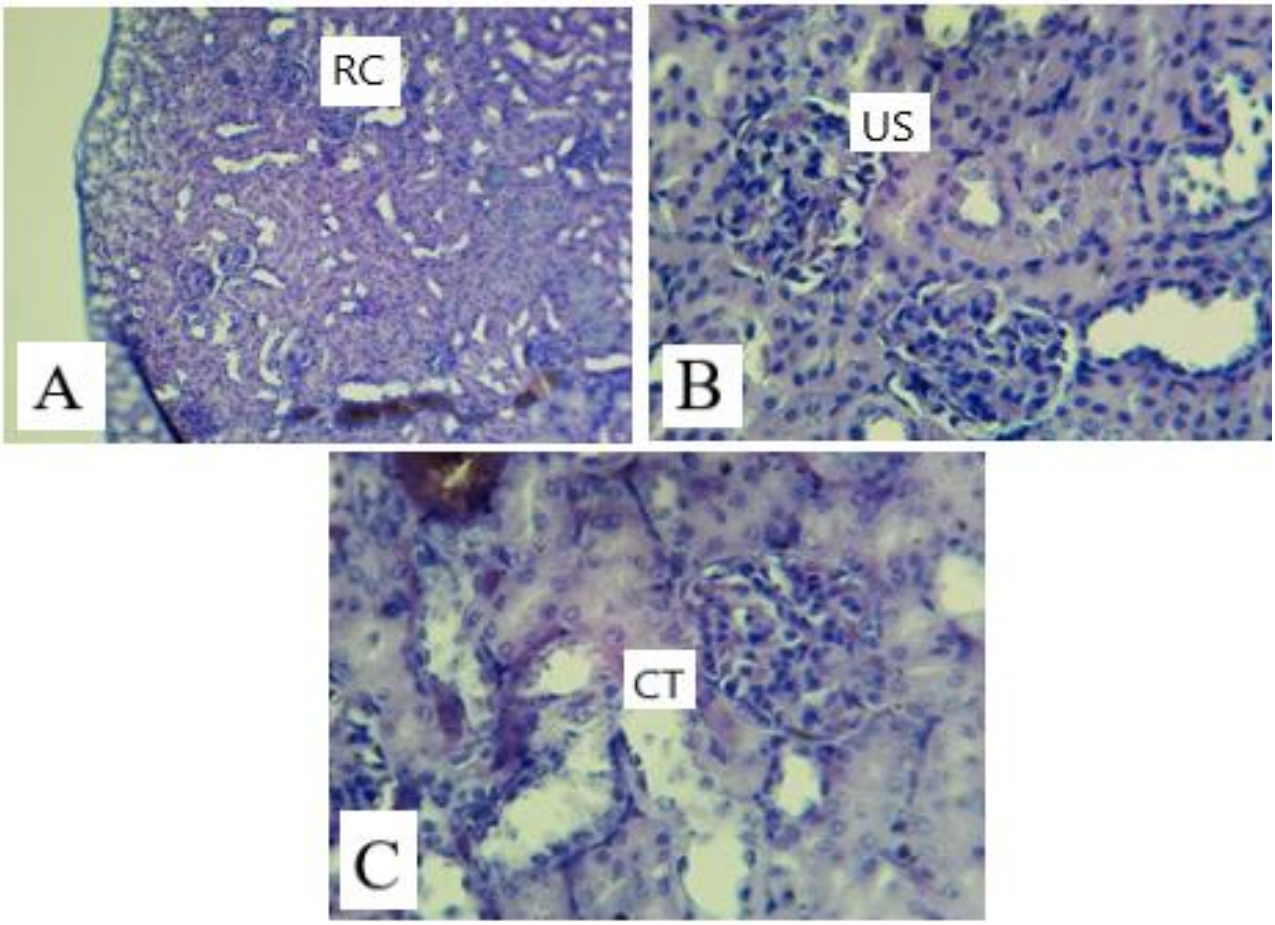

Figure 2. Cross-sections of kidneys in control mice. RC: Renal corpuscle; US: Urinary space; CT: Convoluted tubules; A: 10X, B and C: 40X, (H&E).

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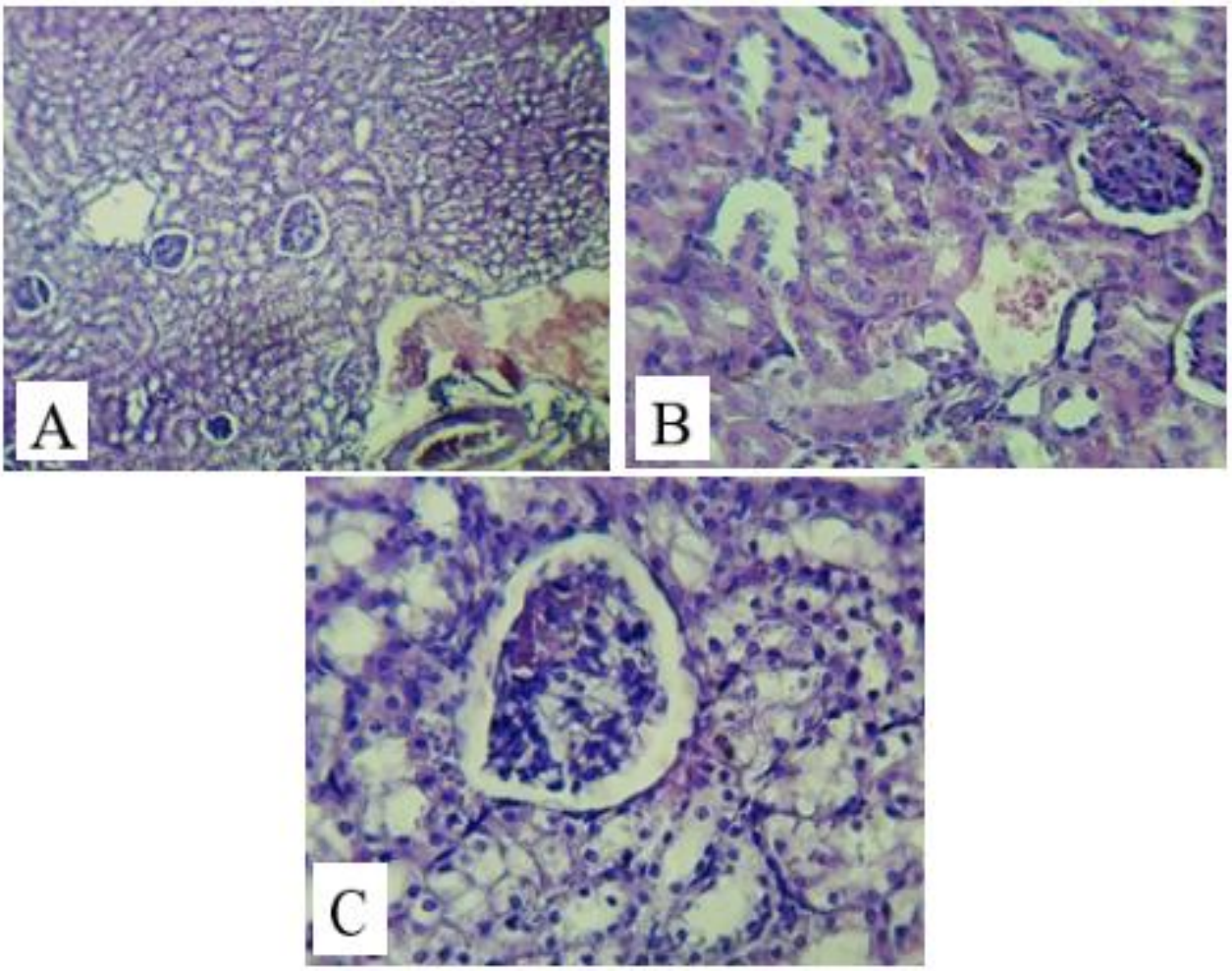

Figure 3. Cross-sections of kidneys of the mice exposed to cadmium and treated with extract of *Tribulus terrestris* fruit. A: 10X; B and C: 40X (H&E).

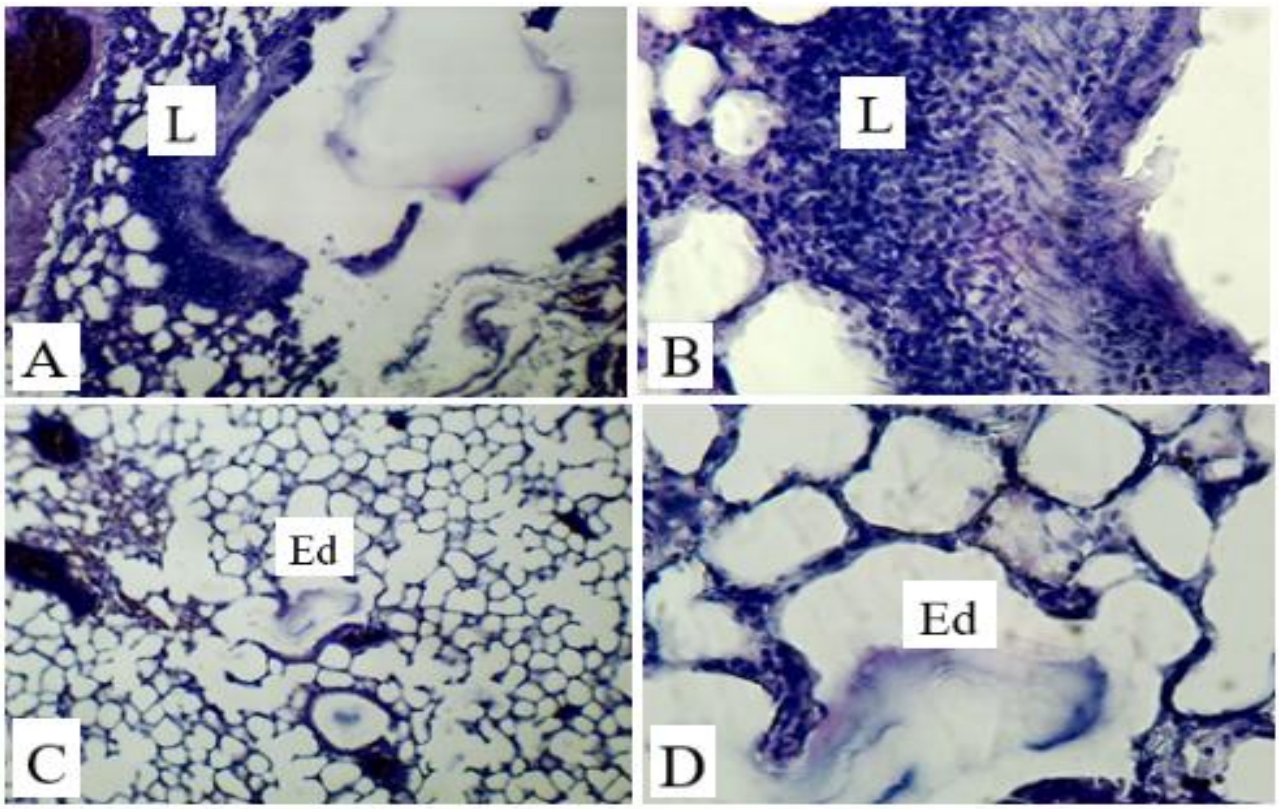

Figure 4. Lung tissues of the mice exposed to cadmium. L: Lymphocytes aggregation; Ed: edema; A and C: 10X; B and D: 40X (H&E).

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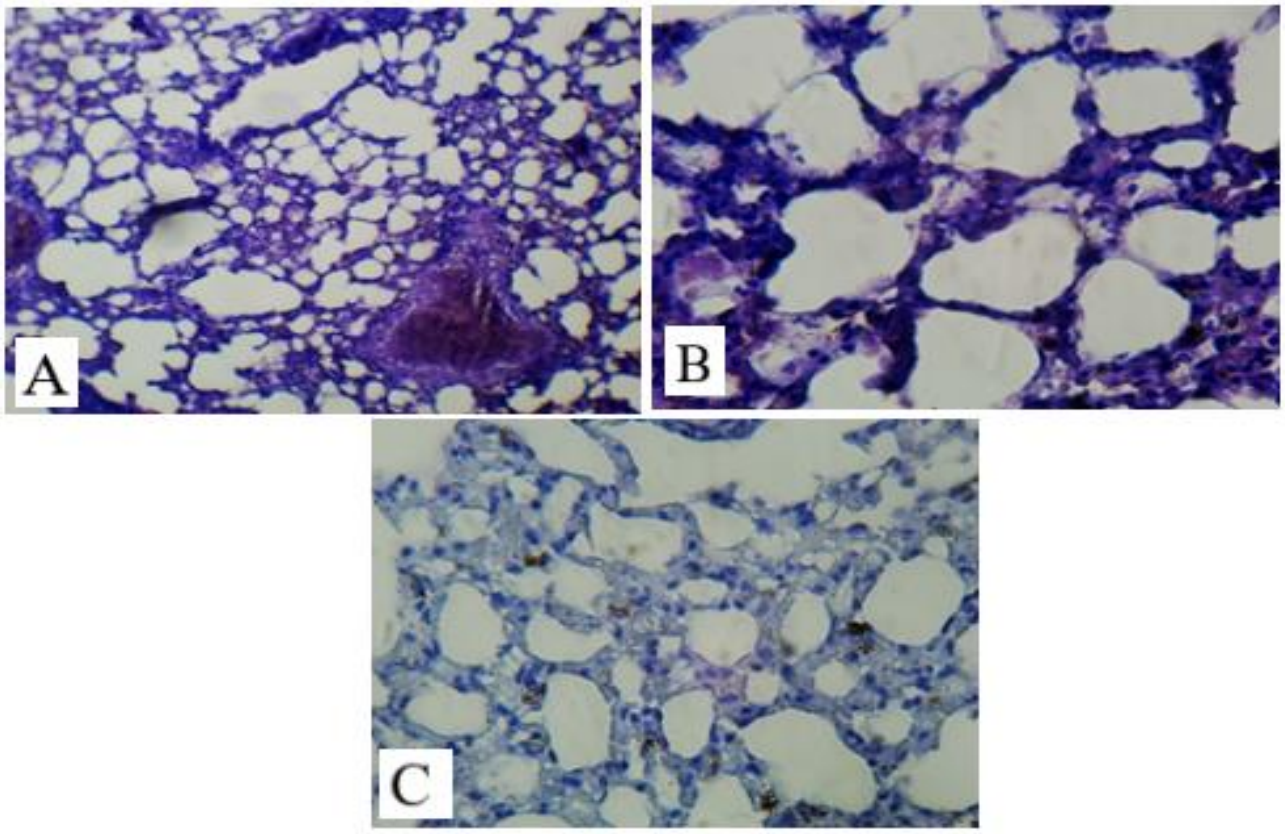

Figure 5. Histopathological examination of lung tissues of the control group. A: 10X; B and C: 40X (H&E).

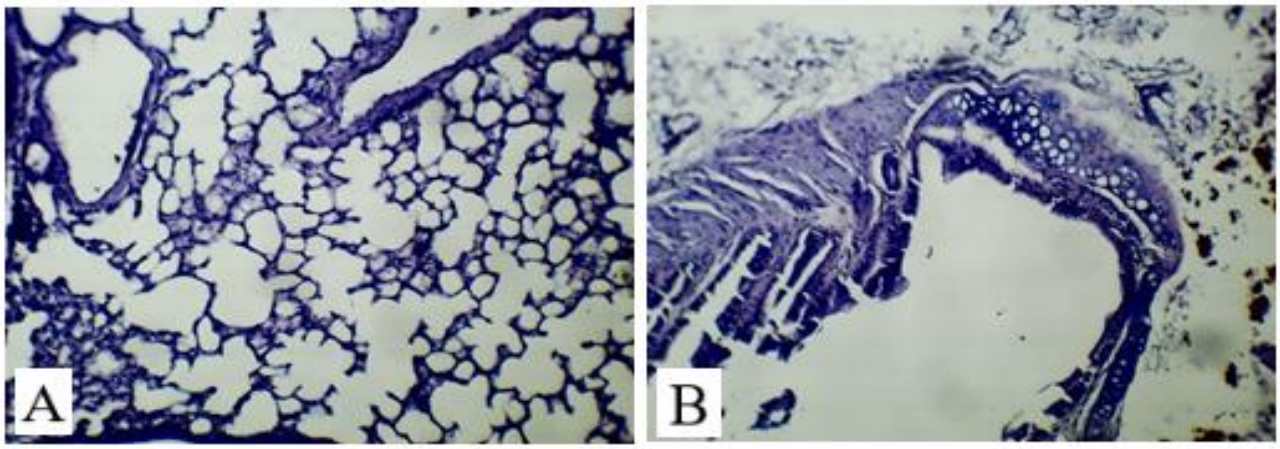

Figure 6. Lung tissues of the mice exposed to cadmium and treated with extract of *Tribulus terrestris* fruit. A: 10X; B: 40X (H&E).

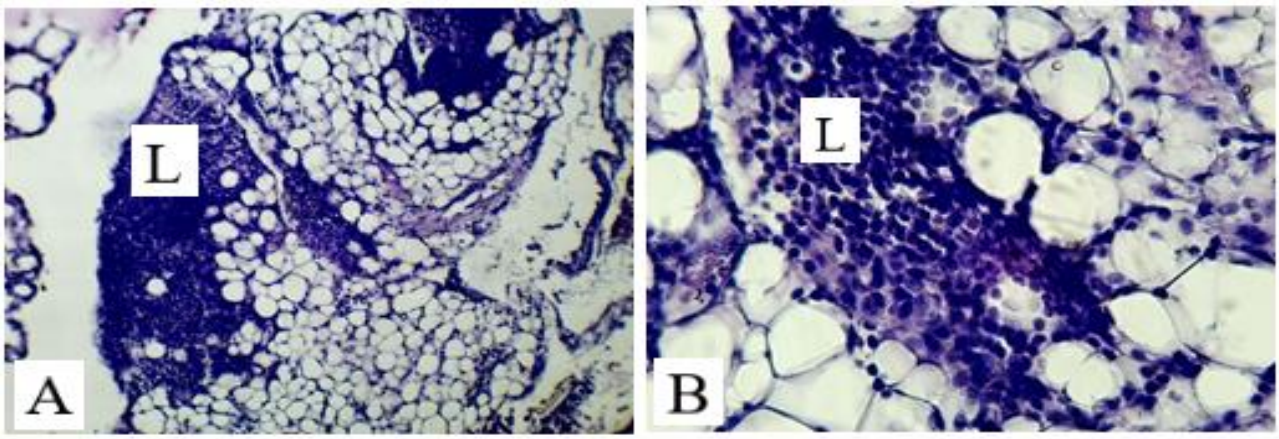

Figure 7. Histopathological examination of lung tissues of the mice exposed to cadmium and treated with extract of *Tribulus terrestris* fruit L: Lymphocytes aggregation; A: 10X; B: 40X (H&E).

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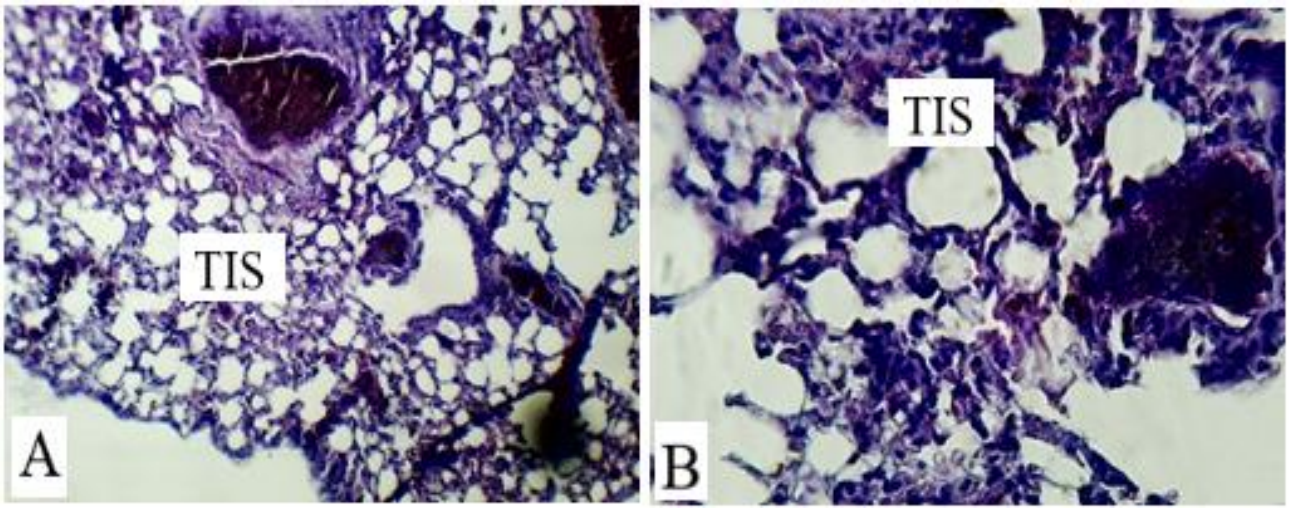

Figure 8. The lung tissues of mice exposed to cadmium and treated with extract of *Tribulus terrestris* fruit. TIS: thick interalveolar septa; A: 10X; B: 40X. (H & E).

DISCUSSION

Cadmium causes many histological changes in both kidney and lung (El-Refaiy and Eissa, 2013). The results of the present study suggested that exposure to cadmium causes toxic effects and histological changes in kidney and lung tissues. The kidney and lung are known as a prominent target of cadmium because of their capability for reducing and transforming toxic materials to less toxic or non-toxic substances (Tam, 2014). Kidney and lung damage could result from cadmium accumulation in the cells. Al-Qizy (2009) showed that renal corpuscle was affected by cadmium. The reason for the breakdown of the kidney cells may be due to the release of cadmium ion from the cadmium-metallothionein complex by lysosomes of convoluted tubular cells (Tohyama et al., 1996). Another study showed that cadmium affects glomerular and tubular structures, and ultrastructure of the kidney by generating free radicals that destroy cellular components (Micali et al., 2018). Cadmium uptake by alveolar macrophage causes immune dysfunction in the lung (Cox et al., 2016). Previous data revealed that edema, cellular infiltration, and immune disturbance occurred in lung affected by cadmium administration (Jelena et al., 2019). Cadmium is a highly oxidant metal and the plants containing antioxidant compounds such as flavonoids, alkaloids, and phenolic compounds have the potential to be used against cadmium toxicity (Kumar et al., 2019).

Tribulus terrestris has numerous antioxidant compounds such as polyphenols and flavonoids (Zheleva-Dimitrova et al., 2012). It has several important therapeutic properties (Sultan and Rubab, 2017). Several studies have shown that this plant had direct effects on the urinary tract as a diuretic and a uricosuric (Akram et al., 2011). The herbal extract of *Tribulus terrestris* improves kidney function and reduces cellular oxidative stress (Najafi et al., 2014). The results of the current work indicated that this plant can reduce the toxic effects of the heavy metal, cadmium, on the kidney and lungs. Similarly, Abdel-Kader et al. (2016) reported that *Tribulus terrestris* has a positive effect on the renal tissue and function. Another study found the capability of this plant to protect kidneys against heavy metals that cause kidney damages (Manikandaselvi et al., 2012). The protective effect of *Tribulus terrestris* could be contributed to its ability to eliminate free radicals and induce the antioxidant enzymes expression as well as the down-regulation of pro-inflammatory markers in cellular injuries (Ali et al., 2018).

DECLARATIONS

Competing interests

The author declares that no competing interests exist**.**

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To cite this paper: Farhan AS (2020). Effects of *Tribulus terrestris* Fruits on Renal and Lung Tissues in Female Mice Administered with Cadmium. *World Vet. J.*, 10 (3): 414-420. DOI: https://dx.doi.org/10.36380/scil.2020.wvj51

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 421-428, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj52

The Use of *Giardia* immunogenic Protein Fraction to Distinguish Assemblages in Humans and Animals

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ORIGINAL ARTICLE
pii: S232245682000052-10
Received: 31 Jul 2020
Accepted: 09 Sept 2020

ABSTRACT

Buffalo calves have a high adverse effect on public health, specifically zoonotic Giardiasis in Egypt. The current study was carried out to investigate the use of enzyme-linked immunoelectrotransfer blot technique (EITB) as a preliminary step for the diagnosis of giardiasis before genotyping. For this purpose, fecal and blood samples were collected from diarrheic calves and workers in closed and non-closed beef buffalo farm. Zoonotic *Giardia* assemblage A was isolated from both diarrheic children and their close contact calves while the calves were infected by animal genotype assemblage E under low contact conditions. This was identified after the amplification and sequencing of a 292 bp fragment of 16S-rRNA ribosomal unit from 20 children and 28 calves *Giardia* isolates using nested PCR. Fractionation of different isolated *Giardia* assemblage and identification of specific fraction versus anti-*Giardia*-IgG antibodies in infected humans or animals using EITB revealed that α-1 giardin antigen with molecular weight ranged 29-34KDa. This specific immunogenic assemblage was fraction-related where it reacted specifically versus antibodies in sera of humans or animals infected by this assemblage and did not cross-react with giardin of other assemblage or with sera of non-infected cases. The results of the current study highlighted the use of EITB as a preliminary investigation before genotyping in the identification of zoonotic giardiasis.

Keywords: Buffalo calves, Genotype, *Giardia*, Human, Immunoblot, Nested PCR

INTRODUCTION

Giardia duodenalis (Syn. *G. intestinalis*, *G. labmlia*) is a worldwide distributed zoonotic protozoan parasite infecting humans as well as domestic and wild animals with high levels of genetic diversity. The parasite is re-emerging as one of the most frequent causes of diarrhea in humans which infects about 200 million people worldwide causing a serious public health concern as each individual eliminates up to 10^8 to 10^{10} cysts per day. The parasite is extensively distributed in children, especially those living in rural communities in developing countries. The high prevalence of this disease among humans in some localities is associated with its high prevalence in some of the surrounding animals (Coffey et al., 2020).

Giardia duodenalis is considered to be a complex species and its members are being morphologically indistinguishable using traditional diagnostic techniques. Genotypic analysis based on Polymerase chain reaction technology has made an enormous contribution to understanding the genetic structure of different *Giardia* species, molecular epidemiology of infection, and zoonotic transmission (Mahmoudi et al., 2020). Based on molecular genetic analyses, *G. duodenalis* are grouped into eight recognized genotypic clusters or assemblages (A-H). Assemblages A and B are zoonotic occurring in humans and many other hosts, assemblages C and D have been diagnosed only in dogs, assemblage E has been reported only in hoofed animals, assemblages F and G have been observed only in cats and rodents respectively, and assemblage H in pinnipeds. The factors affecting host specificity are partially understood where it involves both the host and the parasite (Cacciò et al., 2018). Human isolated assemblages (A and B) have been further sub-grouped using conserved loci into AI, AII, BIII, and BIV (Ramírez et al. 2015). Assemblage AI is the most frequent sub-assemblage that is highly associated with animals while that of AII is limited to humans (Puebla et al., 2017).

Other than the traditional parasitological ways used for diagnosis of different stages of *Giardia* in feces, several enzyme immunoassays are also applicable to use. However, most of these methods are qualitative and unable to distinguish different *Giardia* genotypes (Hooshyar et al., 2019). Characterization of specific protein fraction from the parasite antigens by enzyme-linked immunoelectrotransfer blot technique (EITB) may benefit from an accurate specific way for diagnosis. Several immunogenic protein fractions of *Giardia* have been described, such as cyst wall proteins (CWPs), variable surface proteins (VSPs), giardins, arginine deaminase, and tubulins. The CWPs and proteins α-1 giardin provide marked protection against *G. lamblia* infection (Quintero et al., 2013). Special protein fractions were identified in *Giardia* cysts as specific diagnostic antigens for the detection of the A.G. Abs in sera of infected cases, such as the fractions corresponding to molecular weight (MW) of 26, 28, 38, 42, 46 kDa (Campbell and Faubert, 1994), that

To cite this paper: Ramadan RM, Khalifa MM, Kamel NO, Abdel-Wahab AM and El-Bahy MM (2020). The Use of *Giardia* immunogenic Protein Fraction to Distinguish Assemblages in Humans and Animals. *World Vet. J.*, 10 (3): 421-428. DOI: https://dx.doi.org/10.36380/scil.2020.wvj52

of 29, 75, 88, 102 kDa (Erlandsen et al., 1990), and that of MW of 38, 36, 34, 33, and 29 kDa (Trout et al., 2004). In a study conducted by Quintero et al. (2013), α-1 giardin was identified as a group of protein fractions at MW of 29-33 kDa for *G. lamblia* of humans and these specific fractions could be used for the diagnosis of anti-*Giardia*-IgG antibodies (A.G. Abs) in sera of infected cases.

The present study was conducted due to the presence of an emerging diarrhea problem in a closed buffalo-calves farm. Different stages of *Giardia* were diagnosed in diarrheic fecal samples collected from these calves, the children of the farmworkers, and some villagers with a history of diarrhea living outside this farm. Most people in this village were farmers and lived in intimate contact with these animals.

The current study was intended to investigate the distribution and persistence of giardiasis in human-animal contact in a semi-closed community. Moreover, the study was targeted toward the investigation of the genotyping identification of the obtained *Giardia* isolates. Finally, it aimed to investigate the presence of specific immunogenic antigenic fractions related to the isolated *Giardia* assemblage which can facilitate assemblage identification using EITB as a preliminary method of identification before genotyping.

MATERIALS AND METHODS

Ethical approval

The employed protocol was approved by the Faculty of Medicine Cairo University Ethics Committee, Giza, Egypt, after the agreement of local health authorities for all participants. Procedures used for handling the animals, collection of samples, and the use of the patients' samples during this study were assessed and approved by the institutional review board of the Institutional Animal Care and Use Ethical Committee (Vet. CU20022020132) of Cairo University, Egypt.

Sample collection and examination

Identified fecal and blood samples were collected from diarrheic calves and workers in two levels of human-animal contact. The primary one consisted of 40 calves (one week to 6 months) and 20 children (one month-5 years old) live in intimate contact with closed beef buffalo farm (defined as closed farm samples). The second group composed of 78 calves and 40 children present in 4 non-closed breeding pins; each contained an occasional number of animals moved freely for grazing along with the village with their owners (this group defined as open farm samples). The study was performed from October to December 2019 in Abo-Rawash village, south of Giza Governorate, Egypt. Rectal or fresh deposited fecal samples were collected in identified plastic containers, preserved in ice, and transferred to the Department of Parasitology, Faculty of Veterinary Medicine, Cairo University for examination. *Giardia* cysts or trophozoites were detected using the modified Telemann method according to Fugassa (2014). Serum samples were separated from the collected blood after clotting by centrifugation and stored at -20 °C until use.

Cyst purification and antigen extraction

According to Ehsan et al. (2015), a sufficient amount of each fecal sample was filtered through two layers of surgical gauze and adjusted to yield a volume of 7 ml of filtrate. In the next step, the filtrate was layered over 5 ml of 1M sucrose (1.13 specific gravity) and centrifuged at 800xg for 5 min for the cyst concentration at the sucrose/water interface. The interface layer and the upper layer of the liquid were transferred by a suitable pipette to another clean tube, then re-centrifuged again as mentioned earlier. The supernatant was decanted, the sedimented pellet was re-suspended in 1 ml PBS and stored at 4 °C as concentrated *Giardia* cysts.

For the preparation of the required cyst antigens, the collected purified cysts were disrupted according to the method proposed by David et al. (2011). The cysts were subjected to 6 cycles of freezing and thawing (from -70 °C to +70 °C, 30 minutes in each cycle), then they were selected for use as either DNA isolation or antigen fractionation. For the former, the mixture was incubated with proteinase K (1mg/ml) and lysis buffer (NaCl 0.15M, EDTA 0.1 M, SDS 0.5%, pH 7.8, at 37 °C for 24 hours) where this suspension was stored at -20 °C until use. For the latter, *Giardia* cyst antigen was extracted according to Quintero et al. (2013), the disrupted cyst mixture was centrifuged (15000 rpm for 1 hour at 4 °C). Afterward, the supernatant was aspirated and after the estimation of its protein content, it was used as crude *Giardia* cyst soluble antigen and stored at -70 °C until use.

Fractionation of *Giardia* cyst antigen

Giardia cyst antigen representing human and animal's localities and age groups were fractionated using SDS-PAGE (Laemmli, 1970). Using PROTEAN II Xi cell and Bio-Rad, the antigens were resolved in 12 % polyacrylamide gel slabs under reducing conditions. Prestained MW standard was employed (Bio-Rad Cat. #.161-0318). The comb was adjusted as one small well for the MW standard and a large one for the samples.

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Determination of specific protein fractions

The fractionated proteins were transferred into (0.2 µm pore size) nitrocellulose sheet and determined using EITB according to a study conducted by Towbin et al. (1979). Longitudinal NC strips (0.3 cm wide) containing the fractionated antigens were cut out, treated with known infected human and animal serum (1:100) of known cyst/gram feces in comparison with negative control sera. Anti-human and anti-bovine IgG peroxidase were conjugated (Sigma Immunochemicals, 1:2000 dilution). The color was developed in a 4-chloro-1-naphthol substrate. Specific protein fractions reacted positively versus its *Giardia* infected sera and at the same time did not react versus negative serum samples.

Purification of *Giardia* cyst and DNA extraction

An amount of 100 µl of cyst suspension from *Giardia* positive calves' samples was selected for DNA extraction using a genomic DNA purification kit (Gentra system Instruction, Minnesota USA) according to the instructions manual designed by Hawash (2014). Samples were destructed by exposure to six freezing and thawing cycles in liquid nitrogen. After this, 100 µl of tissue lysogenic buffer was added, samples were incubated at 80 °C for 5 minutes, then centrifuged at 10000xg for 15 seconds. The supernatant was transferred to another clean tube then a 15 µl RNase solution was added to the mixture and it was mixed for 25 times then incubated at 37 °C for 15-60 minutes. After cooling at room temperature, one ml protein precipitation solution was added to the cell lysate, vortices for 20 seconds then centrifuged at 2000x g for 10 min. The supernatant containing the DNA was transferred to another tube containing 3 ml of absolute Isopropanol, mixed well, and centrifuged at 2000x g for 3 minutes. After the removal of the supernatant, 200 µl DNA hydration solutions were added and left for 1 hour at 65 °C. The spectrophotometer quantization of the DNA was performed according to Saiki et al. (1988) and stored at -20 °C for PCR amplification. Representative samples from each *Giardia* positive calves were selected for DNA extraction and genotyping.

Amplification and sequencing

The PCR protocol described by Almeida et al. (2015) was used for the amplification of 16S-rRNA ribosomal unit fragment. Nested PCR protocol was applied by using initial primers Gia2029 (5′-AAGTGTGGTGCAGACGGACTC-3′) and Gia2150c (5′-CTGCTGCCGTCCTTGGATGT-3′) amplifying a 497 bp product and secondary primers RH11 (5′-CATCCGGTCGATCCTGCC-3′) and RH4 (5′-AGTCGAACCCTGATTCTCCGCCAGG-3′) generating a 292 bp fragment.

The PCR reactions consisted of 1–5 µl DNA, 2.0 mM MgCl2, 200 µM each dNTP, 5% DMSO, 12.5 pmol for each of the forward and reverse primer, 0.5 Units of Taq polymerase (Qiagen, Germany), 0.5 Units of TAQ Extender™ (Stratagene, USA) 1× PCR buffer (Qiagen). The polymerization program includes 35 PCR cycles (96 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s) with an initial hot start (96 °C for 4 min) and its final extension as (72 °C for 4 min) was carried out using Perkin-Elmer Gene/ Amp PCR System 2400 (Wouters & Wouters EMS Services, USA). The conditions of the secondary PCR cycle were identical to the primary one except it occurs with increasing the annealing temperature to 59 °C. The purification of the obtained PCR products occurred by Qiagen PCR Agarose Purification Kit (Qiagen, 28004) and sequenced in both directions using ABI Prism™ Rhodamine Terminator Cycle Sequencing Kit (Applied BioSystems, USA) guided by the manufacturer's instructions, with the reduction of the annealing temperature to 59 °C, halved the reaction volumes to 10 µl, and increasing the cycles to 35. The sequences of the samples were analyzed using SeqEdv1.0.3. (Applied BioSystems, USA). Finally, the alignment of sequence information obtained from different calves' isolates was compared with those obtained from GenBank accession numbers AF113902 and AF199446u using Clustal W (Thompson et al., 1994).

RESULTS

Significance of calves' giardiasis on public health

Microscopic examination of the diarrheic fecal samples collected from 40 buffalo calves and 20 intimately contact children in the closed farm revealed different stages of infection induced by *Giardia* in all young calves (less than 2 months old). However, the infection was 40% in older calves (2-6-month-old) with the total percentage of infection reached 62.5%. The infection in the contact children inside this farm was estimated at 75% with a high prevalence in children aged 2-5 years. Investigation of diarrheic calves and their owners in four sporadic other pins revealed that 48.71% of the investigated calves were infected and the highest rate was related to young calves aged less than 2 months (53.33%). The infection in children of the owners of these animals was determined at 60% with a higher percentage in children of 2-6 months old (Table 1).

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Genotypes of diagnosed *Giardia* isolates from children & calves

To genotype the obtained *Giardia* isolates, a total number of 12 calves isolates and 8 human isolates from the 25 infected calves and the 15 human isolates were selected from the infected cases in the closed farm. Moreover, four calves' *Giardia* isolates and three human isolates from each of the other four investigated open pens in the infected groups were selected for genotyping. The sequencing of the samples was applied at the 16S-rRNA locus. All of the sequenced human isolates in the closed and open farm (8 and 12 samples) as well as the 12 isolates of calves derived from infected animals in the closed farm revealed the same genetic sequence with 100% identity as the ones recorded in the gene-bank for *Giardia* assemblage A genotype (AF199446, Figure 1). At the same time, the other calve isolates (i.e., 16 samples) isolated from infected calves in the open pens had sequences computable to previously described isolates for genotype (Assemblage) A sequence except that they had a variation in a single nucleotide, A-G transition at the site of nucleotide position 92. These changes were compatible with the proposed "Hoofed livestock" genotype (AF113902, E) that recorded in the gen-bank for cattle *Giardia* assemblage E. More characterization for the obtained *Giardia* assemblage A in other separate work proved that the isolated human assemblage A recorded in this study was identified as assemblage A1.

***Giardia* specific fraction from human and animal**

Treatment of fractionated *Giardia* cysts assemblage A isolated from infected children versus sera of all assembling A infected children (sample in Lane1), sera of calves live in the closed farm (sample in Lane 3) and sera of calves infected by livestock assemblage E (sample in Lane 2) revealed that the fractions of 55, 46, and 25 KDa reacted as specific fractions, compared to all of the above different infected sera. The fraction at MW of 38 KDa in this fractionated human isolate (assembling A) reacted specifically versus infected calves' sera and did not react versus infected human sera. Broad polypeptides band corresponding to MW ranged 29-34 KDa reacted specifically versus sera of children and calves infected by assemblage A only (Calves infected in the closed farm). Meanwhile, it did not react versus calves' sera collected from the other open-farm and diagnosed as infected by assemblage E (Table 2 and plate 1).

Treatment of fractionated calves' *Giardia* isolates (assemblage E) versus sera of infected calves from open pins (sample in Lane 6 infected by the same assemblage), or those from calves (Lane 7) or children (sample in Lane 8) in the closed farm (infected by assemblage A) revealed specific reaction versus polypeptides at MW of 55 KDa, 46 kDa, 25 KDa, and 18 KDa. Moreover, the fraction of 38 KDa was reacted in these calves isolate versus infected children serum. These calves strain reacted by broadband (45-50 KDa) in the region corresponding to 46 KDa versus infected children sera. The most interesting reaction was recorded versus the fraction of 29-34 KDa, this group of bands was reacted specifically in the calves with assemblage E versus calves with infected sera by this assemblage (Lane 6). At the same time, they did not react versus sera of children or calves infected by assemblage A. Moreover, there was a narrow band at MW of 29 KDa (Lane 7) versus calves from the closed pin. None of the above-mentioned bands reacted positively versus negative human or calves' sera in both types of antigens (Lane 4, 5, 9, and 10) (Plate 1). The fraction corresponding to MW standard at 100, 70, 15, and 12 KDa in fractionated isolates of children and those at MW of 100 KDa and 70 KDa in fractionated isolates of calves were identified as non-specific fractions as they reacted versus infected and non-infected serum samples (Plate 1).

Sample	Base pair region	Sequence	Base pair region
Assemblage A	1	CATCCGGTGGATCCTGCCGGAGCGCGACGCTCTCCCCAACGAC-GAAGCC	49
Livestock	1	CATCCGGTGGATCCTGCCGGAGCGCGACGCTCTCCCCAACGAC-GAAGCC	49
Assemblage A	50	ATGCATGGCCGCTCACCCGGGACGCCGCGACGGCTCAGGACAACCGTTG	99
Livestock	50	ATGCATGGCCGCTCACCCGGGACGCCGCGACGGCTCAGGACGACCGTTG	99
Assemblage A	100	CACCGCCCGCGGCGGTCCCTGCTAGCCGGACACGGCTGGCAACGCGGCGC	149
Livestock	100	CACCGCCCGCGGCGGTCCCTGCTAGCCGGACACGGCTGGCAACGCGGCGC	149
Assemblage A	150	CAAGACGTGCCCGCAAGGGCGGGCGCGCGGGCGAGCAGCGTGACGCAG	199
Livestock	150	CAAGACGTGCCCGCAAGGGCGGGCGCGCGGGCGAGCAGCGTGACGCAG	199
Assemblage A	200	CGACGGCGCGCCCGGGCTTCCGGCGCATCACCCGGGTCGGCGCCGTCGCGG	249
Livestock	200	CGACGGCGCGCCCGGGCTTCCGGCGCATCACCCGGGTCGGCGCCGTCGCGG	249
Assemblage A	250	CGCGCGGAGGGCGCGACGCCTGGCCGAGAATCAGGGTTCGACT	292
Livestock	250	CGCGCGGAGGGCGCGACGCCTGGCCGAGAATCAGGGTTCGACT	292

Figure 1. Genetic sequences of 16-rRNA gene of *Giardia* isolates obtained from infected beef buffaloes' calves in Egypt.

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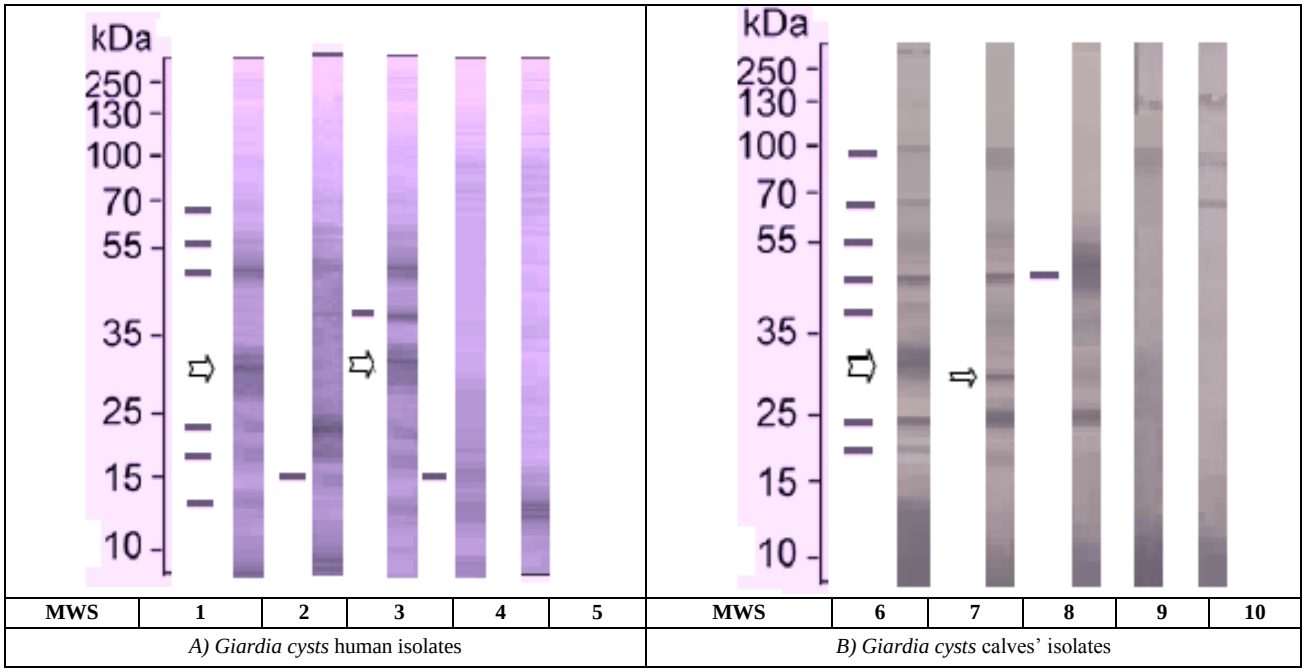

Figure 2. Reaction of different *Giardia* isolates fraction versus infected human and animal sera. **Group (A):** NC strip contain fractionated Human isolates (assemblage A) reacted versus; Lane (1): infected child sera (reaction at specific giardin fraction arrow); Lane (2): infected calve sera (No reaction at. giardin fraction); Lane (3): infected calve sera (from out the farm) (reaction at giardin fraction arrow); Lane (4): negative non-infected child sera; Lane (5): negative non-infected calve sera. **Group (B)**: NC strip contain fractionated Calve isolates (assemblage E) reacted versus; Lane (6): Infected calve sera. Present in the closed farm. (Reaction at giardin fraction, arrow); Lane (7): Infected out farm calve sera (narrow reaction at giardin fraction, arrow); Lane (8): Infected human sera (no reaction at bovine giardin fraction); Lane (9): Non-infected calve sera; Lane (10): Non-infected human sera.

Table 1. Prevalence of *Giardia* cysts in feces of children and their calves in and out of the closed farm in Abo-Rawash village, Giza Governorate, Egypt

Inspected localities		Examined diarrheic calves				Examined diarrheic children			
		Age groups	No. exam	+Ve No.	%	Age groups	No. exam.	+Ve No.	%
Closed farm		< 2 months	15	15	100%	< 2 year	6	4	66.66
		2–6 months	25	10	40%	2 -5 year	14	11	78.57
		Total	40	25	62.5%	Total	20	15	75%
Cases examined outside the closed farm	1	< 2 months	6	3	50%	< 2 year	4	2	50%
		2–6 months	11	5	45.45	2 -5 year	6	4	66.66
	2	< 2 months	8	4	50%	< 2 year	5	2	40%
		2–6 months	12	5	41.66	2 -5 year	5	4	80%
	3	< 2 months	5	3	60%	< 2 year	3	2	66.66
		2–6 months	10	4	40%	2 -5 year	7	5	71.43
	4	< 2 months	11	6	54.54	< 2 year	4	2	50%
		2–6 months	15	8	53.33	2 -5 year	6	3	50%
Total		< 2 months	30	16	53.33	< 2 year	16	8	50%
		2–6 months	48	22	45.83	2 -5 year	24	16	66.66
		Total	78	38	48.71	-	40	24	60.0%

*No. exam=Number examined, (+Ve No.) = Postive Number

Table 2. Specific polypeptides bands in fractionated isolates of *Giardia* cysts from infected children and calves reacted versus different *Giardia* infected sera.

Number of the band in the plate	Specific polypeptides in fractionated *Giardia* cysts isolates (KDa)				
	Human assemblage A versus			Livestock assemblage E versus	
	Assembling A infected		Assemblage E infected Calves	Assemblage E infected Calves	Assembling A infected
	Children	Calves			Calves	Children
3	55	55	55	55	55	55
4	46	46	46	46	46	45-50
5		38	38	38	38	38
6	►29-34	►29-34	--	►29-34	►29	--
7	25	25	25	25	25	25
8				18	18	18

* Other fractions at MW of 100, 70, 15 & 12 KDa in fractionated children isolates and those at MW of 100 KDa & 70 KDa in fractionated calves' isolates were reacted also as non-specific fractions as they react versus infected and non-infected serum samples of children and calves (Plate 1)

To cite this paper: Ramadan RM, Khalifa MM, Kamel NO, Abdel-Wahab AM and El-Bahy MM (2020). The Use of *Giardia* immunogenic Protein Fraction to Distinguish Assemblages in Humans and Animals. *World Vet. J.*, 10 (3): 421-428. DOI: https://dx.doi.org/10.36380/scil.2020.wvj52

DISCUSSION

Giardia infection is still considered as a re-emerging zoonotic disease in the rural community in developing countries due to contamination of public water with raw sewage from animal and human sources, lack of water treatment, and use of manure fertilizer in agriculture (Squire and Ryan, 2017). The increase of contact between humans and animals in the rural community, as the case in the present study, could correspond to the higher prevalence of infection by zoonotic *Giardia* assemblages from humans to animals or vice versa. The recorded percentage of infection in the investigated closed farm where the owners and their children were in continuous daily contact with buffaloes and their calves in the small localized area was higher than that obtained results of a study reported by Ismail et al. (2018) as 10-34.6% infection among children living in Egypt. This percentage of infection in the investigated localities could be attributed to the nature of the study as it focused on a small community and all of them were symptomatically suffering from diarrhea. The higher level of infection in younger than older groups in the current study was in line with the findings of a study performed by Júlio et al. (2012). However, this finding yield had no support to findings of Samie et al. (2020) that indicated no significant correlation between age and gender concerning the distribution of *Giardia* infection. The higher rate of infection in children of 2-5 years old compared to those of young age may be attributed to the active movement and increasing rate of contact of children in this age group with animals inside the farm than the really young children.

As genotyping facilitates the identification of various strains of *Giardia*, the possible public health significance of calves' giardiasis was determined by characterization at a molecular level and genotyping to representative samples from *Giardia cysts* isolates collected from infected children and calves in the investigated localities. The choice of assay depended on the amount of information carried by the genetic locus under the analysis. The locus of 16SrRNA conceded as one of the highly conserved gene regions that could be used in the detection of *Giardia* (Almeida et al., 2015). A number of 28 isolates from infected calves and 20 from infected children representing about 50% of the total isolates were selected and genotyped using nested PCR. Amplification and sequencing of a 292 bp fragment of the 16s-rRNA ribosomal unit revealed infection of children in closed or open farms as well as calves in the closed farm by *Giardia* assemblage A genotype. The findings addressing these isolates were supported in other related studies as assemblage A1 while calves infected in the open farm harbors "Hoofed livestock" genotype (E). These data indicated predominantly distribution for assemblage A over the Hoofed livestock" genotype (E) in closed farms. In contrast, Gillhuber et al. (2013) mentioned that livestock-specific assemblage E of *G. duodenalis* appeared to be the most frequent genotype in calves.

Infection of calves and children in the closed farm by the same genotype (human assemblage A) and simultaneous infection of calves in the open farms by the Hoofed livestock" genotype (E) demonstrated that the workers inside this closed farm considered to be the source of infection to calves in this closed farm. This was supported by the appearance of infection in all suckling calves born inside this farm and did not move outside. In the same villages, the animals in open farms live in semi-free conditions as they freely moved daily from their pins to field during the day and returned to their pins at the end of the day. Those people with less contact level to such animals were infected by hoofed strain Assemblage E while their counterparts with semi-contact were infected by the zoonotic assemblage A. This finding agreed with the results of a study carried out by Ramírez et al. (2015) as in some localities, the high prevalence in humans was associated with high prevalence in some of the surrounding animals. Giardiasis is a self-limited disease and its chronic form usually causes severe digestive disturbances; however, the disease may be asymptomatic in some other cases (Liu, 2019). Infected children in the present study were suffering mainly from diarrhea without other complaints. This mild symptom in association with the diagnosed *Giardia* assemblage isolated from these children was in line with the reports provided by Puebla et al. (2017). They mentioned that there was a relation between the *Giardia* assemblage and the recorded clinical signs diagnosed in infected individuals. Symptoms of the infection in children harboring assemblage B were more prominent than those in children infected with assemblage A. Moreover, assemblage AI appeared as the most frequent sub-assemblage which was highly associated with animals than humans (Puebla et al., 2017; Sánchez et al., 2017).

Infection of calves by genotype assemblage A isolates has a significant public health risk as generally *Giardia* cysts are stable, it can survive for weeks or months in the environment. Moreover, even a single cyst can induce infection and the infected individual can shed up to 900 million cysts per day in feces then end up in the environment and can spread to human or animal foods by irrigation or by direct contact (Ryan and Cacciò, 2013). In this regard, the current study demonstrated that animal as a reservoir can be easily infected and then spread the infection after propagation to their surrounding humans, especially in case they were infected by a zoonotic assemblage of the parasite

Another objective of the current study was to investigate the antigenic composition of each assemblage concerning the presence of specific immunogenic fractions related to each assemblage that may be responsible to the development of specific anti-assemblage Ab in sera of infected human or animals using EITB. The specific fractions in human assemblage A versus infected sera ranged 25-55 KDa. Similarly, the fractions in livestock assemblage E of calves were within the range of 18-55 KDa, and most of these fractions were identified as specific by several authors, such as

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Franzen et al. (2009), Sabry et al. (2009), and Feliziani et al. (2011) using EITB. The fraction of 38 KDa appeared to be more related to calves A.G.Abs and calves *Giardia* Ag. This fraction indicated special relation to the assemblage, but it may be identified as host-related rather than parasite-related. This was in line with a study performed by Ramírez et al. (2015) as the factors determining host specificity of different *Giardia* assemblage entailed both the host and the parasite.

It was important to demonstrate that the protein fractions corresponding to MW of 29-34 KDa in the fractionated *Giardia* assemblage A reacted specifically versus sera of children and calves of the closed farm. Since this group of calves were infected by assemblage A and at the same time, this group of fractions in this assemblage did not react versus sera of calves infected by calves' assemblage E. On the other hand, the fraction group of the same MW (29-34 KDa) in fractionated hoofed strain assemblage E of calve isolates was reacted specifically versus sera of calves infected by this assemblage and did not react versus children or calves' sera infected by *Giardia* assemblage A. The recorded specificity of this protein group toward the produced specific A.G.Abs in infected sera revealed that it had a specific immunogenic nature significantly related to its original genotype assemblage. This specific group of bands was previously identified by Quintero et al. (2013) as α-1 giardin and demonstrated that these fractions were considered to be specific for the diagnosis of A.G.Abs in sera of infected cases. As mentioned by the researchers and supported in the present study, the immunogenic giardins could be considered as a specific group of protein fractions associated with special assemblage and responsible for the production of specifically related Abs in its host species did not cross-react with Abs produced by giardins of other assemblages. These giardins were a parasite, not host related as it reacts versus their Abs in children or calves, which was confirmed by Ramírez et al. (2015).

The presence of a narrow band at MW of 29 KDa in the tested serum sample (Lane 7 in the plate) of one calve from the closed pin may be related to the infection of this calves by zoonotic and non-zoonotic *Giardia* assemblage. From this view, the diagnosis of this group of fractions using EITB can be used for the preliminary determination *of Giardia* assemblage before the application of genotyping. This could facilitate the identification of the isolates and help to reduce the number of samples which can be amplified using PCR. In agreement with Sánchez et al., (2017) the determination of the type of the assemblage's anti-parasite antibodies in the infected hosts helps to understand the molecular epidemiology of *Giardia*. This will contribute to the determination of the disease features, including the clinical association, resistance to treatment, transmission dynamics of the assemblages, and elucidate the zoonotic potential of the disease**.**

CONCLUSION

In conclusion, buffalo calves had a high adverse effect on the public health significance of zoonotic *Giardia* infection in the rural community in Egypt. Immunoblot analyses identified the protein fraction of MW 29-34 KDa as an assemblage specific immunogenic determinant that can be used for assemblage identification using EITB alone or as a preliminary investigation before genotyping assay of the different *Giardia* isolates. This approach obtained an easy method to identify *Giardia* genotype by using EITB as a preliminary investigation before genotyping assay.

DECLARATION

Competing interests

The authors declare that they have no competing interests.

Author`s contribution

Mohamed M. El-Bahy was the leader of the research team, designed the study, supervised the work, contributed in revising the Manuscript. Reem, M. Ramadan; Marwa, M. Khalifa and Nancy, O. Kamel carried out sample collection and examination, cyst purification, antigen extraction, fractionation of *Giardia* cyst antigen, determination of specific protein fractions and DNA extraction. Azza, M. Abdel-Wahab carried out the PCR work and sequencing. All authors checked and approved the final version of the manuscript.

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To cite this paper: Ramadan RM, Khalifa MM, Kamel NO, Abdel-Wahab AM and El-Bahy MM (2020). The Use of *Giardia* immunogenic Protein Fraction to Distinguish Assemblages in Humans and Animals. *World Vet. J.*, 10 (3): 421-428. DOI: https://dx.doi.org/10.36380/scil.2020.wvj52

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 429-436, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj53

Spatial Monitoring of Pyrethroid Residues by RP-HPLC in Raw Bovine Milk in West Delta Region of Egypt

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ORIGINAL ARTICLE
pii: S232245682000053-10
Received: 01 Aug 2020
Accepted: 13 Sept 2020

ABSTRACT

The massive implementation of pesticides in agronomy, public health series, manufacturing, and production can increase pesticides in the environment leading to tremendous effects on human health. Hence, the current study aimed to evaluate the concentration and frequency of pyrethroid residues in raw bovine milk sourced from local producers at West Delta of Egypt carefully chosen from three cities on High-Performance Liquid Chromatography system. A total of 200 of raw bovine milk samples (Alexandria 75, El- Behera 75, and Matrouh 50) were analyzed. The achieved results indicated that Cypermethrin, Cyhalothrin, and Deltamethrin residues were respectively screened at frequencies of 18.66%, 17.33%, and 14.66% in Alexandria, 21.33%, 17.33%, and 16% in El- Behera, and 44%, 28%, and 24 % in Matrouh. Cypermethrin was detected in a mean concentration level of 6.63, 5.98, and 10.74 in Alexandria, El- Behera, and Matrouh, respectively. However, Cyhalothrin and Deltamethrin had lower mean values of 4.66 and 2.45 in Alexandria, 5.14 and 3.05 in El- Behera, and finally 2.84 and 2.69 in Matrouh. There was no α- Cypermethrin in all the investigated samples while Cypermethrin was present with the highest mean concentration and frequency levels in Matrouh, compared to other cities. Concerning the compatibility of examined samples with maximum residue levels (MRLs) set by the European Commission and Codex regulation, indicated only 2.66%, 1.33%, and 0.00 % for Cyhalothrin, and 1.33%, 4%, and 2% for Deltamethrin examined samples in Alexandria, El- Behera, and Matrouh cities exceeded MRLs, respectively. As for Cypermethrin, its concentrations did not exceed MRLs. These results indicated a significant amount of these chemical pollutants in raw bovine milk offered by local producers in the West Delta of Egypt, which can threaten human life. Accordingly, continual assessment of pyrethroid residues in milk is of utmost importance to guarantee Egyptian consumers' health.

Keywords: Bovine milk, Cyhalothrin, Cypermethrin, Deltamethrin, MRLs, Pesticides, Pyrethroids, RP-HPLC

INTRODUCTION

Milk is crucial for human nutrition because it contains a plethora of indispensable compounds especially fat and proteins. Recently, many authorized organizations around the world are effectively acting on how to protect the food supply chain from being contaminated by forbidden hazards, such as pesticide residues in order to save human life. Pyrethroids are known as artificial insect repellents which originated from natural biopesticides called pyrethrin complexes which were extracted from a flowering plant named *tanacetum cinerariifolium* (Gammon et al., 2019). The most prevalent pyrethroids are bifenthrin, deltamethrin, cyfluthrin, cypermethrin, esfenvalerate, cyhalothrin, and permethrin (Gong, 2013). The environmental applications of pyrethroid insecticides include spraying around homes to control flies and ants (Feo et al., 2010), agricultural practices in crop protection against pests (Panseri et al., 2013), medical practices in topical control of lice and scabies and vector-borne diseases such as malaria which transmitted by mosquitoes, and veterinary practices in sprays, pour-on preparations and animal dipping (Corcellas et al., 2014).

Accidently, pyrethroids can get entrance to milk through unrestrained and haphazard use of these repellents for dairy animals or their surroundings such as foodstuffs, crops, contaminated drinking water, contaminated soil, and direct application to the bovine's body for controlling vector-borne diseases and parasites. Generally, the fatty portion of milk acts as an ideal solvent for most pyrethroids due to their lipophilic nature (Ismail and Elkassas, 2016). Although pyrethroids have great effectiveness, safety, high eco-friendly properties, low toxicity, relative stability, lower environmental persistence and selective insecticidal activity when matched with the natural pyrethrins, their extensive implementation has resulted in environmental pollution of ecosystems. In this regard, pyrethroids can either be easily hydrolyzed in the environment for few weeks or continue for more than one year based on some factors such as pyrethroid type, unmannerly usage, dampness, light and oxidation. In general, they are non-persistent contaminants and less hazardous when compared with organochlorine and organophosphorus compounds (Yu et al., 2017). Pyrethroids have hydrophobic and lipophilic characteristics, therefore unremitting consumption of contaminated milk with pyrethroids for long time can cause bio-accumulation of these residues in fatty tissues, in addition to their spreading in other body tissues via blood stream. Although, in most circumstances, pyrethroids can be hydrolyzed to detoxified

To cite this paper: Amer AA and Abou-Alella SA (2020). Spatial Monitoring of Pyrethroid Residues by RP-HPLC in Raw Bovine Milk in West Delta Region of Egypt. *World Vet. J.*, 10 (3): 429-436. DOI: https://dx.doi.org/10.36380/scil.2020.wvj53

metabolites, which generally have low and or non-toxic effects on animals and human, concerns about their tumor causing and endocrine troublesome should be in consideration (Bordoni et al., 2019). Degradable metabolites of pyrethroids such as l-Cyhalothrin, α-Cypermethrin and 3-phenoxybenzoic acid can be detected in animal or human secretions including milk (Wu et al., 2013).

On the other hand, chronic toxicity, carcinogenicity, genetic aberrations, endocrine-unsettlement and improper hormonal functions can be developed in some human bodies (Kampire et al., 2011). Chronic toxicity associated with many symptom complaints like vertigo, headache, vomiting, eye irritation, exhaustion, muscular convulsions and nervous symptoms. Chronic exposure to Cypermethrin has been reported to cause immune system toxicity or dysfunctionality (Wu et al., 2011). Moreover, there are few symptoms may be developed due to acute toxicity like skin itching, gastric, respiratory, convulsions and neurological disorders. Lately, cypermethrin has been reported to cause chronic neurotoxicity and tumors in brain especially in children or reproductive toxicity even at very low doses (Hudson et al., 2014). Moreover, Cypermethrin, has been classified by US Environmental Protection Agency (EPA) as potential carcinogenic agent while, Cyhalothrin was included in the list of group D carcinogens (IARC, 2014).

Globally, licenses about safety guides, corrective usage and withdrawal times of many pesticides have been established such as maximum residue levels (MRLs), for pesticides in foods of animal origin as well as plant products, stated by the European Union (EU) 37/2010 (European Commission, 2010) and the Codex Alimentarius Commission. In this regard, regulation (EC) 37/2010 has established MRL's for Cypermethrin (20 ppb), Deltamethrin (20 ppb) and Cyhalothrin (50 ppb). Furthermore, the Codex Alimentareous recommendations about the upper legal limits of Deltamethrin and Cypermethrin isomers in milk were 100 and 30 ppb, respectively (CAC, 2018). Therefore, the core-tip of the present research based on detection of selected three pyrethroid pesticides (Cypermethrin and its isomer (α-Cypermethrin), Cyhalothrin and Deltamethrin) in raw bovine milk samples collected from local producers at West Delta region of Egypt using High Performance Liquid Chromatography (HPLC). Finally, the detected contamination levels of these pesticides residues were compared with maximum residue levels and in turn this study will be useful for overall community and farmers that use pesticides with caution.

MATERIALS AND METHODS

Materials

Milk collection

Two hundred samples of fresh raw bovine milk, 250 ml in polyethylene bag, were collected randomly from local producers from three cities (Alexandria 75, El- Behera 75 and Matrouh 50) in West Delta, Egypt. Samples were obtained as sold to the public and transferred as soon as possible in an icebox at 4 ± 1^{o}C to the laboratory with a minimum of delay.

Chemicals and reagents

Synthetic Pyrethroid Cyhalothrin 95% and Deltamethrin 98%, Cypermethrin and α- Cypermethrin 93% were purchased from Tedia Company (Fairfield, OH, USA).

Methods

Preparation of standard solutions for pyrethroid pesticides

A typical stock solution was made separately for each type of pyrethroid pesticide in a concentration of 600 ppm by liquefying 60 mg of each pyrethroid with acetonitrile as a solvent then adding to 100 ml of this solvent. From this stock standard solution, the pyrethroids working standard solutions were prepared by diluting separately 0.0625, 0.125, 0.250, 0.500 and 1.0 ml of the pyrethroids stock solution to 50 ml with acetonitrile to obtain solutions of 0.75, 1.5, 3.0, 6.0 and 12.0 ppm, respectively. Such working standard solutions were prepared for each pyrethroid pesticide to determine the linearity of standard curve. All used solvents were HPLC grade.

Determination of pyrethroid pesticides

Sample fortification technique. The quantity of each pyrethroid was analyzed using the method of recuperation by fortification of the samples. The fortification was applied by adding standard concentration solutions to obtain different concentrations of pyrethroids in the samples. These fortifications were done separately for each examined pyrethroids. Calibration curves were constructed with fortified samples.

Extraction and purification technique. Accurately, 10 ml of the sample were put in an Erlenmeyer flask and acidified with 1N HCl to approximately pH 4. Further, 50ml acetonitrile were added and the flask was closed and vigorously shaken for 30 minutes. The sample was filtered in a glass funnel by Wattman filter paper NO.42 and the filtrate was collected in a beaker. The residue in the filter paper was transferred to the same Erlenmeyer flask, 25ml acetonitrile was added, and the flask was sealed and mechanically shaken for 15 minutes. Thus, the sample was filtered again using the same procedure and the same filter paper. The filtered sample was also collected in the same flask with the first filtrate.

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Partitioning

The filtrate (acetonitrile phase) in the beaker was transferred to a separator funnel and 15ml n-hexan were added and shaken for 1 minute. This procedure was repeated twice and acetonitrile phases were collected in the beaker while the hexanic phase was discarded. However, 45 ml of the acetonitrile phase was added and shaken for approximately 1 minute. Finally, the acetonitrile layer was collected in a beaker containing the first acetonitrile phases and evaporated in an exhaustion system under stream of nitrogen as well as heated to dryness at 30^{o}C.

***Column Chromatography Cleanup*.** In this step, dry residue was liquefied in 10 ml n-hexan then elution was performed via a 4g silica gel column. The silica gel was previously treated by hot air at 130^{o}C for 5 hours and cooled then diluted with 5 ml deionized water. The silica gel was put in the chromatographic column and eluted with 1ml n-hexan: diethyl ether (9: 1). The dry residue was diluted with 10ml n-hexan and 7ml n-hexan: diethyl ether (9: 1). The eluate was dried at room temperature in an exhaustion system. The diethyl ether was purified before its use to eliminate the possible peroxides.

RP-HPLC analysis. Actually, the dry residue was re-suspended with 1ml high grade acetonitrile, homogenized in a shaker for few seconds. Accordingly, the homogenate was injected onto High Performance Liquid Chromatography (HPLC). The suitable conditions of HPLC were: HPLC apparatus (Agilent1100) equipped with diode array detector (DAD); Column: Zorbex SBC 18 (150mm x 4.6mm x 0.5um film thickness); Mobile phase: acetonitrile: distilled deionized water (80: 20); Flow rate: 1.0ml/ min.; Detector: 226nm ultraviolet. Firstly, the homogenate was filtered in the sample filtering system of HPLC. After filtration, the mobile phase used in HPLC was acetonitrile: distilled deionized water (80: 20) under isocratic conditions and a flow rate of 1.0 ml/min and the mobile phase was filtered in the solvent filtering system.

Quantitative analysis. A comparison was performed between the injections of pyrethroid residues in the examined samples and other injections obtained by the standard solutions. Quantitative determination of these residues was obtained by the measurement of the peak areas in the chromatogram. Recovery rate (%) of pyrethroids pesticides in the examined milk samples were Cyhalothrin 91.9% and Deltamethrin 97%, Cypermethrin 91 % and α- Cypermethrin 93%

Data analysis

The statistical data analysis of pyrethroid pesticides levels and frequencies in milk samples was exposed to techniques for analysis of variance (ANOVA), two-way test of variance in SPSS (Version 16). Subsequently, Kruskal-Wallis test was used for conducting a nonparametric comparison including all milk samples among cities while, the comparison based on frequencies (%) of detection which was evaluated by Fisher's Exact test.

RESULTS

The present results revealed the existence of selected pyrethroid residues (Cypermethrin, α- Cypermethrin, Cyhalothrin and Deltamethrin) in raw bovine milk samples collected from local producers in West Delta area of Egypt as follows: 75 milk samples collected from Alexandria city and 75 from El- Behera city and finally, 50 samples collected from Matrouh city. All milk samples were examined for detection the presence of selected pyrethroid residues. The data in Table 1 explained that all analyzed milk samples were polluted with selected pyrethroid residues at the maximum rates of detection except α-Cypermethrin which was failed to be detected in all examined samples. In contrast, all selected pyrethroid residues were unnoticeable at the minimum detection values in examined milk samples. The most repeatedly-noticed pyrethroid residues were of Cypermethrin at the mean concentration levels ± (standard deviations) of 6.63 ± (16.54) and 5.98 ± (15.05) ppb among all collected milk samples from Alexandria and El- Behera cities, respectively with the highest level reported in Matrouh- collected milk samples which was 10.74 ± (20.79) ppb of whole milk. By comparing the levels of contamination detection for all examined milk samples, there was a significant difference (P1 = 0.059) among the selected three cities. The percentages of contamination were detected in 18.66 and 21.33 % of the collected milk samples from Alexandria and El- Behera, correspondingly with the highest percent of contaminated samples reported in Matrouh which was 44 %.

Although all contaminated milk samples by Cypermethrin were not exceed the MRL (100 ppb) according to CAC (2018), the frequencies of contamination among contaminated samples were significantly varied (P2 = 0.032). In this regard, the mean contamination values ± (standard deviations) and median levels (Q1-Q3) for contaminated samples were 34.57± (21.79) and 29.15 (17.14 – 53.0) for Alexandria- contaminated milk samples while, the values among El-Behera- polluted milk samples were 26.91± (16.07) and 36.30 (20.52 – 51.0). Finally, for Matrouh- contaminated samples, the values were 36.63 ± (23.01) and 30.55 (15.25 – 52.02), respectively. The magnitude of Cyhalothrin detection levels showed clear differences among selected cities with the mean concentration levels ± (standard deviations) in the following order: 5.14 ± (12.62) > 4.66 ± (12.09) > 2.84 ± (6.42) for El- Behera > Alexandria > Matrouh- collected milk samples, respectively.

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Table 1. Levels and frequency of detection of selected Pyrethroids pesticides in raw bovine milk samples collected from local producers in West Delta, Egypt

Insecticide (ppb)	Governorate	All samples				Contaminated samples			
		N	Min - Max	Mean (SD)	Median (Q1 - Q3)	N (%)	N (%) > MRL	Mean (SD)	Median (Q1 - Q3)
Cypermethrin	Alexandria	75	nd – 75.61	6.63 (16.54)	0 (0 - 0)	14 (18.66)	0 (0.0)	34.57 (21.79)	29.15 (17.14 – 53.0)
	El- Behera	75	nd – 65.73	5.98 (15.05)	0 (0 - 32)	16 (21.33)	0 (0.0)	26.91 (16.07)	36.30 (20.52 – 51.0)
	Matrouh	50	nd – 84.11	10.74 (20.79)	0 (0 – 13.51)	22 (44)	0 (0.0)	36.63 (23.01)	30.55 (15.25 – 52.02)
	P1 = 0.059, P2 = 0.032								
Cyhalothrin	Alexandria	75	nd – 71.32	4.66 (12.09)	0 (0 - 0)	13 (17.33)	2 (2.66)	26.88 (15.97)	21.34 (15.76 – 36.07)
	El- Behera	75	nd – 70.59	5.14 (12.62)	0 (0 - 0)	13 (17.33)	1 (1.33)	27.18 (15.81)	20.62 (16.22 – 35.02)
	Matrouh	50	nd – 24.32	2.84 (6.42)	0 (0 - 0)	14 (28)	0 (0.0)	16.18 (4.13)	13.96 (13.25 – 18.07)
	P1 = 0.056, P2 = 0.783								
Deltamethrin	Alexandria	75	nd – 37.65	2.45 (6.68)	0 (0 - 0)	11 (14.66)	1 (1.33)	16.31 (8.53)	13.95 (11.14 – 17.53)
	El- Behera	75	nd – 39.26	3.05 (7.84)	0 (0 - 0)	12 (16)	3 (4.0)	18.83 (9.18)	14.80 (12.40 – 25.57)
	Matrouh	50	nd – 38.64	2.69 (6.08)	0 (0 - 0)	12 (24)	1 (2.0)	16.83 (7.22)	15.41 (13.16 – 17.64)
	P1 = 0.112, P2 = 0.502								
α-Cybermethrin was failed to be detected in all examined raw milk samples									

Min – Max: minimum – maximum; Q1: 25th percentile, Q3: 75th percentile; MRL: Maximum Residue Level as per the European Commission (EC) Regulation No 37/2010 (EU) for Cyhalothrin: 50 ppb, and (CAC, 2018) for Cybermethrin: 100ppb, α- Cybermethrin: 100ppb; Deltamethrin, 30ppb, SD: Standard Deviation; nd: non-detectable. P1 = Probability values result from the nonparametric comparison among cities (Kruskal-Wallis test) including all samples. P2 = Probability values result from the comparison among frequencies (%) of detection (Fisher's Exact test).

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Moreover, when the rates of contamination detection for all analyzed milk samples were matched together, a significant difference (P1 = 0.056) was recognized amongst the chosen three cities. Although, Cyhalothrin residues were equally recognized in 17.33 % of milk samples related to Alexandria and El- Behera cities, the percentage of compatibility with the MRL, as per the European Commission (EC) Regulation No 37/2010 (EU) for Cyhalothrin which is 50 ppb, showed quite change. Accordingly, there were 2.66 and 1.33 % of Cyhalothrin-contained milk samples from Alexandrian and El- Behera exceeded the MRL. By analyzing the frequencies of Cyhalothrin detection, all the mean contamination values were settled above the median levels for contaminated samples. Consequently, the mean values ± (standard deviations) > median levels (Q1-Q3) were as follows: 26.88 ± (15.97) > 21.34 (15.76 – 36.07) and 27.18 ± (15.81) > 20.62 (16.22 – 35.02) for Alexandria and El- Behera- contaminated samples, respectively. Despite the percentage of contaminated milk samples related to Matrouh city was somewhat high (28%), none of them went beyond the MRL of the (EC) Regulation No 37/2010, while the mean value ± (standard deviations) > median level (Q1-Q3) were 16.18 ± (4.13) >13.96 (13.25 – 18.07), respectively.

Finally, there was insignificant difference (P2 = 0.783) in the percentages of frequency detection levels among contaminated samples. Deltamethrin residues were noticed at the highest mean level among El- Behera- collected milk samples when compared with the other selected cities where mean level ± (standard deviations) was 3.05 ± (7.84) ppb, followed by Matrouh then Alexandria- collected milk samples in which the values were 2.69 ± (6.08) and 2.45 ± (6.68) ppb of whole milk, respectively. All examined milk samples among the three cities were significantly different (P1 = 0.112) based on the contamination detection levels. Concerning the frequency percentages of Deltamethrin detection amongst contaminated milk samples, there were 14.66, 16 and 24 % of collected samples from Alexandria, El- Behera and Matrouh cities were contaminated, respectively. Furthermore, when the contaminated samples compared with MRL of CAC (2018) which is 30 ppb for Deltamethrin, the percentages of incompatible samples were 1.33 and 2 % for Alexandria and Matrouh cities, while, the highest percent of contaminated samples was related to El- Behera city which was 4%. The data of frequency detection levels revealed a significantly difference (P2 = 0.502) between contaminated samples. Regarding the variability in the frequencies of Deltamethrin detection among contaminated milk samples, the arithmetic means ± (standard deviations) were in this order: 18.83 ± (9.18) > 16.83 ± (7.22) >16.31± (8.53) for El-Behera > Matrough > Alexandria- contaminated milk samples. Subsequently, robust medians with interquartile ranges (Q1-Q3) reached 13.95 (11.14 – 17.53), 14.80 (12.40 – 25.57) and 15.41 (13.16 – 17.64) in Alexandria, El- Behera and Matrouh- contaminated milk samples, respectively.

DISCUSSION

Diverse pesticides could get entrance into milk and dairy products via presence of their residues in dairy animal feed stuffs and or improper application on farm animals, environmental pollution and unintentional falls. Therefore, inhibiting the potential sources of contamination is the first step for milk protection system against the pesticides residues. Recently, synthetic pyrethroids are commonly used due to their prohibited rule against many types of pests in farms, mammals and public health commitments Gao et al. (2013). In this study of the pyrethroid residues in raw bovine milk, Cypermethrin was the prevalent pyrethroid with the highest occurrence of positive marks being existing in milk samples collected from local producers in West Delta region of Egypt, followed by Cyhalothrin and then Deltamethrin residues. In addition, the highest percentage of contaminated samples by Cypermethrin residues as well as the mean values, ppb ± (standard deviations) were related to Matrouh city (44% and 10.74 ppb ± (20.79) when matched with Alexandria (18.66% and 6.63 ppb ± (16.54) and El- Behera (21.33% and 5.98 ppb ± (15.05), in turn this specifies its frequent use to control the pests on a wide scale in different areas of Matrouh. This result is in strong agreement with Muhammad et al. (2012) who found the highest concentration level of Cypermethrin in milk than rest of the inspected pyrethroids in Pakistan. In addition, these mean levels are higher than those reported by Hassan et al. (2014) in Pakistan and Ismail and Elkassas (2016) in Egypt in which the mean concentrations of Cypermethrin in raw milk were 0.23 and 0.1985 ppm while, the frequencies were 21 and 13%, respectively. On the other hand, the frequency of Cypermethrin detection measured in present investigation was lower than that reported in a study conducted in Brazil by Dallegrave et al. (2018), in which the frequency of Cypermethrin in raw milk reached 92 % but mean concentration was 2.75 ppm which is lower than the value of the present study. Furthermore, the present result was higher than that of Goulart et al. (2008) who found the concentration level of 0.75 ppm for Cypermethrin in raw milk. On the contrary, there was a study in South Africa conducted by Sereda et al. (2009) with no detection levels for any Cypermethrin residues in bovine milk. All samples showed concentrations lower than the MRL established by CAC (2018) for Cypermethrin: 100ppb and this data is in the same line with Bedi et al. (2018) in India and Dallegrave et al. (2018) in Brazil. Present result was nearby those reported by Bedi et al. (2015) who found 99% of analyzed milk samples in India were complied with MRLs for Cypermethrin except one sample but with lower mean concentration (ppb) and frequency % which were 0.9 ± 5.0 ppb and 4.1%, respectively. This incidence might constitute a public health hazard due to presence of α- cyano group in the structure of Cypermethrin as one of type II pyrethroids which produces a toxigenic impact especially on liver and kidneys in addition to the stimulation of genetic damage on a long-term exposure (Vardavas et al., 2016).

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In the current study, the concentration and frequency levels for Cypermethrin residues were found at high abundancy. This can be attributed to its rapid solubility with high capability to form covalent bonds with some milk proteins, thus predominates in the milk (Gao et al., 2010). In Egypt, people especially farmers widely use Cypermethrin as agricultural and anti-parasitic pesticide to counteract external parasites of animals such as ticks. This fact may be an explanation for its incidence of 44% in Matrouh- contaminated milk samples. Being toxic to marine and aquatic organisms, Cypermethrin, is included in the list of restricted pesticides by EPA, however in Egypt, it is widely assimilated to prevent a broad range of pests in agronomy and veterinary divisions. Also, it is used to control the pests at home and buildings. Therefore, the high concentration levels of Cypermethrin may constitute adverse health impressions. As regards the Cyhalothrin and Deltamethrin mean values of detection level among examined milk samples, the highest mean values were associated with El- Behera city when compared with other cities in which mean values, ppb ± (standard deviations) were 5.14 ppb ± (12.62) and 3.05 ppb ± (7.84) for Cyhalothrin and Deltamethrin–contained samples, consequently. However, the highest percentages of contaminated samples were reported in Matrouh city with 28% and 24% for Cyhalothrin and Deltamethrin- contaminated milk samples, respectively. The results of detection levels for Cyhalothrin and Deltamethrin residues in this study were higher than those reported by Dallegrave et al. (2018) who reported the mean concentration values of Cyhalothrin and Deltamethrin in Brazilian raw milk of 0.28 and 0.20 ppm, respectively but with higher frequencies than the present result (76 for Cyhalothrin and 13 % for Deltamethrin). The mean residue level and frequency of Deltamethrin in the current study were high in comparison with Hassan et al. (2014) where the mean amount was 0.21 ppm and frequency was only 7% for analyzed milk samples in Pakistan. Similarly, Bedi et al. (2015) reported lower mean (ppb) and frequency (%) levels which were 0.8 ± 4.3 ppb and 4.5% for Cyhalothrin while for Deltamethrin were 0.5 ± 3.4 ppb and 2.2%, respectively.

Consequently, in the current study, Deltamethrin residues showed the least mean concentration levels within other pyrethroid pesticides in the examined milk samples. This could be attributed to its low persistence, therefore diminish quickly in milk. The similar findings were reported by Ahmad et al. (2012) in Pakistan and Dallegrave et al. (2018) in Brazil Notwithstanding, Deltamethrin is identified less frequently than Cypermthrin, however the toxigenic effect of Deltamethrin may be more dangerous than Cypermethtrin as confirmed by Shen et al. (2012). On the contrary, some Egyptian authors failed to detect Deltamethrin in any examined raw milk samples for example, Nasr et al. (2007) in Gharbia City and similarly, Ismail and Elkassas (2016) in Kafr El-sheikh City due to the restricted usage of this pesticide. The present results for Cyhalothrin and Deltamethrin are in the contrary with the Indian study by Bedi et al. (2018) who found all milk samples were free from both residues. Moreover, about 2.66 and 1.33% of Alexandria and El-Behera- contaminated milk samples, respectively were above the MRL of European Commission (EC) Regulation No 37/2010 Cyhalothrin: 50 ppb. Concerning the MRL of CAC (2018), for Deltamethrin which is 30 ppb, there were 1.33, 4 and 2% of Alexandria, El- Behera and Matrouh- contaminated milk samples, respectively exceeded the MRL. There were significant differences in the detection levels of examined pyrethroid residues in all milk samples among selected cities as well as among frequencies of detection except for Cyhalothrin frequency percentages among contaminated milk samples which was (P2=0.783).

Usually, pyrethroid which possesses *trans* conformation are hydrolyzed more readily by mammalian esterases with reduced toxicity than those with the *cis* conformation like Deltamethrin. Although presence of Deltamethrin residues in milk may constitute a great public health hazard**,** it could be rapidly absorbed and this proved in a study performed by Castillo et al. (2013) who found a very low level of Deltamethrin residues in milk (<1%) after their initial application, and maximum levels were achieved in milk after 2 days with undetectable residue level in milk after 8 days of treatment. In Egypt, the bad habit of consuming raw milk directly after milking can be done by some people especially farmers which in turn can lead to conceivable adverse health impacts. Additionally, pesticide containers might be left nearby or even in the same area of milking places that may lead to unintentional leakage and milk pollution. Furthermore, application of pyrethroid sprays on crops neighboring to dairy housing may result in spreading and accumulating of their aerosols on dairy animal premises, water and foods which finally causing milk to be contaminated. Finally, feeding of dairy animals on pyrethroids- polluted crops can cause their residual descending in milk. So, great attentions should be implemented about proper raw milk handling and processing, protection against exposure via feed or from animal environment in addition to continual monitoring the withdrawal time of pyrethroid residues in milk which is very crucial to protect the public health and assess the food safety. Therefore, the recognition of synthetic pyrethroid residues is the head point in relations of preventive actions for ensuring food safety and public health. Every year, programs are held by EU to display and prevent objectionable contamination of foods and raw materials to safeguard consumers.

CONCLUSION

Presence of pyrethroid residues in evaluated milk samples signposts either the use of these pesticides in the past or their continual application till now in many fields such as agricultural and medical purposes. Contamination of such milk samples with pesticides may constitute a noticeable hazard to Egyptian consumers particularly in Matrouh which was the

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most contaminated city with examined pyrethroid residues owing to the haphazard use of pesticides on growing crops with the lack of notification about corrective application regimens. Additionally, the grazing habit of dairy animals especially in Matrouh which feed on growing crops. Although the pyrethroids are detected in possible quantities but the unlimited increase of these residues may cause harmful impacts on human health. Additionally, concentration level of some pyrethroids went beyond to (MRL) in a number of milk samples which is also an issue of worries about consumer health. Therefore, monitoring strategies should be continually applied to ensure pesticide residues level in milk as well as in the areas where enormous quantity of pesticides are applied every year without scheduled monitoring for to achieving the safety of consumer health and ensuring the food safety criteria in country.

DECLARATION

Authors' contributions

Amer Abd El-Moamen Amr designed the study, conceptualized, conducted the practical part of research, prepared the final draft, performed the statistical analysis of the obtained data. Abou-Alella Abd-Elmohsen Sarah visualized and investigated the experiments, interpreted the results, collected the research papers, collaborated in writing of the final manuscript. All authors checked the submitted manuscript and approved it for publication present journal.

Competing interests

The authors declare that they have no conflict of interest

Acknowledgments

The authors are grateful to the stuff members of Food Hygiene and Control Department, Faculty of Veterinary Medicine, Alexandria University for their financial support and academic help to complete this work.

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2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 437-445, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj54

The Role of Afferent C-Fibers in Muscle Contraction of Trachea and Bronchi in Rats

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ORGINAL ARTICLE
pii: S232245682000054-10
Received: 09 Aug 2020
Accepted: 21 Sept 2020

ABSTRACT

The present experimental study on some basal neurophysiological systems aimed to evaluate the effect of the non-adrenergic non-cholinergic system on muscle contraction of the trachea and bronchi. Moreover, the study was targeted toward the investigation of the effect of the local intramural ganglion. The obtained results indicated that C-fibers, which represent the excitatory non-adrenergic non-cholinergic system, caused smooth muscle contraction by the realization of reflex through local intramural ganglia. Furthermore, it was observed that C-fibers affected the muscle by releasing tachykinins for constricting effect. The constricting influence of fibers was greater in the case of involving local reflex through the ganglion, and less significant in the case of activating of the humeral mechanism related to tachykinins. This finding became apparent as a result of the comparison of contractile muscle responses in Krebs-Henseleit's solution with atropine, and with activating C-fiber capsaicin applications. It was also observed that in the rat trachea and bronchus the elimination of NO-ergic mechanisms led to an increase in the contraction, and the dilatation effect of nitric oxide was associated with preganglionic and postganglionic nerve structures of the intramural ganglia. In conclusion, it is identified that afferent C-fibers increase the contractions of the smooth muscle of the trachea and bronchi of the rat mainly with the involvement of ganglion neurons. The humoral mechanism of C-fibers performs a minor and additional role in muscle contraction by the release of tachykinins. The obtained data can contribute to the study of the interaction of the autonomous nervous system and non-adrenergic non-cholinergic system.

Keywords: Bronchi, Intramural ganglion, Non-adrenergic non-cholinergic system, Tachykinins

INTRODUCTION

The smooth muscle of the trachea and bronchi is controlled by sympathetic, parasympathetic and non-adrenergic non-cholinergic innervation (Stretton, 1991). Sympathetic innervation has the least effect on the development of smooth muscle contraction of the lower respiratory tract (Stretton, 1991). Mediators of sympathetic nerve endings (noradrenaline) influence on beta-2-adrenoreceptors, mediating the muscle relaxation (Sergio and Angelina, 2010). Parasympathetic innervation has the most significant influence on the development of smooth muscle contractile response. The mediator of parasympathetic nerve fibers (acetylcholine) excites muscarinic receptors - M3R - and leads to an increase in the contractile response (Carlson and Kraus, 2020). However, in certain conditions, acetylcholine can influence on muscarinic receptors M2R, which leads to inhibit the subsequent release of acetylcholine (Soukup et al., 2017). In such conditions, contractile muscle responses are reduced. The role of non-adrenergic non-cholinergic system (NANC) in the overall muscle contraction or relaxation has not been studied enough. Therefore, considerable attention in modern physiology is paid to the role of NANC system (Fedin et al., 1997). An important area of attention is C-fibers involved in the mechanism of hyperresponsiveness and pathogenesis of chronic obstructive pulmonary disease. C-fibers are afferent fibers of NANC, performing functions - generation of nervous excitation and the release of biologically active substances. This question still remains to be clarified: which mechanism of C-fibers, namely the release of tachykinins or realization of afferent transmission through local intramural ganglion, has a leading role in the realization of muscle contraction of respiratory tract (Kryukova et al., 2001). Bronchoconstriction and bronchodilation of the respiratory passages are mediated by different divisions of nervous system (Figure 1). The cholinergic and excitatory are responsible for bronchoconstriction, while the adrenergic and inhibitory (NANC) are responsible for bronchodilation. One musclular cell can receive impulses from different neurons and both excitatory and inhibitory potentials can occur there (Fedin et al., 1997). The NANC afferent fibers extend from the neurons of the jugular and nodose ganglia (Jacoby, 2003). In addition, the lungs innervated by fibers coming from the dorsal root ganglia and they are similar to fibers of the jugular ganglia (Undem and Kollarik, 2005). These are capsaicin sensitive C-fibers connected with epithelium and neurons of intramural ganglia of the respiratory passages. Tachykinins (neurokinin A) released from C-fibers cause airway constriction due to a direct effect on smooth muscle via neurokinin receptors of the second type (NK2 receptors) (Kwong et al., 2001) through increased activity of ganglionic neurons which have receptors for substance P (SP). Substance P is a neuropeptide from the family of tachykinins produced by C-fiber structures.

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Substance P influences on the neurokinin receptors of the first type (NK1 receptors) and leads to an increase in contraction of the smooth muscle. Capsaicin-sensitive C-fibers form a plexus right beneath the epithelium and cause bronchoconstriction in reaction to thermal stimuli, cold, mechanical or chemical influence. They may also react to substances released as a result of tissue damage or inflammation (Undem and Kollarik, 2005; Vanhoutte, 2013). The bronchopulmonary C-fibers of the NANC system constitute the majority of afferent fibers innervating the respiratory tract. These fibers primarily react to chemical stimuli and present low-threshold, slowly adapting receptors (Kubin, 2016). Almost all C-fibers are capsaicin-sensitive and they tend to have a double function: the release of biologically active substances and participation in the form of afferent pathway in the local reflex with the involvement of intramural ganglion (Elekes et al., 2007). C-fibers are activated by small doses of capsaicin and produce tachykinins causing constriction (through neurokinin A, SP) or relaxation (as a result of vasoactive intestinal peptide (VIP); nitric oxide (NO) effects (Mazzone and Undem, 2016). C-fibers are found around capillaries in the lung parenchyma (Undem and Kollarik, 2005). The main inhibitory mediators of the neurons of the functional module are nitric oxide and vasoactive intestinal peptide. These mediators modulate cholinergic neurotransmission at the level of the smooth muscle or through presynaptic inhibition of acetylcholine release. The density of NO-containing fibers in the smooth muscle of a human decreases from the trachea to the periphery and is completely absent in the bronchioles. In ganglia, on the contrary, the number of NO-containing neurons increases from 57% (in the trachea) to 83% (in small bronchi). Furthermore, different mediators produce bronchodilation through nitric oxide synthesized by epithelial cells (Hennel et al., 2018). Vasoactive intestinal peptide also modulates the cholinergic transmission reducing the SP-induced release of acetylcholine. In chronic bronchitis, the level of VIP is reduced and it correlates negatively with the severity of bronchial obstruction. This mediator has an inhibitory effect on smooth muscle at low concentrations in a presynaptic way and at high concentrations in a postsynaptic one. The frequent co-localization of NO and VIP in neurons was marked. Considering the fact that, C-fibers are anatomically and physiologically connected with NO-structures, the question of NO-mediated contraction of the smooth muscle of the trachea and bronchi is interesting. The role of C-fibers in NO-mediated contraction is not clarified. The question is not fully investigated - with which structures (nerve or muscle) is the NO-mediated effect predominantly associated (Zhu and Dey, 2001; Hennel et al., 2018). The purpose of this study is to determine the participation of C-fibers in excitatory and inhibitory mechanisms of the non-adrenergic non-cholinergic system in the tracheal and bronchial smooth musculature contraction in the rat. Also, the goal was to study the role of C-fibers in NO-mediated muscle contraction and in establishing the structures with which the dilatation effect of nitric oxide is mainly associated.

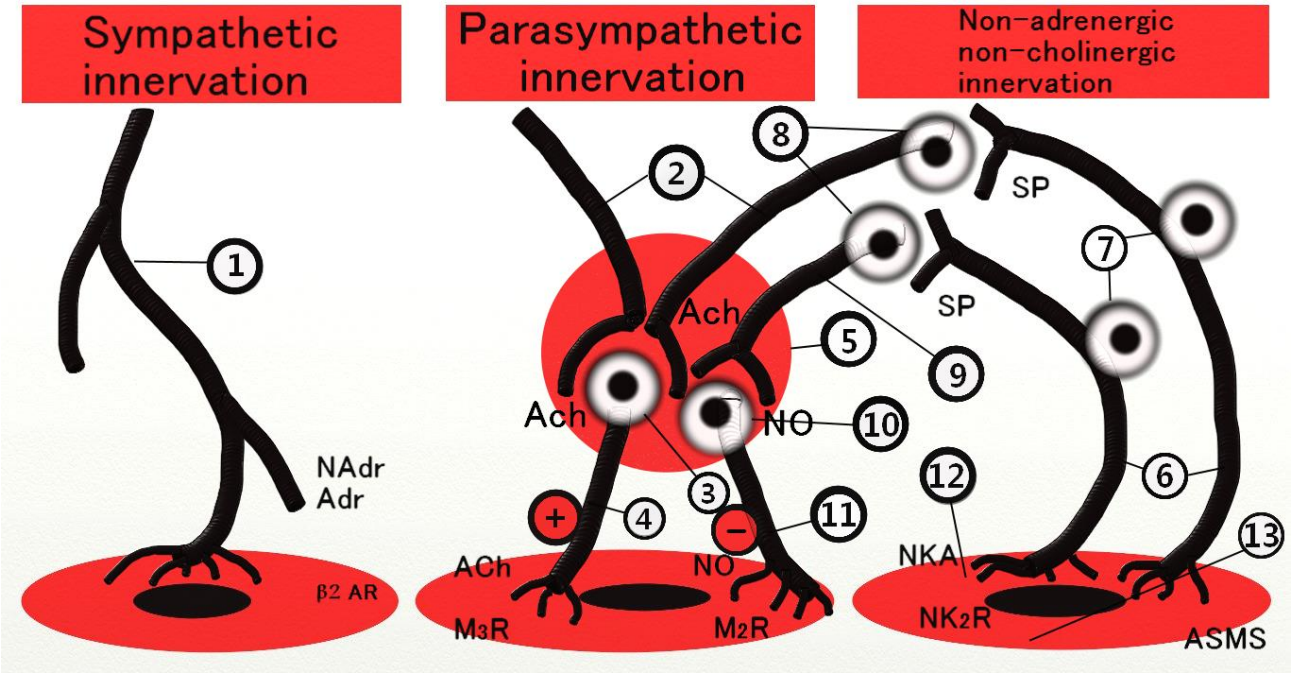

Figure 1. Scheme of innervation of smooth muscle of trachea and bronchi in mammals: sympathetic innervation, parasympathetic innervation, non-adrenergic non-cholinergic innervation (participation of C-fiber in the trachea and bronchi smooth muscle innervation). ASMS: airway smooth muscle cells. NAdr: noradrenaline. Adr: adrenaline. B2AR: beta-2-adrenoreceptor. Ach: acetylcholine. M3R, M2R: muscarinic receptors. NO: nitric oxide. NKA: neurokinin A. NK2R: neurokinin receptor. SP: substance P. « + »: nervous pathway leading to smooth muscle contraction. « ─ »: nervous pathway leading to the relaxation of smooth muscle. 1: sympathetic fiber, 2: preganglionic parasympathetic fibers, 3: cholinergic neuron, 4: postganglionic parasympathetic fiber, 5: ganglion, 6: C-fiber, 7: body of sensory neuron of the jugular ganglia, 8: body of neuron of the brain ganglia, 9: preganglionic NO-ergic fiber, 10: NO-ergic neuron, 11: postganglionic NO-ergic fiber, 12: focus of ejection of local tachykinins, 13: body of the muscle cell.

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MATRIALS AND METHODS

Animals

Twenty five wister rats of both sexes with average body weight of 230 g were used. There were no differences in the contractile responses of the muscles of males and females, because females were taken for experiments in the period of diestrus (not in estrus). Animals were kept in vivarium, which owned all the requirements for animals. Diet of animals was standard formulated rodent diets, with *ad libitum* daily access. Anesthesia procedure was carried out by placing the animal in a chamber with 4-5% chloroform. The narcotic effect causes a fairly rapid loss of consciousness. After the animal lost consciousness, decapitation was applied. This approach provided quick and painless euthanasia of the animal (Recommendations for euthanasia of experimental animals, European commission) (Close et al., 1997). The animal was fixed on the dissection table. After that, the thorax was opened and then the operation was performed with the extraction of the respiratory tract of the animal. The parenchymal tissue of the lungs was removed mechanically with a wooden spatula (Hatziefthimiou et al., 2005). The respiratory tract was washed in the Krebs-Henseleit solution, and then the trachea and bronchi preparations were made, according to Kolahian et al. (2010). The preparation was 0.4 – 0.6 cm long and 0.5 – 0.7 cm wide. The incision line of the trachea and bronchi passed through the cartilaginous half-rings. The smooth muscle remained intact. The preparations of the trachea and bronchi were placed in a chamber with Krebs-Henseleit solution, where one edge of the preparation was fixed by needles and the second edge of the preparation was mounted by hooks-holders attached to an electromechanical sensor which registered the magnitude of the contractile response (measured in mN) (Kolahian et al. 2010; Noller et al., 2019).

Ethical Approval

All of process was done in acoording to ethical regulations of Ministry of Education and Science of the Russian Federation within the framework of the applied scientific research and experimental development on "Research and development of cross-cutting technology of production of functional food products to ensure food security of the Northern territories of the Russian Federation" (Ethical certificate; project ID – RFMEFI57717X0264).

Equipment

In the experiments, a physiological complex was used, including special chambers for the trachea and bronchi, an ultratermostat, aerator, peristaltic pump (ML0146/C-V, Multi Chamber Organ Baths, Panlab, Germany), electromechanical sensors (Grass FT-03 force displacement transducer, Astro Med, West Warwick, RI, USA), an electrostimulator (direct-current stimulator, Grass S44, Quincy, MA, USA), a personal computer, special software (Chart v4.2 software, Power Lab, AD Instruments, Colorado Springs, CO, USA).

Electrophysiological experiments

The electrical field stimulation was applied in all experiments. For this purpose, two silver electrodes were put into the dishes with the preparations. During the work the electric stimulation of preganglionic nerve fibers (frequency of stimuli:8 Hz, duration : 0.5 ms, amplitude : 20 V, duration of stimulation: 10 s), postganglionic nerve fibers (frequency of stimuli : 30 Hz, duration : 0.5 ms", amplitude: 20 V, duration of stimulation: 10 s) and muscle (frequency of stimuli : 30 Hz, duration : 2 ms, amplitude : 20 V, duration of stimulation: 10 s) was applied. Electrical stimulation simulated the natural conduction of electrical impulses through the preganglionic and postganglionic link of reflex chain or muscle. The contractile response of smooth musculature of the trachea and bronchi of the rat was studied in the case of using electrical stimulation and pharmacological agents. First, electrical stimulation was given to the chambers with trachea and bronchi. Then the contractile muscle responses were recorded. These answers were taken as basal level (or 100%). After this, pharmacological substances were added and contractile muscle responses were recorded. Thus, the responses of the tracheal and bronchial muscles were recorded taking into account electrical stimulation and pharmacological preparations. The magnitude of the contractile responses to the drug usage largely depended on the original tonus of smooth muscle as well as the control contractile responses in the case of application of electrical stimulation in connection with the physiological saline. Despite the fact that all animals were the same age and the sampling was homogeneous, the variability of the original tonus and control responses (measured in mN) of such organs as the trachea and bronchi was rather high, and this fact determined the accounting of contraction in percentage (counted in percentage of the basal level of activity taken at 100 %). The methods of electrical stimulation of preganglionic, postganglionic nerves and muscles are taken from the research methods proposed by Fedin et al. (1997).

Statistical analysis

Statistical analysis was carried out with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA, version 17.0.2). A comparison between the groups of control and experimental results was performed with

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the use of independent sample t-test. Data were expressed as mean ± standard deviation. The value of $P<0.05$ was considered statistically significant.

Pharmacological procedure

Perfusion was performed with Krebs-Henseleit solution of the following composition: sodium chloride (118 mmol/l in the form of perfusion, Sigma-Aldrich, Germany); potassium chloride (4.8 mmol/l in the form of perfusion, Sigma-Aldrich, Germany); magnesium sulphate (1.18 mmol/l in the form of perfusion, Sigma-Aldrich, Germany); potassium dihydrophosphate (1,2 mmol/l in the form of perfusion, Sigma-Aldrich, Germany); calcium chloride (2.5 mmol/l in the form of perfusion, Sigma-Aldrich, Germany); sodium bicarbonate (25.0 mmol/l in the form of perfusion, Sigma-Aldrich, Germany); glucose (5.5 mmol/l in the form of perfusion, Sigma-Aldrich, Germany). The necessary level of oxygen, temperature (37^0C) and pH (6.9 – 7.1) was maintained in the dishes with the preparations, (Kryukova et al., 2001). The flow of fresh Krebs-Henseleit solution was provided regularly as well as the outflow used in Fedin et al. (1997) study. During the experiments, the following substances were exogenously administered: capsaicin (3 nmol in the form of application with volume 0.2 ml, the duration of capsaicin application 1 minute, Sigma-Aldrich, Germany) for the activation of C-fibers, capsaicin (3 nmol in the form of perfusion during 30 minutes, Sigma-Aldrich, Germany) for the inactivation of C-fibers, atropine (0.2 nmol in the form of perfusion during 30 minutes, Sigma-Aldrich, Germany). Activation of C-fibers or their inactivation depended on the time of exposure to capsaicin. With a short exposure time, the fibers were excited, and with prolonged exposure, their blockade occurred. Also, in the study, we used atropine (0.2 nmol in the form of perfusion) for the interruption of neuromuscular transmission, a drug inhibitor of NO-synthase L-NAME (0.4 nmol, in the form of perfusion, Sigma-Aldrich, Germany). The study needs to eliminate the influence of the epithelium so its physiological effects were inhibited by indomethacin (3 nmol, in the form of perfusion during 30 minutes, Sigma-Aldrich, Germany) in all experiments. Indomethacin had no effect on the smooth muscle contractions of the trachea and bronchi of rats. This finding was tested previously, before proceeding with the main series of experiments (Saeideh et al., 2019). Moreover nitric oxide induces relaxation of airway smooth muscle through COX-2-derived PGI2 (COX-2/PGI2) pathway (Dorris and Peebles, 2012). Doses of drugs were selected on the basis of preliminary experiments (Vanhoutte, 2013).

RESULTS

The following results were obtained during the study to define the physiological significance of afferent C-fibers on the contractile activity of smooth-muscle system. This series of experiments involved the study of the contractile responses of smooth muscle in conditions of C-fibers activation by low doses of capsaicin (in the form of applications) and their inactivation due to long-term capsaicin perfusion which led to their long-term depletion and loss of ability of afferent transmission and the release of tachykinins. The excitation of C-fibers led to the realization of local reflex. It is the cause for an increase in the frequency of contractile responses after the application of capsaicin (Figure 2). The magnitude of the contractile responses of tracheal smooth muscle during the excitation of C-fibers increased to 130.11 % ± 3.53 ($n = 8$, significant differences in comparison with response in the Krebs-Henseleit solution, $P = 0.01$). The responses of the bronchial muscle were also increased and reached 121.12 % ± 3.01 ($n = 8$, significant differences in comparison with response in the Krebs-Henseleit solution, $P = 0.02$). With the inactivation of C-fibers, the contractile responses of the tracheal muscles were 95.31 % ± 2.11 ($n = 8$, no significant differences) and the responses of the bronchial muscle – 84.02 % ± 1.92 ($n = 8$, significant differences in comparison with response in the Krebs-Henseleit solution, $P = 0.04$) (Figure 2 and Table 1).

Due to the reason that the C-fiber of the non-adrenergic/non-cholinergic system are capable to be active without the participation of metasympathetic intramural ganglia, an important stage of the research will be experiments to define the role of activated C-fibers in isolation of the ganglion by interrupting atropine neuromuscular transmission. In such experimental conditions, smooth muscle responses are associated mainly with the tachykinins released from the fiber and their local effect on neuromuscular structure. Compared to the smooth muscle responses recorded in Krebs-Henseleit solution, the responses with blocked cholinergic receptors were strongly decreased (Table 2). The magnitude of the contractile responses of tracheal muscle decreased to 15.61 % ± 2.23 ($n = 8$) and bronchial muscle to 18.44 % ± 1.87 ($n = 8$). Further, capsaicin in the form of applications was placed in the dishes with the preparations and there was an increase of contractile responses of the trachea to 27.33 % ± 1.67 ($n = 8$, significant differences in comparison with atropine, $P = 0.04$) and bronchi to 32.29 % ± 2.46 ($n = 8$, significant differences in comparison with atropine, $P = 0.04$).

We examined the effects of NO-ergic system on the smooth muscle of the rat by applying the inhibitor of NO-synthase L-NAME. Different types of electrical stimulation of preganglionic and postganglionic nerve fibers as well as muscles were used during this series of experiments. The highest value of contraction of preparations was noted during the stimulation of preganglionic and postganglionic nerve fibers. During the stimulation of preganglionic fibers, the

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tracheal responses were 123.82 % ± 5.11 (n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.02). The contraction responses of the bronchi were 121.92% ± 4.82 (n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.02) (Table 3).

With electrical stimulation of postganglionic nerves, the contractile responses of the trachea were 113.01 % ± 5.01 (n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.04). The bronchial muscle responses were 117.02 % ± 4.71 (n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.03) (Table 4).

During electric stimulation of the muscle the tracheal responses were 87.83 % ± 4.51 (n = 9, no significant differences) and for bronchus up to 79.22 % ± 4.01 (n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.02) (Table 5).

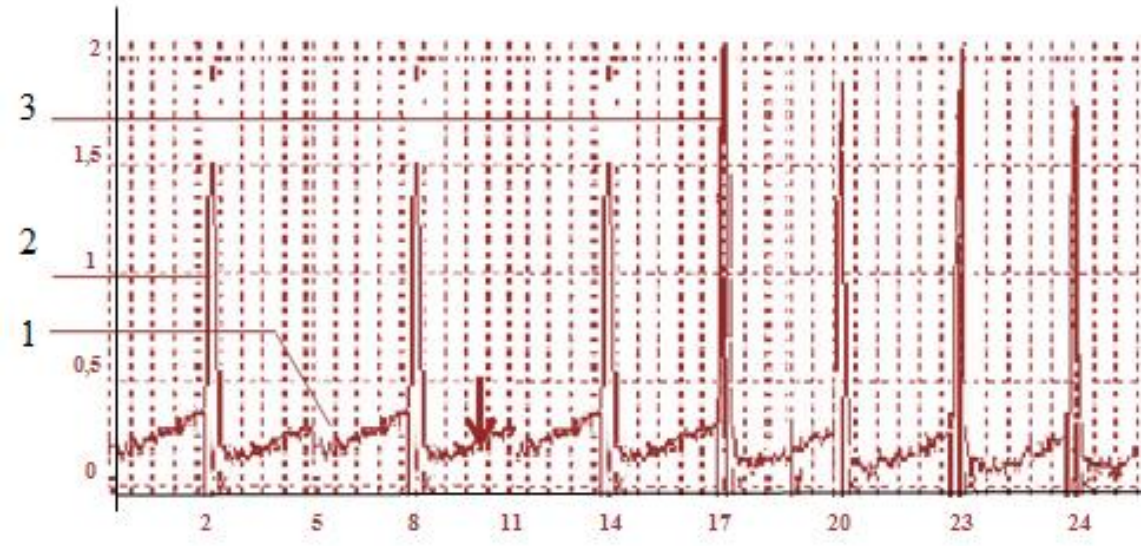

Figure 2. Sample of representative recording of the contractile response of the trachea in control (left part illustration) and after capsaicin application (right part illustration); by the abscissa axis the time is indicated (in seconds). By the ordinate axis the changes of smooth muscle responses are indicated (in mN). The brown arrow indicates the introduction of a drug into the chamber with a trachea. 1: basal level of responses (natural muscle tone); 2: control contractile responses to electrical stimulation of postganglionic nerve fibers; 3 : contractile response after capsaicin application.

Table 1. The magnitude of the tracheal and bronchial muscle contraction in millinewton and percentage.

Drug application	Trachea		Bronchi	
	Magnitude (mN)	Magnitude (%)	Magnitude (mN)	Magnitude (%)
Krebs-Henseleit solution perfusion	1.52 mN ± 0.02	100.00 % ± 1.32	1.48 mN ± 0.05	100.00 % ± 3.39
Capsaicin (activation). 3 nmol. application	1.98 mN ± 0.07	130.11 % ± 3.53	1.81 mN ± 0.05	121.12 % ± 3.01
Capsaicin (inactivation). 3 nmol. perfusion	1.45 mN ± 0.03	95.31 % ± 2.11	1.24 mN ± 0.03	84.02 % ± 1.92

Table 2. The magnitude of the contraction of tracheal and bronchial muscles in millinewton and percentage.

Drug application	Trachea		Bronchi	
	Magnitude (mN)	Magnitude (%)	Magnitude (mN)	Magnitude (%)
Krebs-Henseleit solution, perfusion	1.49 mN ± 0.03	100.00 % ± 2.01	1.46 mN ± 0.03	100.00 % ± 2.05
Atropine, 0.2 nmol, perfusion	0.23 mN ± 0.01	15.61 % ± 2.23	0.27 mN ± 0.01	18.44 % ± 1.87
Atropine, 0.2 nmol, perfusion + Capsaicin(activation), 3 nmol, application	0.41 mN ± 0.01	27.33 % ± 1.67	0.47 mN ± 0.01	32.29 % ± 2.46

Table 3. The magnitude of the contraction of the tracheal and bronchial muscles in millinewton and percentage during stimulation of preganglionic nerve fibers

Drug application	Trachea		Bronchi	
	Magnitude (mN)	Magnitude (%)	Magnitude (mN)	Magnitude (%)
Krebs-Henseleit solution, perfusion	1.54 mN ± 0.03	100.00 % ± 1.95	1.51 mN ± 0.03	100.00 % ± 1.99
L-NAME, 0,4 nmol, perfusion	1.91 mN ± 0.09	123.82 % ± 5.11	1.84 mN ± 0.08	121.92 % ± 4.82

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Table 4. The magnitude of the contraction of the tracheal and bronchial muscles in millinewton and percentage during stimulation of postganglionic nerve fibers

Drug application	Trachea		Bronchi	
	Magnitude (mN)	Magnitude (%)	Magnitude (mN)	Magnitude (%)
Krebs-Henseleit solution, perfusion	1.54 mN ± 0.03	100 % ±1.95	1.51 mN ± 0,03	100.00 % ± 1.99

By the abscissa axis used preparations are indicated. By the ordinate axis the changes of smooth muscle responses are indicated in %. *: significant differences (n = 9, P = 0.04) of the tracheal contractile response in comparison with a control response. *: significant differences (n = 9, P = 0.03) of the bronchial contractile response in comparison with a control response. After administration of the drug, the responses were recorded for 5 minutes.

Table 5. The magnitude of the contraction of the tracheal and bronchial muscles in millinewton and percentage during stimulation of muscles

Drug application	Trachea		Bronchi	
	Magnitude (mN)	Magnitude (%)	Magnitude(mN)	Magnitude (%)
Krebs-Henseleit solution, perfusion	1.54 mN ± 0.03	100.00 % ± 1.95	1.51 mN ± 0.03	100.00 % ± 1.99

By the abscissa axis used preparations are indicated. By the ordinate axis the changes of smooth muscle responses are indicated in %. *: significant differences (n = 9, P = 0.02) of the bronchial contractile response in comparison with a control response. After administration of the drug, the responses were recorded for 5 minutes.

DISCUSSION

Having analyzed the results of experiments, which had provided the contractile responses of the smooth muscles of the trachea and bronchi in the physiological solution, during activation and inactivation of C-fibers (tables 1 and 2), a constricting role of C-fibers of the non-adrenergic non-cholinergic system became apparent. During their excitation by application of capsaicin, the contractile responses of the trachea and bronchi increased. There was no significant difference between the responses of the trachea (130.11 % ± 3.53; n = 8) and bronchi (121.12 % ± 3.01; n = 8). The P value was 0.06 which is not significant. During inactivation of C-fibers affected by prolonged capsaicin perfusion, the decrease of contractile responses was occurred. This decrease was more apparent in bronchi (84.02 % ± 1.92; n = 8, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.04) than in the preparations of the trachea (95.31 % ± 2.11; n = 8, no significant differences). Perhaps in the bronchi the constrictor effect of C-fibers is more pronounced.

Reynolds and Doherty (2008) conducted similar experiments on the effect of capsaicin on the tracheal muscle of guinea pigs. In their experiments, prolonged perfusion with capsaicin reduced contractile responses of the tracheal muscle (Reynolds et al., 2008). Our experiments, in contrast to the studies of Reynolds et al. (2008), were conducted on rats and allowed us to evaluate the effect of capsaicin not only on the trachea, but also on the bronchi. The novelty of our study is that in the inactivation of C-fibers, reduction of contractile responses from the control value was more pronounced in the bronchi than in the trachea. Probably, the constrictor effect of C-fibers is more pronounced in the bronchi than in the trachea. This may be due to the higher density of C-fibers in the bronchi compared to the trachea or large release of excitatory tachykinins in the bronchi unlike the trachea. At the moment, this assumption has not yet been confirmed by other research methods and it should be clarified in more details. The present assumption is currently based on a study of the contractile effects of the muscle. To identify the role of the metasympathetic intramural ganglion in contractile activity of the smooth musculature of the trachea and bronchi mediated by C-fibers, the experiments were carried out with the interruption of atropine neuromuscular transmission (Figure 3). Once atropine, an anticholinergic drug, was placed in the dishes with the preparations, the contractile responses of the trachea and bronchi reduced to the minimal value. The responses started to increase as soon as capsaicin was put into the dishes and C-fibers were activated. The contraction responses of the trachea increased to 27.33 % ± 1.67 (n = 8, significant differences in comparison with responses in the atropine, P = 0.04) and bronchi to 32.29 % ± 2.46 (n = 8, significant differences in comparison with responses in the atropine, P = 0.04). Activation of C-fibers which leads to muscle contraction, is represented by two mechanisms in the respiratory tract of rats. The first mechanism leading to muscle contraction is a local reflex through the ganglia and the second mechanism is the release of tachykinins. In the present experiments, atropine blocked cholinergic transmission is the main pathway leading to muscle contraction through the ganglia. Blocking cholinergic transmission to the muscle leads to such experimental conditions, when the influence of ganglia is eliminated. In such experimental conditions at excitation of C-fibers can be assessed humoral mechanism of activation of muscle contraction through the release of tachykinins. Taking into account the fact that after cholinergic blockade the activation of C-fibers led to an increase in muscle responses, it can be assumed that C-fibers release primarily excitatory tachykinin, leading to increased contraction of the muscles. It is possible to assume that excitatory tachykinins can activate muscle cells

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bypassing the intramural ganglion. Information about excitatory influence of tachykinins of C-fibers (in particular about neurokinin A) on neurokinin receptor with the subsequent development of muscle construction is confirmed in the works by Kwong et al. (2001), Elekes et al. (2007), and Majkowska-Pilip et al. (2019). However, in these works and other similar studies the experiments were conducted without using any postganglionic stimulation, which increases the importance of the present results obtained under conditions closest to natural ones. The comparison of the muscle responses during activation of the C-fibers under the influence of the intramural ganglion (figure 2) and elimination of the effects by atropine (figure 3) allows monitoring the fact that activated C-fibers in the presence of the ganglion cause stronger contractile responses. This result may indicate that the mechanism of muscle contraction mediated by activation of C-fibers occurs mainly with the involvement of intramural ganglion and to a lesser extent with the involvement of excitatory tachykinins. Establishing the contribution of intramural ganglion and C-fibers to the local reflex of smooth muscle contraction is the novelty of this study.Nitric oxide is one of the main inhibiting mediators of the non-adrenergic non-cholinergic system (Aleksandrov et al., 2015). Maarsingh et al. (2005) provides report on the role of nitric oxide in regulating the activity of tracheal smooth muscle in a guinea pig, however most studies have been conducted without the use of electrical stimulation of nerve fibers. Its synthesis in the non-adrenergic non-cholinergic system of the respiratory tract is carried out by specific intramural ganglion neurons. The inhibition of the NANC-induced generation of nitric oxide leads to a decrease in relaxation of smooth muscle in the mouse trachea (Elekes et al., 2007). The highest value of contractions of the preparations were noted during the stimulation of preganglionic (123.82 % ± 5.11 for tracheal responses and 121.92 % ± 4.82 for bronchial responses; n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.02 for the trachea and bronchi, figure 4) and postganglionic nerve fibers (113.01 % ± 5.01 for tracheal responses and 117.02 % ± 4.71 for bronchial responses; n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.04 and P = 0.03 respectively, for the trachea and bronchi, Figure 5), which indicates a significant role of NO-synthesizing neurons of the intramural ganglia in the mediation of the relaxation effect on smooth muscle. Probably the relaxation effect of nitric oxide is associated with preganglionic and postganglionic nerve structures of the intramural ganglia. As a result of experiments with L-NAME, it was found that in the lower respiratory tract of the rat the dilating role of the NO-ergic system is related to nervous structures and is not associated with muscle structures. It was found out that in the rat trachea and bronchus the elimination of NO-ergic mechanisms leads to an increase in the contraction. NO-ergic mechanisms are associated with preganglionic and postganglionic nerve fibers. Information on the association of NO-mediated contraction with neural, but not muscular structures represents the novelty of our study. However, at the moment, this assumption has not yet been confirmed by other research methods and it has yet to be clarified in more details. Also, this study contains some new information on the role of C-fibers in the contraction of the muscle of the trachea and bronchi, taking into account the physiological effect of the ganglion. Vanhoutte (2013) confirms in his research that nitric oxide can be produced not only from the epithelial cells but also from the intermediate cells in airway smooth muscle, and nitric oxide production from the intermediate cells is stimulated by acetylcholine. Based on the data of Vanhoutte (2013) and the present investigation, we can make an assumption about some reciprocal effect on the smooth muscle from the neuro-humoral system. On the one hand, acetylcholine is produced for neuromuscular transmission, and on the other hand, in response to acetylcholine production, nitric oxide is released as a compensatory mechanism. Electrical stimulation of preganglionic fibers always excites C-fibers (Noller et al., 2019). In present studies, when preganglionic fibers were stimulated, contractile responses of the tracheal muscles against the background of L-NAME were higher than those of the trachea against the background of the same preparation, but under conditions of stimulation of postganglionic nerves (123.82 % ± 5.11 - tracheal responses in terms of electrical stimulation of the pregaglionic nerves and 113.01 % ± 5.01 - tracheal responses in terms of electrical stimulation of the postgaglionic nerves; n = 9, significant differences, P = 0.04; Fig. 4 and Fig. 5). Taking this fact into account, it can be concluded again about the exciting role of C-fibers and assume their contribution to the NO-mediated contractile reactions. It can be assumed that excitation of C-fibers and NO-mediated relaxation are two opposite mechanisms that, under the conditions of the physiological norm, form a certain balance between constriction and dilatation effects on the tracheal smooth muscle. Probably in this balance there is a definite relationship between the effects of C-fibers and NO-influences on the muscle.Under conditions of electrical muscle stimulation inhibition of NO-synthase resulted in reduction of bronchial contractile responses (79.22 % ± 4.01 bronchial responses; n = 9, significant differences in comparison with responses in the Krebs-Henseleit solution, P = 0.02, figure 6). This may indicate a constrictor effect of nitric oxide in the conditions of electrical stimulation of bronchial muscles. However, these results have not yet been confirmed by other studies and require further study. Different responses of the bronchial muscles to the blockade of nitric oxide under various types of stimulation can be explained by the fact that the dilative function of nitric oxide is associated mainly with neural structures and constrictor-effect associated with inhibition of NO-synthase, is related to muscle structure of the bronchi of the rat.

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CONCLUSION

As a result of the conducted studies it was found that the leading role in the contraction of the muscle of the trachea and bronchi is associated with the ganglion. Afferent C-fibers play a smaller role in contraction compared to ganglia. So, it is necessary to conclude what is the novelty of this study. The constricting influence of C-fibers is far greater in the case of involving local reflex through the ganglion, and far less in the case of activating of the humoral mechanism related to tachykinins. It was also established that relaxation effect of nitric oxide is associated with preganglionic and postganglionic nerve structures of the intramural ganglia, but not with the smooth muscle structures of the trachea and bronchi of the rat. We found that in the bronchi of the rat, compared with the trachea, there is a more pronounced excitatory (constrictor) effect of C-fibers.

DECLARATIONS

Acknowledgements

The study indicates the results conducted with the financial support of the Ministry of Education and Science of the Russian Federation within the framework of the applied scientific research and experimental development on "Research and development of cross-cutting technology of production of functional food products to ensure food security of the Northern territories of the Russian Federation" (project ID – RFMEFI57717X0264).

Acknowledgments

Authors are thankful to Petrozavodsk State University for their special supports.

Authors' contributions

Authors have similar role in all process of study and writing of manuscript. The manuscript was approved by all authors.

Competing interests

The authors declare that they have no competing interests.

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To cite this paper: Kirilina VM, Smirnova OE, Blazhevich LE and Chepurina IV (2021). The Role of Afferent C-Fibers in Muscle Contraction of Trachea and Bronchi in Rats. *World Vet. J.*, 10 (3): 437-445. DOI: https://dx.doi.org/10.36380/scil.2020.wvj54

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 446-450, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj55

Dog as a Potential Source of *Helicobacter pylori* in Egypt: Public Health Significance

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ORGINAL ARTICLE
pii: S232245682000055-10
Received: 05 Aug 2020
Accepted: 19 Sept 2020

ABSTRACT

Helicobacter species are a group of Gram-negative, microaerophilic bacteria, which are known to colonize the gastrointestinal and biliary tracts of humans and various animal species. The objective of the present study was to determine the prevalence of *Helicobacter pylori* in owned dogs and their role in the transmission of *H. pylori* to the dog owners. For this purpose, 60 gastric biopsy samples from dog owners and 80 stool samples from owned dogs were collected and examined for the presence of *H. pylori 16*s *rRNA* gene by nested PCR. The PCR positive samples from human and dog isolates were further subjected to partial *Helicobacter* genus-specific *16s rRNA* gene sequencing. Phylogenetic analysis based on partial sequence of this gene was performed to determine the relationship between human and dog isolates. *H. pylori* was detected in 62.5% and 91.6% of dog and human samples, respectively. The nucleotide sequence of *Helicobacter* genus-specific *16s rRNA* gene of human and dog isolates were similar. In conclusion, this study indicated a high prevalence of *H. pylori* in both dogs and dog owners in Egypt. Zoonotic transmission of *H. pylori* between dogs and humans is probable and represents a public health concern.

Keywords: Dogs, *Helicobacter pylori*, Humans, Phylogenetic analysis, 16s rRNA sequencing

INTRODUCTION

Helicobacter pylori is a spiral, Gram-negative microaerophilic bacterium that prefers commonly the acidic medium, because of its ability to produce urease (Siqueira et al., 2007). It is considered to be the second predisposing cause of cancer-associated deaths and the fourth cancer-causing factor all over the world (The Globocan Project, 2010). It is reported that above half of the world's people with a higher percentage of adults are affected by *H. pylori* infection, especially in developing countries (Frenck and Clemens, 2003).

Helicobacter species inhabits the gastric and intestinal mucosa of humans, pet animals including dogs and cats, avian species, as well as wild animals such as monkeys (Abdi et al., 2014; Hong et al., 2015). In humans, *H. pylori* mainly invade the mucosa of the stomach. The majority of *Helicobacter* infections may develop to asymptomatic gastritis, nevertheless, 10% of infections may progress to cause gastric or duodenal ulcers, and 1% may develop into gastric carcinoma (Beswick et al., 2006). It is well known that there is close contact between humans and companion animals, which poses a great risk of transmitting many zoonotic diseases. Most of these infections in humans initiate from animals, including dogs, through direct contact (Meining et al., 1998; Haesebrouck et al., 2009).

In dogs, spiral-shaped bacteria are commonly found in the stomach. They are present in 67-86% of clinically healthy dogs and 61-100% of dogs presenting chronic vomiting (Hwang et al., 2002; Recordati et al., 2007). Many studies have reported that the majority of dogs affected with gastric ulcers may act as a reservoir of *H. pylori*. These studies have been reported in many countries such as Belgium (Van den Bulck et al., 2005), Thailand (Pirarat et al., 2003), Italy (Recordati et al., 2007), Iran (Torkan and Shahreza, 2016), and Egypt (Abdel-Raouf et al., 2014). The prevalence of gastric *Helicobacter* infection in dogs has been reported to be between 61 to 100% (Eaton et al., 1996; Happonen et al., 1998; Yamasaki et al., 1998; Wiinberg et al., 2005). Also, a relationship has been found between pet ownership or frequent exposure to dogs and infection with different gastric *Helicobacter* species (Chung et al., 2013).

Therefore, domestic animals, especially dogs, are charged to be a common source of *Helicobacter* (Abdi et al., 2014; Okubo et al., 2017). The isolation of *Helicobacter* spp. from saliva, dental plaque, and feces of dogs reinforces the hypothesis of transmission from these animals through oral-oral or fecal-oral routes (Souza et al., 2004). The same condition is observed in humans with *H. pylori* infection, i.e., oral–oral and fecal-oral are considered possible routes of transmission (Brown, 2000). The occurrence of *H. pylori* in dogs and humans enhances the need for *Helicobacter* detection and companion animal treatment (Nowroozilarki et al., 2017). Therefore, eradication of *Helicobacter*

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infections in dogs that have close contact with humans should be considered as one of the methods to control this zoonotic infection.

The aim of this research was to study the prevalence of *H. pylori* infection in owned dogs and dog owners of Egypt and its public health significance.

MATERIALS AND METHODS

Ethical approval

This study was conducted according to ethical guidelines approved by the Faculty of Veterinary Medicine, Cairo University, Egypt. The signed consent for the use of samples was obtained from each patient who participated in the study.

Sample collection

Gastric biopsy samples (n=60) of patients with the history of dog ownership were collected from different hospitals in Giza, Egypt. Canine stool samples (n=80) were collected from the hospital of the Faculty of Veterinary Medicine, Cairo University, and other private veterinary clinics in the Giza governorate. Biopsy and stool samples were collected in sterile tubes containing Brain Heart Infusion (BHI) broth (Merck, Germany) and 5% non-activated fetal calf serum and transferred on ice to the laboratory.

Molecular identification of *Helicobacter pylori* by nested PCR targeting *16S rRNA* gene

DNA was extracted from stool samples using QIAamp DNA Stool Mini Kit, (Germany) according to the manufacturer's instructions. While for gastric biopsies, DNA was extracted by a modification of the method described by (Marais et al., 1999)**.** The extracted DNA was stored at -20 °C until required. The nested PCR assay targeting the *16S rRNA* gene of *H. pylori* was performed using primer pairs Hp1, Hp2, and Hp3 (Table 1) (Hamza et al., 2018)**.** The temperature profile was as follows: 30 s at 95 °C, 30 s at 55 or 60 °C, and 30 s at 72 °C. For nested PCR, 25 cycles were used for each round of amplification. PCR products were analyzed on 2% agarose gel electrophoresis stained with ethidium bromide.

Sequencing of *Helicobacter* genus-specific *16S rRNA* gene

To study the relationship between *H. pylori* isolated from human and dog, the extracted DNA from PCR positive samples were amplified for *Helicobacter* genus-specific *16s rRNA* gene using C97 and C05 primers (Table 1) (Elhariri et al., 2017). The temperature profile was as follows: 94 °C for 1 min; 55 °C for 2.5 min; 72 °C for 3 min (35 cycles). The PCR products were purified using a Qiaquick purification kit (Qiagen, Germany) and sequenced using Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, Waltham, MA, USA). The obtained nucleotide sequences from human and dog isolates were submitted to the GenBank under accession numbers MN901212 and MN901172, respectively.

Phylogenetic analysis

The nucleotide sequences in the current study were compared with those available in GenBank databases using the NCBI-BLAST server. Sequences were downloaded and imported into the BioEdit program version 7.0.1.4 for multiple alignments using the BioEdit Clustal W program. Phylogenetic analysis was performed with the MEGA program version 7 using the neighbor-joining approach.

Table 1. Primers used in this study

Target genes	Primers	Sequences (5'-3')	PCR product size (base pair)
Helicobacter genus-specific 16s rRNA	C97-F C05-R	GCT ATG ACG GGT ATC C ACT TCA CCC CAG TCG CTG	1200
Helicobacter pylori-specific-16s rRNA	HP1-R HP2-F HP3-F	CTGGAGAGACTAAGCCCTCC ATTACTGACGCTGATTGTGC AGGATGAAGGTTTAAGGATT	109

F: forward, R: reverse

RESULTS

H.pylori was detected by nested PCR assay targeting the *16S rRNA* gene of *H. pylori* in 62.5% (50/80) and 91.6% (55/60) of owned dogs and humans, respectively. The nucleotide sequences of *Helicobacter* genus-specific *16s rRNA* from human and dog isolates were located in the same cluster with a bootstrapping value 99% (Figure 1), indicating that they are highly related to each other. The similarity was found between *H. pylori* isolated from both dogs and human cases.

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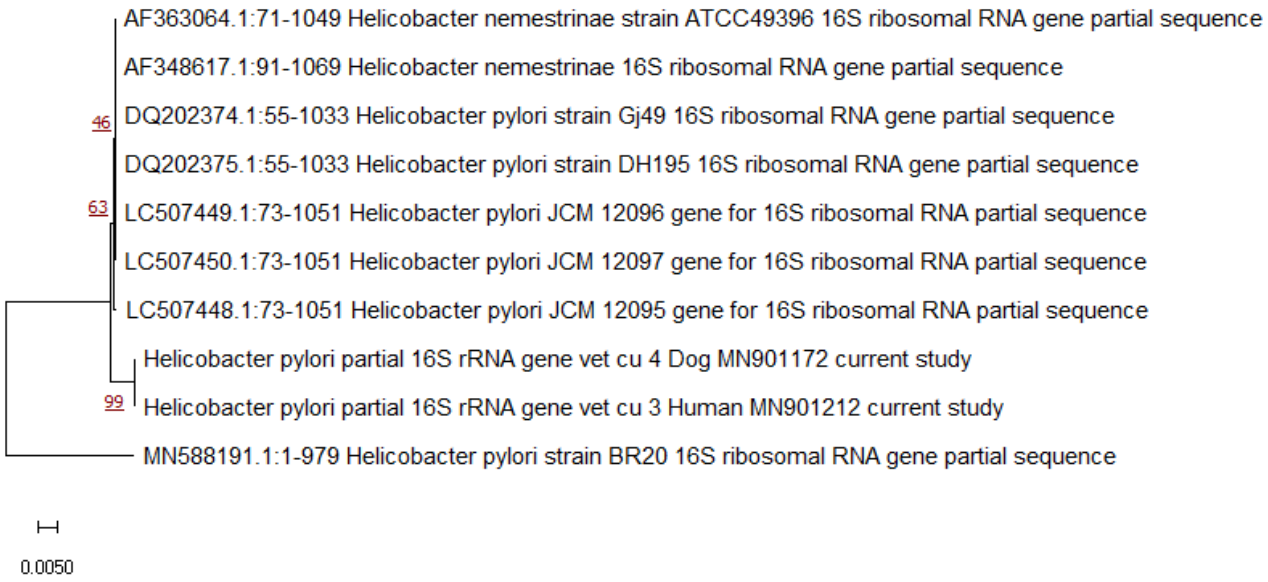

Figure 1. Neighbor-joining tree based on the nucleotide sequences of the partial coding regions of *16s rRNA* gene of *H. pylori* from human and dog isolates. The Evolutionary analysis was performed with MEGA version 7.

DISCUSSION

Previous findings revealed that dogs may play an important role in the transmission of *H. pylori* to humans (Recordati et al., 2007; Abdel-Raouf et al., 2014). Many researches indicated that the prevalence of *H. pylori* is high in dogs and may reach 100% (Wiinberg et al., 2005; Okubo et al., 2017). In the current study, 62.5% of dog stool samples were positive for *H. pylori* by PCR, which is similar to the findings of Hong et al. (2015) who detected *Helicobacter* spp. DNA in feces of laboratory dogs. In a study carried out by Zou et al. (2019) in China, it was found that dogs were the main home-reared animals and the positive rate of *H. pylori* infection was higher in people who breed dogs. Epidemiological data revealed that ownership of dogs is a risk factor for *H. pylori* infection in children in the rural areas and it was concluded that dogs could be a potential source of bacteria (Dore et al., 2002).

The investigations revealed that dogs, especially those suffering from gastric ulcer, may be the reservoir of *H. pylori* and/or might be the original host of this bacterium (Torkan and Shahreza, 2016). A study conducted by Hamza et al. (2018) showed that higher rates of *H. pylori* detection in gastric biopsies of dogs using PCR (76.6%) and cultivation (89.1%).

The prevalence rate of *H. pylori* infection was found to be lower in developed countries than in developing countries. The rate of *H. pylori* infection is generally lower than 30% in developed countries, while it may be as high as 50-70% in developing countries (Pounder and Ng, 1995). Some studies have pointed out that the poor economic status and a lower degree of culture may induce higher *H. pylori* infection rates (Hu, 2008). Poor hygiene conditions and close contact with stray animals could be risk factors that increase the prevalence of *Helicobacter* infections (Bolandi et al. 2017). Moreover, the research conducted by Bolandi et al. (2017) on household dogs raised under hygienic conditions and fed with cooked food revealed a low prevalence (8.66%) of *H. pylori* infection. Although there is a significant presence of *Helicobacter* in dogs, it is not possible to relate it with gastric alterations in these animals (Rossi et al., 1999; Moutinho et al., 2007; Takemura et al., 2007). *H. pylori* infect about 50 % of the world's human population associated with gastric and extra gastric diseases (Bravo et al., 2018). *H. pylori* is responsible for peptic ulcer, gastritis, lymphoma, duodenal ulcer, and gastric cancer (Atapoor et al., 2014; Ghorbani et al., 2016).

In this study, *H. pylori* was detected in 91.6% of dog owners. This differs from El-Shenawy et al. (2017) who reported that the prevalence rate of *H. pylori* in Egyptian patients was 53.1%, but similar to the finding of Abu-Zekry et al. (2013) that *H. pylori* infection was detected by culture method in 70% of gastric biopsy specimens. This difference may be due to a variety of contributing factors, including socioeconomic status, living conditions, and location of each population even in the same country.

In Egypt, serological detection of *H. pylori* was done by Elhariri et al. (2017) in dogs and humans and found that 37.2% and 44.4% were positive, respectively. This indicates the zoonotic importance and the possibility of transmission of disease between dogs and their owners. It is clear that some animals, including cats, dogs, and sheep may be infected by *H. pylori*, but their roles in the transmission to humans are not proved (Mladenova-Hristova et al., 2017).

To cite this paper: Elhelw R, Elhariri M, Ragab E, Kadry M and Hamza D (2021). Dog as a Potential Source of *Helicobacter pylori* in Egypt: Public Health Significance. *World Vet. J.*, 10 (3): 446-450. DOI: https://dx.doi.org/10.36380/scil.2020.wvj55

H. pylori infection in Human probably resulted from a host jump from a different animal (Dewhirst et al., 2005). Host jump is not impossible, in light of the fact that the stomachs of different animals are contaminated with various *Helicobacter* species, whose phylogeny is incongruent with that of their hosts. In fact, the closest known relative of *H. pylori* is *H. acinonychis*, which invade large cats and appears to have emerged by a host jump from humans (Eppinger et al., 2006).

The theory of host jump of *H. pylori* was reinforced by making the phylogenetic relationship between human and dog samples which showing 100% homology between each other and this reflects the possibility of the transmission of this bacteria between dogs and their owners. This finding has been documented previously from all around the world, including Egypt, where (Abdel-Raouf et al., 2014) suggested that *Helicobacter* colonizes the stomachs and intestines of humans and several animal species such as cats, dogs and might have jumped quite recently from animal hosts to people.

CONCLUSION

The high prevalence of *H. pylori* in dog owners proved the role of the owned dogs in the transmission of this pathogen. The evidence of *H. pylori* transmission from dogs to humans enhances the need for *Helicobacter* detection and treatment in pet animals.

DECLARATION

Authors' contributions

Rehab Elhelw, Mahmoud Elhariri, Eman Ragab, Mona Kadry, and Dalia Hamza contributed to the collection of samples, isolation of strains, performing the molecular detection of target genes, analysis and interpretation of the data as well as writing the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors declare that they did not have any funding source or grant to support their research work.

Competing interests

The authors declare that they have no competing interests.

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To cite this paper: Elhelw R, Elhariri M, Ragab E, Kadry M and Hamza D (2021). Dog as a Potential Source of *Helicobacter pylori* in Egypt: Public Health Significance. *World Vet. J.*, 10 (3): 446-450. DOI: https://dx.doi.org/10.36380/scil.2020.wvj55

2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 451-456, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj56

Capacity of *Mentha spicata* (spearmint) Extracts in Alleviating Hormonal and Folliculogenesis Disturbances in a Polycystic Ovarian Syndrome Rat Model

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ORIGINAL ARTICLE
pii: S232245682000056-10
Received: 02 Aug 2020
Accepted: 14 Sept 2020

ABSTRACT

Polycystic ovary syndrome, a common cause of infertility among women in the reproductive age, is associated with high levels of androgens. Recognizing the anti-androgenic effects of spearmint, the present study aimed to evaluate the effects of its hydroalcoholic extract on the levels of luteinizing hormone, follicle-stimulating hormone, and testosterone and ovarian folliculogenesis in normal and letrozole-induced polycystic ovary syndrome rats. Female mature rats were divided into six groups (n=8 per group), as follows: Normal rats (I or Control), normal rats which received 250 mg/kg spearmint extract (II) or 500 mg/kg spearmint extract (III), and PCOS-induced rats (IV), PCOS-induced rats which received 250 mg/kg spearmint extract (V), or 500 mg/kg spearmint extract (VI). At the end of the experiment the animals were euthanized, and then mentioned parameters were evaluated. Administration of spearmint extract to PCOS rats resulted in a decrease of body weight and testosterone level, higher corpus luteum, and lower ovarian cysts and atretic follicles, compared to PCOS rats which received no spearmint. Accordingly, the spearmint can attenuate polycystic ovarian syndrome-related problems, such as a high testosterone level and ovarian cysts.

Keywords: Folliculogenesis, *Mentha spicata*, Ovary, PCOS, Rat

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common hormonal disorder among women in the reproductive age. It has been demonstrated that genetic factors, hormonal disorders, lifestyle, environmental factors and stress contribute to the development of this syndrome (Shaikh et al., 2014a; Krishnapillai et al., 2015). PCOS is often associated with a high level of androgen hormones, obesity, insulin resistance and oligomenorrhea or anovulation (Hatirnaz et al., 2015). Since a high level of androgens is considered the essential factor in PCOS, the animal model of PCOS is frequently created by androgenizing of animals (Van Houten and Visser, 2014). Medicinal plants have been traditionally used as natural medications, and played vital roles in disease prevention and their promotion (Monsefi and Masudi, 2014; Sharangouda et al., 2015; Namavar Jahromi et al., 2019). *Mentha spicata*, known as spearmint, is a medicinal plant which in Iran's traditional medicine is mainly recommended for digestive system disorders as a carminative and antispasmodic agent, and also for alleviating hirsutism and menstrual pain (Vejdani et al., 2006). The antioxidant, anticancer, anti-inflammatory, antifungal, antimicrobial, and antidiabetic properties of *Mentha spicata* have been shown in some studies (Guimarães et al., 2011; Alaee et al., 2016).

It is known that spearmint is beneficial in decreasing free testosterone level and hirsuitism in women with mild hirsutism with PCOS, and its adverse histopathological effects on kidney, liver and uterine tissue in animals were observed (Akdogan et al., 2003; Akdogan et al., 2004; Akdogan et al., 2007; Guney et al., 2006; Grant, 2010). The effects of this herbal plant on folliculogenesis of ovarian tissue in normal and PCOS conditions were not determined. Present study was designed to evaluate the effects of spearmint extract on follicle stimulating hormonr (FSH), luteinizing hormone (LH), testosterone hormones and ovarian folliculogenesis in the animal model of PCOS induced by letrozole.

MATERIALS AND METHODS

Preparation of *Mentha spicata* hydroalcoholic extract

Mentha spicata was purchased from Pursina Pharmaceutical Company, Tehran, Iran. Hydroalcoholic extract was prepared using the maceration method (Monsefi et al., 2015). The dried leaves of the plant were powdered and

To cite this paper: Alaee S, Jafar Bagheri M, Sadeghi Ataabadi M and Koohpeyma F (2021). Capacity of *Mentha spicata* (spearmint) Extracts in Alleviating Hormonal and Folliculogenesis Disturbances in a Polycystic Ovarian Syndrome Rat Model. *World Vet. J.*, 10 (3): 451-456. DOI: https://dx.doi.org/10.36380/scil.2020.wvj56

macerated in ethanol for 3 days. Then the solution from the total extract was filtered with filter paper, concentrated by evaporation and stored in refrigerator until being used for the experiments. The yield (w/w) of the solution was 13% (g/g).

Animals

Forty-eight mature Wistar albino female rats were obtained from animal house of Shiraz University of Medical Sciences, Shiraz, Iran. Prior to use in the study, rats were kept in cages in temperature-controlled rooms with constant humidity and 12 hr/12 hr light/dark cycle with free access to standard diet and water. For selection of rats with normal estrus cycle, daily vaginal smears were carried out and immediately evaluated with a light microscope (Monsefi et al., 2013). Wistar female rats with two normal estrus cycles were weighed and allocated into the six groups (n: 8) as below; Group I (control): Received 1 ml distilled water orally for 20 days; Group II: Received spearmint extract (250 mg/kg) for 20 days; Group III: Received spearmint extract (500 mg/kg) for 20 days; Group IV: Received letrozole orally for 28 days; Group V: Received letrozole orally for 28 days, and then received spearmint extract (250 mg/kg) for 20 days; Group VI: Received letrozole orally for 28 days, and then received spearmint extract (500 mg/kg) for 20 days. Polycystic ovary syndrome induction was carried out by treating rats daily with letrozole (Femara®, made by Novartis Pharmaceuticals Corp., Basel, Switzerland) orally (1 mg/kg) for 28 days, and confirmed by persistent estrus phase and high number of ovarian cysts in ovarian sections via hematoxylin and eosin staining (Neisy et al., 2019).

After treatment duration, animals were weighed, euthanized by inhalation of ether, and a blood sample was taken for hormonal analysis. In addition, the ovarian tissues of all rats were removed and prepared for the histological evaluation (Sadeghi et al., 2017).

Hormonal assay

Blood samples were collected from the heart, and were centrifuged at 3000 rpm for 15 min. Serum portions were separated and frozen until being evaluated. Serum concentrations of testosterone (Padtan Elm Company, Tehran, Iran), LH and FSH were measured with their specific kits (Hangzhou Eastbiopharm Co., Ltd., Hangzhou, China) (Sadeghi et al., 2017).

Histological analysis

Ovarian tissues were removed, fixed in 10% buffered formalin solution, and the paraffin blocks were prepared. The blocks were sectioned at 5µm thickness, and were stained in the hematoxylin and eosin method (Alaee et al., 2014). The number of primordial follicles, primary follicles, secondary follicles, Graafian follicles, atretic follicles, corpus luteum and cysts were counted in ovarian sections using a light microscope (Olympus, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 16 software (IBM, Armonk, USA). For data analysis, the One-Way ANOVA test was used, followed by the Tukey test to compare the means. P value of ≤ 0.05 was considered statistically significant.

Ethical approval

The study protocol was approved by the Animal Ethical Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S1066), and was carried out in accordance with the university's Guideline for the Care and Usage of Laboratory Animals.

RESULTS

The results are presented in two parts; **A**: Evaluation of the effects of spearmint extract on normal female rats (comparing groups I, II and III). At the end of the experiment, the body weight of the animals that received spearmint extract was not different from that of the control group ($p>0.05$, table 1). The level of LH, FSH and testosterone did not change among spearmint extract–administered groups in comparison to the control group ($p>0.05$, table 1). Administration of spearmint extract in two doses of 250 and 500 mg/kg (groups II and III) led to a significant decrease in the number of primordial follicles ($P<0.001$). In addition, the number of primary follicles, secondary follicles, Graafian follicles and corpus luteum decreased in these groups compared to the control group, but it was not statistically significant ($p>0.05$). However, the number of atretic follicles was significantly higher in the groups administered spearmint extract compared to the control group ($p < 0.001$, table 2). B: Evaluation of the effects of spearmint extract on PCOS-induced rats comparing with groups C (control), IV, V and VI. At the end of the study, the body weight in the PCOS-induced group was significantly higher than those in the control group (P: 0.031).

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At the end of the experiment, in both PCOS-induced groups that received spearmint extract, the rats' weights were not different from that of the control group (P > 0.05). In the PCOS-induced group that was administered high doses of spearmint extract, weights were significantly less in comparison to those of PCOS-induced rats (P: 0.001, table 1). The level of LH and FSH showed no significant alteration among control, PCOS-induced groups and PCOS-induced groups that received spearmint extract (p > 0.05), but the level of testosterone in the PCOS-induced group was significantly higher in comparison to the control group (p < 0.001). In PCOS-induced groups that took two doses of spearmint extract, testosterone level was significantly lower in comparison to the PCOS-induced group (p < 0.001, table 1).

The number of primordial follicles was significantly lower in the PCOS-induced group, and in the PCOS-induced groups which received spearmint extract compared to control group (P < 0.001). The number of primary follicles was not different in PCOS-induced rats by comparing to the control group and the PCOS-induced rats which took spearmint extract (P > 0.05). The number of secondary follicles was meaningfully lower in the PCOS-induced group and those which received spearmint extract as compared to the control group (P: 0.033). There were no Graafian follicles in the PCOS-induced group, and in those which had spearmint extract, but this was not statistically significant comparative to the control group (P > 0.05). The number of atretic follicles and cysts was considerably higher in the PCOS-induced rats in contrast to the control group (P < 0.001), and in the PCOS-induced group compared to PCOS-induced groups which received spearmint extract (P < 0.001) (Figure 1). The number of corpus lutea in PCOS-induced rats was also meaningfully lower than the control group (P: 0.001), and its number increased significantly in PCOS-induced rats who took the spearmint extract (250 and 500 mg/kg) in comparison to PCOS-induced rats (P < 0.001).

Table 1. Body weight at the beginning and end of the experiments, and the level of luteinising hormone, follicle stimulating hormone and testosterone of female rats in studied groups

Groups	Weight at the beginning (g)	Weight at the end (g)	LH (ng/dl)	FSH (ng/dl)	Testosterone (mIU/ml)
(I) Control	152.37 ± 10.04	199.25 ± 13.54	23.00 ± 1.90	15.80 ± 1.30	0.40 ± 0.09
(II) Extract (250 mg/kg)	158.00 ± 12.66	207.87 ± 17.23	22.50 ± 1.70	13.41 ± 2.04	0.29 ± 0.08
(III) Extract (500 mg/kg)	160.37 ± 13.00	195.12 ± 12.71	22.90 ± 1.9	14.90 ± 2.29	0.30 ± 0.06
(IV) PCOS	158.12 ± 9.17	223.00 ± 4.035*, †	24.70 ± 1.50	13.86 ± 0.60	3.70 ± 0.90*,††
(V) PCOS + Extract (250 mg/kg)	154.12 ± 7.19	210 ± 19.27	23.00 ± 1.80	13.23 ± 0.43	1.27 ± 0.43
(VI) PCOS+ Extract (500 mg/kg)	151.75 ± 5.99	190.25 ± 17.63	22.40 ± 1.40	14.90 ± 1.20	1.05 ± 0.2

Data are shown as mean ± SD. P ≤ 0.05 is considered statistically significant. *: Significant differences between PCOS-induced group (IV) and control group. †: Significant differences between PCOS-induced group (IV) and group VI. ††: Statistically significant differences between PCOS-induced group (IV) and groups V and VI.

Table 2. The number of primordial, primary, secondary, Graafian and atretic follicles, corpus lutea and cysts in ovarian tissue of the studied groups

Groups	Primordial follicles	Primary follicles	Secondary follicles	Graafian follicles	Atretic follicles	Corpus luteum	Ovarian cysts
(I) Control	7.81±3.22*, ††	8.62±1.08	5.56±1.09§	0.31±0.40	2.12±1.14*†	5.93±1.52†	0†
(II) Extract (250 mg/kg)	3.12±1.99	7±2.06	4.18±2.07	0.25±0.44	10.06±3.60	4.93±1.84	0
(III) Extract (500 mg/kg)	0.87±0.61	7.18±1.79	4.65±2.06	0.43±0.72	7.18±4.47	4.37±1.70	0
(IV) PCOS	2.93±1.34†	7.37±1.50	3.31±1.50	0	12.56±1.96•	1.31±1.07•	10.18±3.01•
(V) PCOS + Extract (250 mg/kg)	2.12±1.08	6.43±1.78	2.25±1.73	0	7.93±2.73	4.06±1.98	0.18±0.54
(VI) PCOS + Extract (500 mg/kg)	2.18±2.1	8.00±1.15	2.5±1.54	0	7.56±2.73	5.18±1.79	0.12±0.34

Data are shown as mean ± SD. P ≤ 0.05 is considered statistically significant. *: Significant differences between group II, III and control group. †: Significant differences between PCOS-induced group (IV) and control group. ††: Significant differences between control and groups V and VI. §: Significant differences between control and groups IV, V and VI. •: Significant differences between PCOS-induced group (IV) and groups V and VI.

To cite this paper: Alaee S, Jafar Bagheri M, Sadeghi Ataabadi M and Koohpeyma F (2021). Capacity of *Mentha spicata* (spearmint) Extracts in Alleviating Hormonal and Folliculogenesis Disturbances in a Polycystic Ovarian Syndrome Rat Model. *World Vet. J.*, 10 (3): 451-456. DOI: https://dx.doi.org/10.36380/scil.2020.wvj56

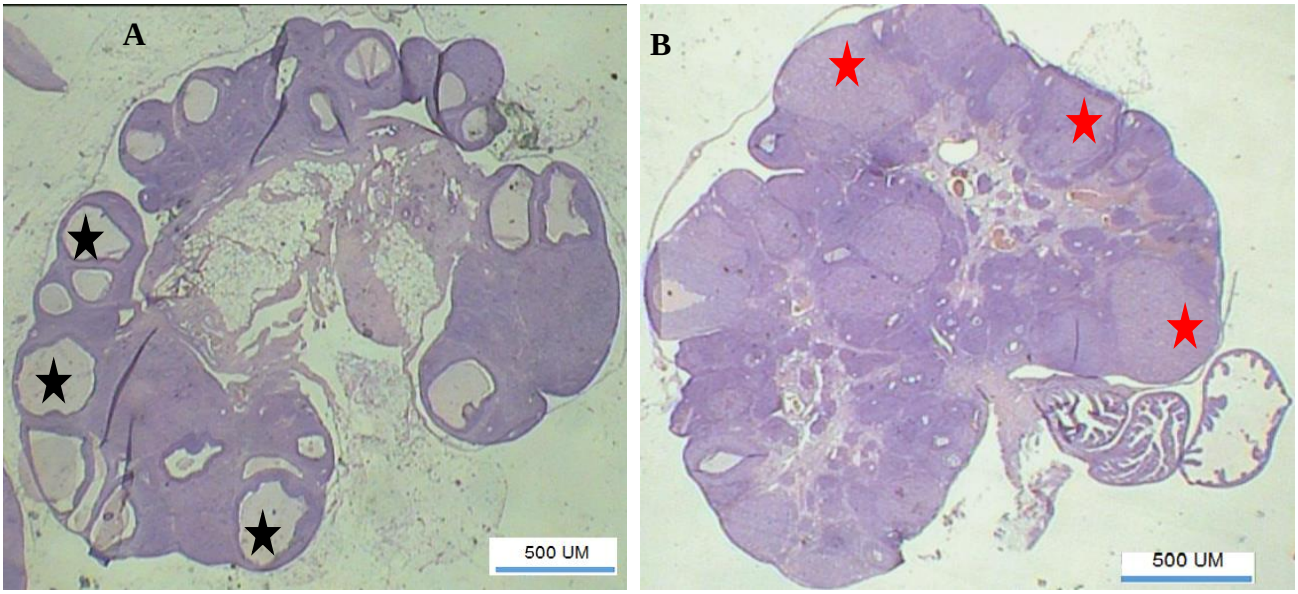

Figure 1. Light photomicrograph of ovarian tissue of the rats. Hematoxylin and eosin staining, 40× magnification. **A**: Ovarian tissue of polycystic ovary syndrome rats displays a considerable number of ovarian cysts. **B**: Ovarian tissue of polycystic ovary syndrome rats received spearmint extract (500 mg/kg) demonstrates no ovarian cyst. Treatment with spearmint extract has increased corpora lutea in this group compared with the polycystic ovary syndrome group. The red stars show corpora lutea, and the black stars show cystic follicles.

DISCUSSION

Nowadays, infertility is an important major concern of many couples which affects both men and women. PCOS is a condition that affects a woman's hormone levels that may lead to infertility related problems. Women with PCOS tend to have higher levels of androgens (Akdogan et al., 2007; Alaee et al., 2019). According to anti-androgenic effects of *Mentha spicata* and its beneficial effect in women with mild hirsutism and PCOS (Akdogan et al., 2007; Grant, 2010), in the current study, spearmint extract was administered to an animal model of PCOS to determine the effects of this herbal plant on LH, FSH and testosterone hormone levels, and also on folliculogenesis of ovaries. In addition, the effects of the extract on mentioned parameters were studied in normal rats. Measurement of body weight changes was one of the established methods to evaluate the toxicity of plant extracts (Gupta and Sharma, 2006). Since there was no significant change in the body weight of normal rats after administration of spearmint extract, it seemed that this medicinal plant has no general toxicity effect, which was also confirmed in other studies (Nozhat et al., 2004; Sadeghi et al., 2017). Furthermore, the spearmint at the level of administered doses had no effect on LH, FSH and testosterone levels in normal rats. Given the significant increase in the number of atretic follicles in normal rats which received spearmint extract, this agent may have detrimental effects on the ovarian folliculogenesis. Other studies have also demonstrated detrimental effects of spearmint tea on uterine, kidney and liver (Akdogan et al., 2003; Akdogan et al., 2004; Guney et al., 2006). Androgens are essential drivers of early and intermediate stages of follicular maturation. Locally produced androgens facilitate follicular development and serve as a substrate for estrogen production in the later stages of folliculogenesis (Pan et al., 2015). The optimum level of this hormone is crucial, because an excess level of androgens overrides follicular development, resulting in follicular arrest, follicular atresia and disturbance of ovulation (Gleicher et al., 2011).Therefore, the higher number of atretic follicles may be attributed to anti-androgenic effects of spearmint, but it should be determined whether the levels of testosterone and the number of corpus lutea, which are a manifestation of ovulation, did not decrease in the normal rats receiving spearmint extract. The disruption of follicular development in these groups may be related to the alteration in the level of other kinds of androgens that were not evaluated in our study, such as dihydrotestosterone and androstenedione, which are also involved in the growth and development of ovarian follicles in mammals (Cupisti et al., 2008, Lebbe and Woodruff, 2013). PCOS is a metabolic disease usually accompanied by insulin resistance, visceral obesity and elevated body mass index, all of which are correlated with an elevated level of oxidative stress and androgen production of the ovaries and adrenal glands, thus it contributes to the disturbed follicular development, oocyte maturation, and, ultimately, infertility (Shaikh et al., 2014b; Hussain et al., 2015; Papalou et al., 2016). The results showed that administration of spearmint extract to PCOS rats significantly reduces body weight and testosterone level.

In the present study, the weight did not change in normal rats that received spearmint extract. Current study found that spearmint has no effect on body weight in a normal condition. However, in PCOS condition, it may control and sustain body weight which may occur thorough metabolic mechanisms that were disturbed in the condition of PCOS.

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Therefore, spearmint extract may initiate a complicated mechanism that results in control of body weight, and also reduction of testosterone. It was demonstrated that spearmint leaves decrease cholesterol, and in type II diabetes, decrease oxidative stress, and improve activity of antioxidant enzymes (Rajeshwari et al., 2012). Al-Rekabi (2015) showed that administration of phenolic compounds of *Mentha spicata* leaves extract to diabetic male rats ca significantly enhance the antioxidant defense system, and reduce body weight and levels of glucose and cholesterol. Grant (2010) showed that administration of spearmint tea for 30 days significantly reduced free and total testosterone in PCOS women. A significant decrease in free testosterone level was also observed in women with hirsutism after receiving spearmint teas (Akdogan et al., 2007). It was shown that in the PCOS condition, ovarian steroidogenic enzyme deficiencies, such as aromatase deficiency, induced a hyperandrogenemic environment in the ovary, contributing to follicular maturation arrest and oligoovulation or anovulation (Rajeshwari et al., 2012). Reducing the body weight of anovulatory obese women decreased testosterone concentration, restored ovulation, and improved menstrual function and conception rates (Moran et al., 2003). However, in this study, although administration of spearmint led to a significant decrease in the body weight and testosterone level of PCOS-induced rats, the attenuated number of follicles caused by PCOS induction was not improved. Yet, similar to an identical study in which the effects of spearmint oil on PCOS were evaluated, the number of corpus luteum increased after the spearmint extract was administrated, reflecting the higher rate of ovulation in this group (Sadeghi et al., 2017). The high number of atretic follicles and ovarian cysts observed in PCOS-induced rats was thought to be associated with a high level of androgen. In PCOS animals that took spearmint extract, the number of atretic follicles and ovarian cysts decreased considerably, which could be associated with the anti-oxidant and anti-androgenic effects of the spearmint.

CONCLUSION

Spearmint as an anti-androgenic herb is believed to reduce testosterone level in PCOS condition, resulting in destruction of ovarian cysts and restoration of ovulation. It is suggested for future studies to evaluate ovarian antioxidant capacity and apoptosis status, and also fertility potential of PCOS-induced female rats after receiving spearmint extract at the level of mentioned dose used in this study.

DECLARATIONS

Acknowledgments

The authors of present article thank the Student Research Committee, Shiraz University of Medical Sciences, for the ethical approval and financial support of this research (grant number: 1396-01-21-15581).

Competing interests

The authors declare that there are no conflicts of interest.

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To cite this paper: Alaee S, Jafar Bagheri M, Sadeghi Ataabadi M and Koohpeyma F (2021). Capacity of *Mentha spicata* (spearmint) Extracts in Alleviating Hormonal and Folliculogenesis Disturbances in a Polycystic Ovarian Syndrome Rat Model. *World Vet. J.*, 10 (3): 451-456. DOI: https://dx.doi.org/10.36380/scil.2020.wvj56

2020, Scienceline Publication
World's Veterinary Journal
World Vet J,10(3): 457-464, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj57

Comparison of Serological Tests in Cattle and Ovine Brucellosis; An Abattoir Study in Algeria

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ORGINAL ARTICLE
pii: S232245682000057-10
Received: 03 Aug 2020
Accepted: 21 Sept 2020

ABSTRACT

Despite the paucity of data, brucellosis is considered as a major problem in Algeria. The aim of present study was to assess the presence of bovine and ovine brucellosis in the areas close to the capital city (Algiers) where its vaccination is not implemented. A total of 402 cattle and 203 ovine sera were collected from two slaughterhouses, and examined by the Rose Bengal Test (RBT). Positive samples were then tested by Complement Fixation Test (CFT) and Hypertonic Double Gel Diffusion (DDG) with a smooth lipopolysaccharide, and the extract of native hapten was also tested by Indirect Enzyme Linked Immuno Sorbent Assay (iELISAs) with smooth lipopolysaccharide and polyclonal or protein G conjugates. Twenty-four bovine sera (5.97%) were RBT positive. Of these, 23 were positive in CFT, DDG, and 16 samples were also positive in iELISA when the assay was adjusted to 100% specificity. Only two ovine sera were RBT positive; one was CFT and DDG positive, and the other one had a CFT-titer of 1/4, and was DDG negative. This preliminary study confirmed that bovine brucellosis is a major problem in Algeria, and indicated that some field studies are needed to determine the prevalence of Brucellosis in Algeria urgently. Similarly, other studies are necessary in areas with dominance of ovine breeding system. Further studies in the areas with a dominance of ovine breeding system are necessary. The results of this work showed that simple tests like RBT and DDG are not outperformed by CFT or iELISA for assessing the apparent prevalence of brucellosis in the absence of vaccination. Finally, isolation and typing of the involved *Brucella* species are also necessary in order to have a complete epidemiological picture of brucellosis in Algeria.

Key words: Abattoirs, Algeria, Brucellosis, Cattle, Serology, Sheep, Prevalence

INTRODUCTION

Bacteria of the genus *Brucella which* causes brucellosis is a highly contagious zoonosis affecting primarily domestic livestock and a variety of wild-life species. Brucellosis in animals is characterized by a reproductive failure, and the human form of this disease is a grave and debilitating condition requiring prolonged and costly antibiotic treatments. Thus, this disease presents a serious threat to the animal industry and public health (McDermott et al., 2013). Brucellosis is considered as one of the most widespread zoonosis in the world (Hull and Schumaker, 2018) and it has a great impact on emerging economies and resources of poor countries (Grace et al., 2012). Although it has been eradicated in ruminants in most European countries, the USA, Canada, Australia and a few other countries, brucellosis is still a endemic disease in many parts of the world, especially in North and East Africa, the Middle East, South and Central Asia and Central and South America (Corbel, 2006). Moreover, intensification of breeding caused by raising food demand increased the risk of spread of this and other zoonosis (Jones et al., 2013).

Humans contract the disease from animals or their products, but they are not considered as a source of contagion. Thus, under most circumstances, human brucellosis is the best indicator of the existence of the disease in animals. Regarding, the high number of cases for human brucellosis reported in Algeria is indicative of the important impact of this zoonosis in the country, 819 human cases were recorded in first four months of 2016 (Robert, 2016). The intervention against brucellosis includes implementation of dairy food sanitation, specific measures to protect risk groups (farmers, abattoir workers and veterinarians), and control and eradication of the disease in livestock. In Algeria, despite the control program initiated in 1970 and strengthened in 1995 which was based principally on a test-and-slaughter strategy, bovine brucellosis is a serious problem in dairy herds in all likelihood as suggested by the consistent presence of clinical symptoms compared with the disease and occasional serological testing. About 30 years ago, Benelmouffok et al. (1984) summarized the previous evidence indicating high (up to 25%) apparent seroprevalence as determined by the Standard Serum Agglutination Test (SAT). More recently, apparent herd seroprevalence of 26.3% using Rose Bengal Test (RBT) and 15.7% by complement fixation test (CFT) were reported for cattle in the Western area of Algeria (Tiaret)

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(Aggad and Boukraa, 2006). Although these reports suggest a high prevalence of bovine brucellosis, the paucity of data and their long-time span clearly indicated the need for additional studies. Similarly, there is a paucity of works on brucellosis in small ruminants (Hamdi-Chérif et al., 1999; Meskoud-taibi and Benzadi, 2009). However, while cattle are usually infected by *B. abortus*, sheep and goats are infected by *B. melitensis*, the *Brucella* species are considered to be more infectious for human (O'Callaghan, 2020). Accordingly, a first goal of the research described here was to obtain additional data on the status of brucellosis in Algeria. To this end, a serological study was carried out on two slaughterhouses that process animals coming from different areas of the country.

Because of the absence of pathognomonic symptoms, the diagnosis of brucellosis requires laboratory tests. Bacteriological culture is cumbersome and dangerous, but serological tests are routinely used. However, there is a bewildering list of serological tests (Ducrotoy et al., 2016), and most new developments are focused on tests suitable for surveillance of large numbers of animals in *Brucella*-free areas that require conditions not met in resource-limited settings (McGiven, 2013; Ducrotoy et al., 2015). Thus, a second aim of the present work was to compare apparent prevalence figures obtained with simple RBT, more sophisticated and highly-sensitive tests like indirect ELISA (iELISA) with tests of higher specificity that also differ in the degree of sophistication (CFT and double gel diffusion (DDG) with smooth-lipopolysaccharide (S-LPS) and native hapten polysaccharide (NH).

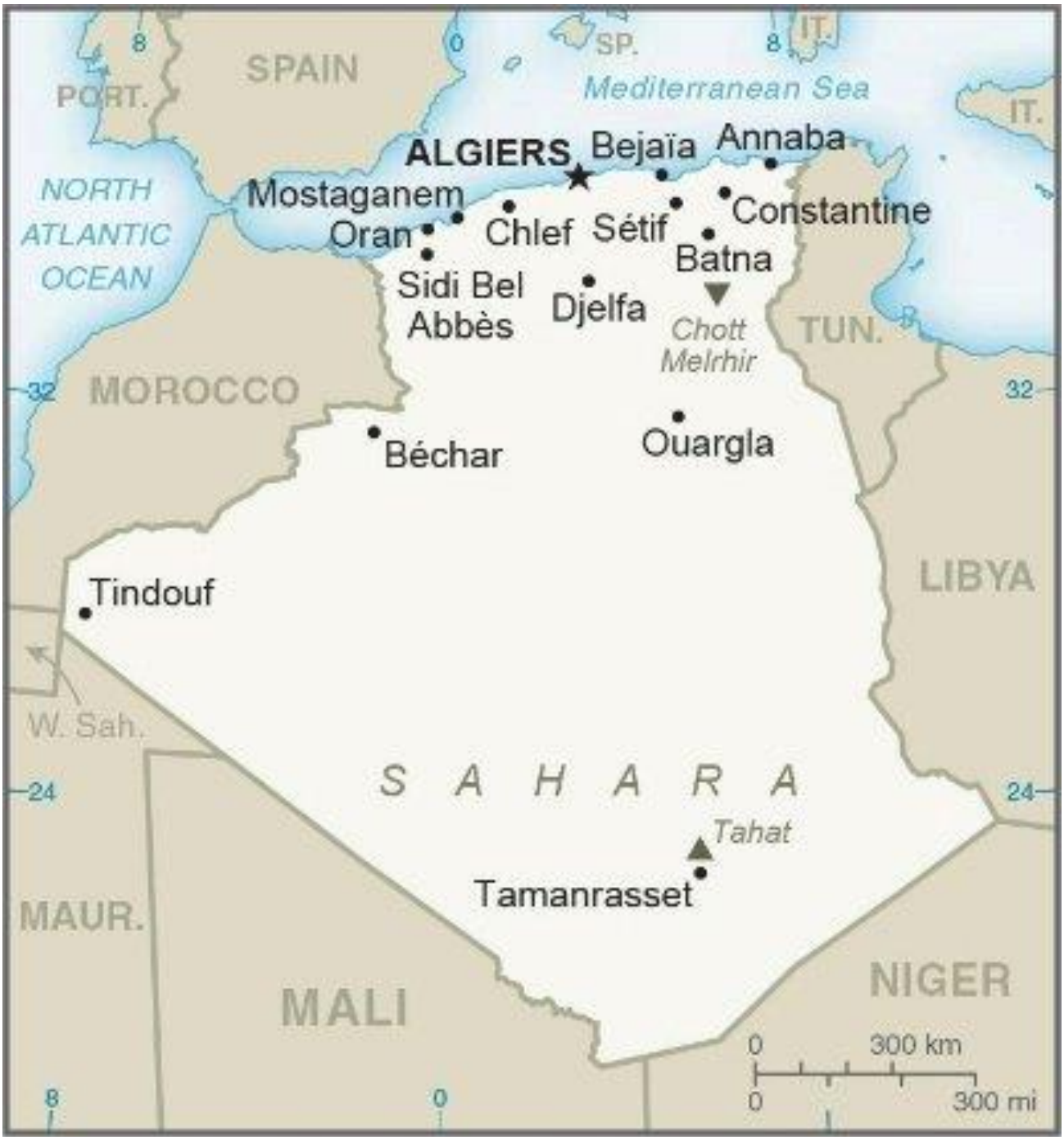

Figure 1A. General map of Algeria (http://www.state.gov/p/nea/ci/ag/)

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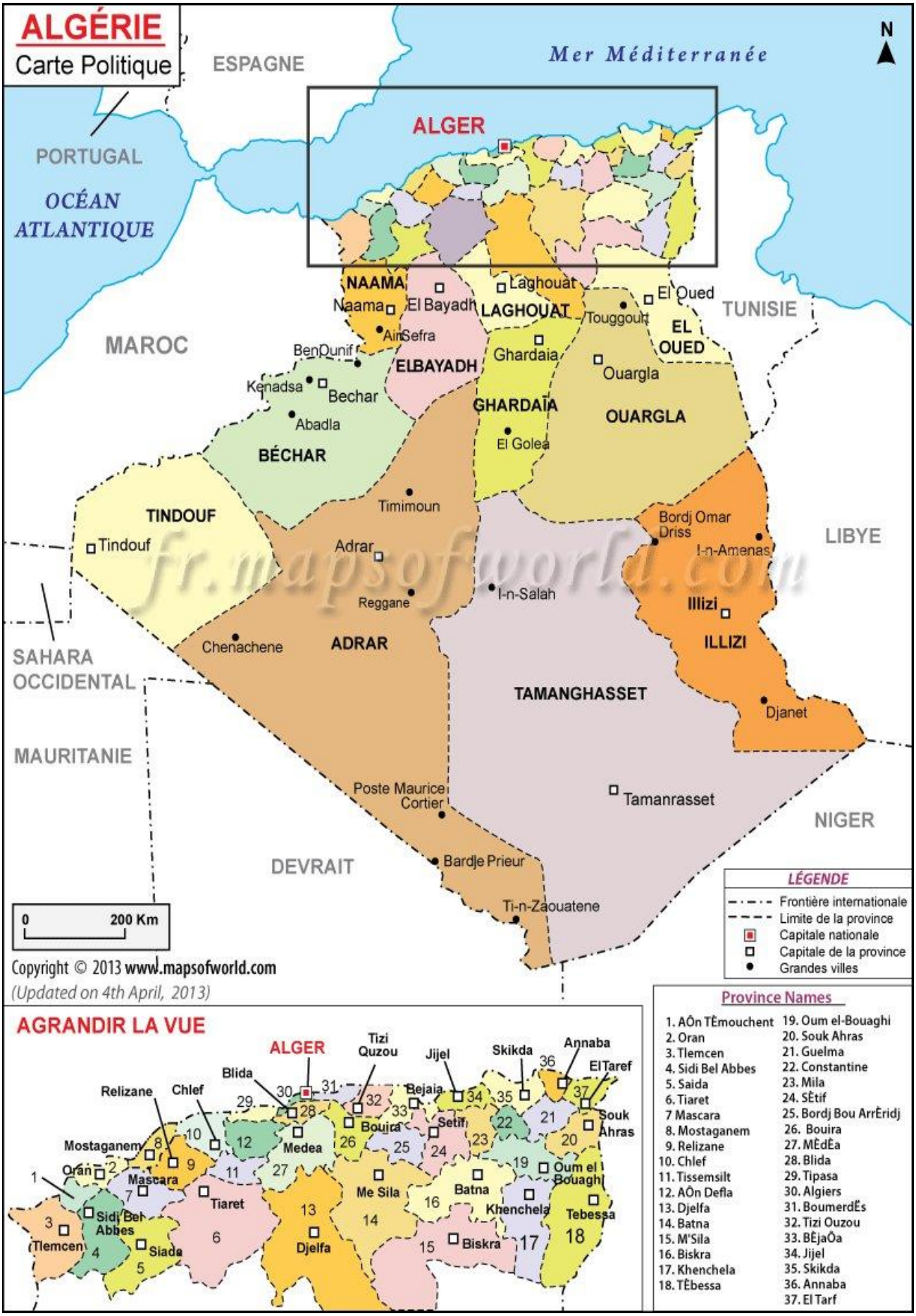

Figure 1B. The *Wilayates* close to Algiers (http://www.algerieprofonde.net/algerie/cartes-dalgerie/). The caption shows the total number of cattle tested from each place and the prevalence percent:Ain-Defla, n: 74 (0.00%); Alger (Algiers), n: 86 (1.16%); Blida, n: 58 (13.80%); Bouira, n: 151 (7.28%); Boumerdes, n: 27 (7.40%); Chlef and Relizane, n: 5 (20.00%); Médéa, n: 1 (100.00%).

To cite this paper: Khames M, Zúñiga-Ripa A, Pérez Gómez S, Oumouna M and Moriyón I (2021). Comparison of Serological Tests in Cattle and Ovine Brucellosis; An Abattoir Study in Algeria. *World Vet. J.*, 10 (3):457-464. DOI: https://dx.doi.org/10.36380/scil.2020.wvj57

MATERIALSAND METHODS

Ethical approval

Samples were collected as per the standard sample collection procedure without any stress or harm to the animals, according to the rules of the Veterinary School of Algiers, Algeria.

Study site, period and sample size

The study was carried out in two slaughterhouses in Algiers, Algeria. Originally in the outskirts, the Rouiba slaughterhouse is now in the urban area. This slaughterhouse is working each day of the week, and animals from different areas are processed. So, it made it possible to trace the geographical origin of the cattle. El-Harrach slaughterhouse (about 2600 m^2) is located in the urban area, and it was not possible to trace the origin of the animals due to the work's conditions there. For the Rouiba slaughterhouse, the study included the entire cattle population (402heads: 264females and138males) of every breed (local, cross-breed and montbeliarde) and age sacrificed from 26 February to1 April 2012, and63 non-vaccinated sheep (19 females and 44 males) were sacrificed from1to 28 February 2012; for El-Harrach slaughterhouse, 140 non-vaccinated sheep of different breeds like ouled djellal and el rembi, and different age categories (86 females and 54 males) were sacrificedfrom1 to 31 March 2012.

Ante mortem inspection

An inspection to determine the sex, age and identification status on both ears was performed, and three types of cattle were defined; imported (Montbeliarde cattle; foreign identification tag), local (small, uniform colored dress - black or brown - no tag), Magpie black or red magpie crossbreed cattle (with or without tag (deleted or removed)).

Sample collection

Five milliliters of blood were collected from the jugular or the caudal vein with Vacutainer-type tubes, or directly at the moment of scarification, and all data (breed, age, sex and pregnancy) were recorded. The blood was let to clot at the room temperature, the sera were collected by centrifugation, 3000 tours per minute for 15 minutes, and finally stored at -20°C until being analyzed.

Serological tests

All cattle's serum samples were analyzed by the RBT using the official Spanish antigen (kindly provided by Dr. J.M. Blasco, Centro de Investigación en Tecnologia Agraria, Gobierno de Aragón, Spain) according to the standard procedure (OIE, 2016). Briefly, after equilibrating the antigen suspension at the room temperature, an equal volume (25µl) of antigen and serum were mixed on a white tile with a toothpick, and shaked by hands for 4 minutes. No matter the intensity, definite clumping/agglutination was considered as a positive reaction. Sheep's serum samples were analyzed by the modified RBT (mRBT) (Blasco et al., 1994;Diaz-Aparicio et al., 1994;OIE, 2016). A volume of 25µl of antigen and 75µl of serum were used. In all cases, RBT-positive serum samples were then retested for CFT which was performed by the standard micro method technique (OIE, 2016)(a titer equal to 4 contained 20 international CFT units per ml). In addition, RBT-positive sera(or MRBT for sheep) were analyzed by the DGD immunoprecipitation test with a hot extract of *B. melitensis*16M rich in native hapten (NH)and Smooth lipopolysaccharides (S-LPS) in 10% NaCl hypertonic gels (Diaz et al., 1981). Two antigen concentrations were used: 1 and 5mg/ml. Finally, an in-house iELISA with polyclonal and protein G conjugates and smooth *B. abortus* S-LPS was performed as described before (Alonso-Urmeneta et al., 1998). Plates included both a positive and a negative control, and the results were expressed as the optical density (%OD) of the positive control. The iELISA and DDG antigens were supplied by the Brucellosis Laboratory, Institute of Tropical Health, University of Navarra. Complement fixation test (CFT) was performed at the laboratory for food quality, Government of Navarra, Navarra, Spain. The Laboratorio de Calidad Agroalimentaria, Gobierno de Navarra, Villaba, Navarra, Spain.

Statistical analysis

The results were analyzed using the Chi square test (SPSS, version 19) and P values < 0.05 were considered significant. Confidence intervals were calculated according to Toma et al. (1996)

RESULTS AND DISCUSSION

Cattle

Twenty-four samples of bovine sera were positive by RBT (Apparent prevalence: 5.97%, 95% confidence interval, 5.95 – 5.99). Of these, 23 were positive by CFT, DDG (i.e. apparent prevalence: 5.72% CI (5.7–5.74in all cases), and 16 samples by iELISA (i.e. apparent prevalence: 3.98% CI (3.96–4)). By CFT, 18 samples had titers equal to or higher

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than1/256; 2, 1/128; 1, 1/64; 1, 1/16 and 1, 1/4. By DDG with the antigen at 1mg/ml, 21 serum samples developed both the LPS and NH precipitin lines, one only with the S-LPS precipitin line, one only with the NH precipitin line, and one was negative. The serum that developed only the S-LPS precipitin line which reacted weakly in RBT had a CFT titer equal to 1/4, and was positive in iELISA. The serum that was negative by DDG was also negative in the iELISA, but positive in the RBT and the CFT. For the DDG, the results were not improved by increasing the antigen concentration to 5mg/ml.

It has been consistently observed that a positive result of immunoprecipitation tests with extracts rich in S-LPS and NH correlated with a positive CFT (i.e. ≥1:4 (20 international units) or higher, so that the former can be substituted for the latter in the classical diagnostics scheme that started with RBT screening. However, while CFT is technically demanding, the DDG with these extracts represented a much simpler and repeatable test (Diaz et al., 1979;Jones et al., 1980;Díaz et al., 1984;Diaz-Aparicio et al., 1993). Moreover, it has been observed that a positive result in the DDG test correlates with the shedding of *Brucella* which makes this test particularly useful in difficult epidemiological situations when identification of shedders becomes necessary to control brucellosis (Jones et al., 1980). The results presented here confirmed the previous investigations that demonstrated that a combination of RBT and DDG can be implemented without a loss of performance with respect to that obtained with more sophisticated CFT and iELISA (Diaz et al., 1979; Jones et al., 1980; Diaz et al., 1981; Diaz et al., 1983; Muñoz et al., 2005). It has been argued that the immunoprecipitation tests with S-LPS and NH are not sensitive enough to be useful in the diagnosis of brucellosis, and that they are outperformed by immunoenzymatic tests with diagnostic sensitivity and specificity adjusted according to the Youden or performance indexes (Nielsen, 2002). However, because brucellosis does not spread epizootically, early detection requiring 100% diagnostic sensitivity is not as critical as in highly transmissible epizootic diseases. Moreover, the diagnosis of brucellosis should be interpreted on a herd/flock rather than on an individual basis, and when the objective is the culling of positive animals in eradication contexts, it is repeated frequently over time which facilitates the detection of infected animals. Thus, 100 percent of DSe is not strictly necessary for brucellosis tests, and low DSp generates unnecessary culling with the ensuing conflicts with farmers, needless quarantines, and trade and policy problems (Ducrotoy et al., 2018). Quantitative tests such as iELISA are usually adjusted according to the Youden index which maximizes the DSe/DSp balance. Yet, as shown here, when the DSp was adjusted to 100%, the DSe decreased with regard to the simpler RBT or DDG. As these last two tests are robust, and can be performed with relatively little equipment, they can be recommended for use in laboratories where manpower is not the limiting factor, and access to automatized systems is not available. In fact, these immunoprecipitation tests have been used extensively and successfully as confirmatory tests after a RBT screening in eradication campaigns in northern Spain (Asarta, 1989; Blasco et al., 2016). Indeed, a problem of DGD is the availability of the NH and S-LPS extract. Although there are simple extraction protocols (Díaz et al., 1984), the antigen is not presently commercially available.

In cattle, the close parallelism among the apparent prevalence figures obtained with the different tests is remarkable. Compared to the CFT or iELISA tests, it has been shown that the immunoprecipitation test with NH is only not affected by false positive reactions caused by bacteria cross-reacting with *Brucella* at the LPS level (Muñoz et al., 2005). Taken together, this high specificity and the absence of S19 vaccination in the origin areas of the tested animals support the conclusion that the apparent prevalence figures obtained with this test actually represent real infections by either *B. abortus* or *B. melitensis*. The latter species was known to be a cause of infections in cattle in areas where they are raised and kept with the small ruminants (Verger et al., 1989), and as serological tests cannot differentiate infections by those two species (Ducrotoy et al., 2016), bacteriological studies are necessary for a full understanding of the epidemiology of brucellosis in Algeria. Cattle identification system allowed us to trace back their origin, thereby, all livestock markets were represented at the Rouiba abattoir (i.e. Bouira (Center), Médéa (Center), Blida (Center), Ain El-Defla (West), Algiers (Center), Chlef and Relizane (West), Boumerdès (Center); supplemental figure S1), only one (Ain El-Defla) did not yield any seropositive animal. This illustrated that cattle brucellosis is widespread in the country.

Concerning other epidemiological factors, table 1 summarizes the results broken down according to sex, age, breed and other characteristics recorded. As can be seen, the proportion of seropositive individuals was much higher in females than in males. The literature contained contradictory reports on the influence of sex as a risk factor in cattle brucellosis. Whereas some authors reported that males are at a higher risk (Boukary et al., 2013), others found opposite results (Kebede et al., 2008; Mohammed et al., 2011; Rahman et al., 2011; Olabode et al., 2012). However, it is known that the management determined the rates of infection in males. Artificial insemination is extended in Algeria, and in the present study, all138 males were slaughtered for meat when they were less than two years old, instead of using them as breeding males which prevented them from getting infected from the females. Among the females, only one was less than 2 years old. This bias in the sample reflected the management of the animals in the dairies of Algeria where old females are removed from the farms when the productivity decreases. Similarly, most of the tested animals belonged to the crossbreed category, and this also reflected the preference of the farmers for these animals because of the better milk yields and comparative lower market prices. However, the data showed that there were no statistically significant differences in apparent seroprevalence among this and the other two groups identified in the study.

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Out of 46 identified cattle samples, 10 (21.73%) were positive. Ten out of the 305 (3.27%) non-identified cattle and 4out of the 51(7.84%) cattle samples that presented are moved ear-tag were found positive (Table 1). Statistically, there was a significant association ($p<0.05$) between the apparent prevalence and the identification status. Supposedly, identified animals were screened regularly for brucellosis by Veterinarian Services, and those found positive should be sacrificed. However, owners most often postpone culling, and send the animals to the market according to their own needs. Thus, the sub-sample of identified animals was biased and should contain a higher proportion of seropositive animals, as the results confirmed. The same explanation was likely to apply to those animals from which the ear tag was removed before being sold. Also, in present study, a high proportion (305 out of 402) of cattle was unidentified. These observations were not anecdotal because they reflected the difficulties existing in controlling the disease in Algeria. Indeed, since a serological follow up is strictly necessary, proper animal identification is essential to apply control measures based on the individual vaccination (Blasco and Molina-Flores, 2011; Blasco et al., 2016).

Three out of the 21 (14.28%) pregnant females and 20 out of the 235 (8.51%) non- pregnant females were found positive (Table 1). Statistically, there was a significant association ($p < 0.05$) between apparent prevalence and the pregnancy. Presence of pregnant females in the slaughterhouse could be explained by misdiagnosis of the pregnancy status by the veterinarians who delivered the slaughter certificates. However, of these three animals, one belonged to the identified group and another had the removed ear tag. Thus, an alternative explanation was an owner decision based on an awareness of the disease. The existence of infected pregnant animals was significant because these animals play a very important role in the transmission of the disease (Blasco et al., 2016).

Table 1. Results of brucellosis by serological tests in cattle according to sex, age, breed and identification status in Algeria

Items		Number of positive results (%) in the indicated tests				
		N° of animals	RBT	CF	DDG-NH	iELISA
Sex	Female	264	23(8.71%)	22(8.33%)	22(8.33%)	16(6.06%)
	Male	138	1(0.72%)	1(0.72%)	1 (0.72%)	0(0.00%)
Age (females)	< 2 years	1	0(0.00%)	0(0.00%)	0(0.00%)	0(0.00%)
	>5years	263	23 (8.74%)	22(8.36%)	22 (8.36%)	16(6.08%)
Breed	Local	33	1 (3.03%)	1(3.03%)	1(3.03%)	1(3.03%)
	Cross-breed	344	22 (6.39%)	21(6.10%)	21 (6.10%)	14 (4.07%)
	Montbeliarde	25	1 (4.00%)	1(4.00%)	1 (4.00%)	1(4.00%)
Identification status	Yes	46	10(21.73%)	10(21.73%)	10 (21.73%)	8(17.39%)
	No	305	10 (3.27%)	10(3.27%)	10 (3.27%)	6(1.96%)
	Ear tag removed	51	4 (7.84%)	3(5.88%)	3 (5.88%)	2(3.92%)
Pregnancy (females)	Pregnant	21	3 (14.28%)	2(9.52%)	2(9.52%)	2(9.52%)
	Non-pregnant	235	20 (8.51%)	20(8.51%)	20(8.51%)	14(5.95%)

N°: number; RBT: Rose Bengal Test; CF: complement fixation; DDG-NH: double gel diffusion-native hapten; iELISA: indirect Enzyme Linked ImmunoSorbent Assay

Sheep

Concerning the ovine sera, two samples (0.98%) were positive by mRBT. By CFT, one sample had a titer of 1/4, and the other one had a titer of 1/16. This last serum was found positive by DDG-NH, but only with antigen at 1mg/ml. This was consistent with previous observations that the NH-S-LPS antigen concentration needed to be comparatively reduced for optimal sensitivity in immunoprecipitation with sera from small ruminants (Diaz-Aparicio et al., 1993).These two animals belonged to the larger sheep sample (El-Harrach abattoir).

The apparent seroprevalence found in sheep was lower than 3.08 % reported by Hamdi-Chérif et al.(1999) in the east of Algeria (Setif and M'sila, 1999), or the 7.2% found by the National Institute of Veterinary Medicine of Algiers in 2007 (Meskoud-taibi and Benzadi, 2009), and several circumstances could explain the differences. First, these two surveys which were conducted before the benefits of Rev 1 vaccination campaigns (started in the spring of 2006) could be detectable. It was worth noting that those few positive results cannot be the result of post-vaccinal antibodies. None of the sheep carried the green tag was used to label vaccinated animals in Algeria. An alternative explanation is that the tested sheep came from a few intensive farms surrounding Algiers where the animals which were kept for fattening, and then sold to the slaughterhouse which favored control. The two seropositive sexually mature rams introduced from an infected breeding area, but this practice did not seem a significant risk. In cattle, as in sheep, males play no relevant role in spreading the disease by direct sexual contact (Alton, 1990; Nielsen and Duncan, 1990), while artificial insemination can be a risk, which is not practiced in Algeria. However, the survey in sheep revealed deficiencies in the implementation of those measures (permanent tagging, movements of animals of unknown status) that are essential to carry out brucellosis control and eradication programs. Finally, since the present study was carried on ovine brucellosis

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in a slaughterhouse in the capital city Algiers, the results did not reflect the epidemiological situation in Algeria, and more studies in areas with dominance of ovine/caprine breeding are necessary to determine the origin of the human disease.

CONCLUSIONS

The results of this survey confirmed the importance of cattle brucellosis in Algeria, and that a combination of Rose Bengal test (RBT) and double gel diffusion in gel (DDG)RBT and DDG can be implemented instead of the more sophisticated like complement fixation test (CFT) and indirect Enzyme Linked Immuno Sorbent Assay (iELISA) to determine the apparent prevalence figures. As RBT and DDG are robust and technically simple, they should be useful in laboratories with basic equipment and where manpower is not limiting. Further studies including bacteriological methods, are urgent to clarify the epidemiology of brucellosis in Algeria. Finally, the study highlights a significant absence of proper implementation of general measures (identification and tagging, control of animal movements) that are necessary for successful brucellosis control.

Acknowledgements

We thank Alberto Delgado López for skillful technical assistance and the veterinarians of the slaughterhouses of Rouiba and El-Harrach for their help. The authors of this study would like to thank the Institute of Tropical health for hosting Maamar Khames to perform the serological tests and the School of Sciences and Technology of the University of Medea (Algeria) for funding MK stay in Spain.

Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article. The authors declare that they have no competing interests.

Author's contribution

Maamar Khames, Amaia Zúñiga-Ripa and Ignacio Moriyon designed the experiment and laboratory analyses. Ignacio Moriyon and Mustapha Oumouna designed article writing, revision and approval. Maamar Khames, Ignacio Moriyon and Mustapha Oumouna designed tabulation of experimental data, manuscript writing, commenting and approval. Sagrario Pérez Gómez helped in field study, collected data, laboratory analyses, statistical analysis, manuscript writing. All authors have read and approved the final manuscript.

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To cite this paper: Khames M, Zúñiga-Ripa A, Pérez Gómez S, Oumouna M and Moriyón I (2021). Comparison of Serological Tests in Cattle and Ovine Brucellosis; An Abattoir Study in Algeria. *World Vet. J.*, 10 (3):457-464. DOI: https://dx.doi.org/10.36380/scil.2020.wvj57

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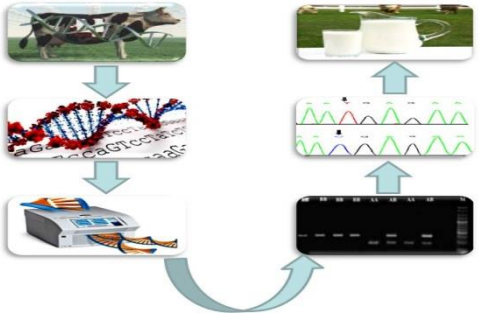

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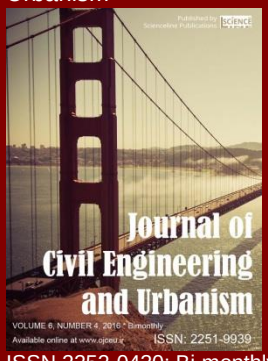

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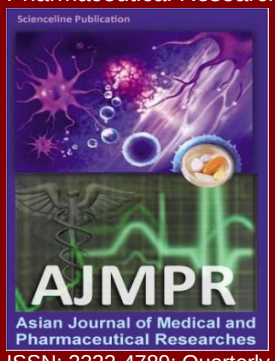

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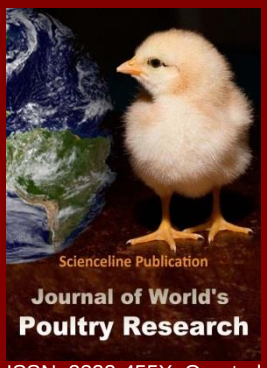

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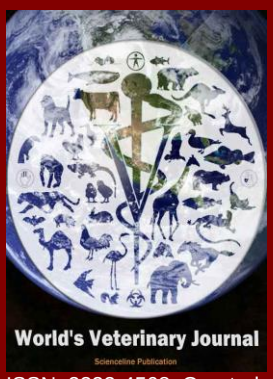

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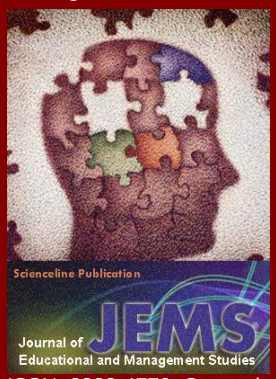

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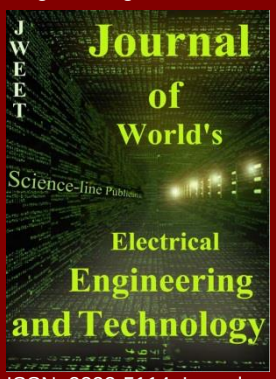

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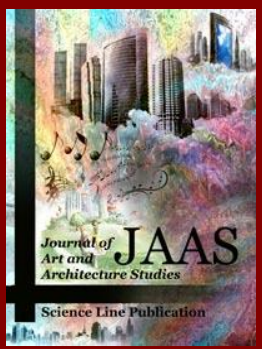

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