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Molecular Detection of *Streptococcus* Species Isolated from Cows with Mastitis

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ABSTRACT

Streptococcal mastitis is considered as one of the most common infectious diseases in the dairy cattle, which threatens the dairy industry all over the world. The aim of this study was to determine the prevalence of *Streptococcus* species in mastitic cows with molecular investigation to detect the presence of some virulence genes of the recovered isolates by PCR. A total of 150 milk samples were collected from dairy cattle with clinical and subclinical mastitis from different areas in El- Gharbia governorate, Egypt. *Streptococcus* species were isolated with an incidence of 38%. *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. pyogenes*, *S. pneumoniae* and *S. faecalis* were isolated from the milk samples of the examined cows with the percentage of 14.7%, 6%, 9.3%, 4.7%, 1.3% and 2%, respectively. Molecular investigation of virulence associated genes revealed that sip, cfb and bca genes of *S. agalactiae* were found with the percentage of 77.7%, 88.8% and 33.3%, respectively. The mig gene of *S. dysgalactiae* was found with an incidence of 77.8%. Of the examined *S. uberis* isolates, 55.5%, 22.2% and 33.3% were carrying the cfu, oppF and has A genes, respectively. The present study revealed the prevalence of *Streptococci* and distribution of virulence associated genes among the isolates. The high frequency of virulence genes in the isolates suggests an important role of these virulence genes in the pathogenesis of *Streptococci* in cattle mastitis.
Key words: Mastitis, Cows, *Streptococci*, Virulence genes

INTRODUCTION

Bovine mastitis is one of the most frequent diseases in dairy cattle which causes major economic losses due to the reduced milk quantity and increased costs of treatment (Koskinen et al., 2009). Mastitis is an inflammation of the mammary gland that occurs as a response to injury. This disease is mainly caused by microorganisms usually bacteria, including Gram-negative and Gram-positive bacteria, mycoplasma, yeasts and algae (Zadoks et al., 2011).

Streptococci are Gram-positive bacteria widespread in the environment and are also commensal organisms of the cow's udder, mucosa and skin. Some of the commensal *Streptococcus* are opportunistic bacteria causing infection if the balance between bacteria and host is broken. Others are considered primary pathogens of mastitis (Cleary and Cheng, 2006). Among *Streptococcus* species, *S. agalactiae* (contagious agent), *S. uberis* (environmental agent) and *S. dysgalactiae* are the predominant group of organisms isolated from mastitis (Kuang et al., 2009).

Streptococcus agalactiae, the lone member of the Lancefield group B, is an important cause of chronic and contagious bovine mastitis. Its presence is frequently associated with high somatic cell counts in milk and decreased milk yield (Jain et al., 2012). However, *S. agalactiae* possesses several virulence factors including structural components, toxins and enzymes that play an important role in the intra-mammary infections (Krishnaveni et al., 2014). *Streptococcus dysgalactiae* is considered as a contagious pathogen, which adapts to the udder environment and can be spread from cow to cow during milking (Yanliang et al., 2016). However, this bacterial pathogen may contain several putative virulence associated genes which are not fully understood (Calvinho et al., 1998). One of these genes, a surface- expressed M-like protein, named mig, has been found to bind to the Immunoglobulin G (IgG), K2-macroglobulin (K2-M) (Jonsson and

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Muller, 1994) and Bovine Immunoglobulin A (B-IgA) and plays a role in anti-phagocytosis by bovine neutrophils in the presence of bovine serum (Song et al., 2001).

Streptococcus uberis is one of the prime causative agents among the environmental pathogens and is predominantly associated with the clinical and subclinical mastitis in both lactating and non lactating cows (Reinoso et al., 2011). Despite the great economical loss due to high prevalence of *S. uberis*, several virulence factors associated with the pathogenesis are not well understood. Among these factors, resistance to phagocytosis conferred by hyaluronic acid capsule (Ward et al., 2001), Christie, Atkins and Munch Peterson (CAMP) factor (Jiang et al., 1996) and Opp proteins (Smith et al., 2002) have been found. The identification and characterization of *Streptococci* virulence associated genes causing bovine mastitis will open the way for the development the new strategies to prevent and control this bacterial pathogen in dairy herds (El-Behiry et al., 2015). Few reports exist on the prevalence of *Streptococci* as well as the occurrence of virulence-associated genes among *Streptococcus* isolates recovered from cattle with mastitis in Egypt. Therefore, the aim of the present study was to determine the prevalence of *Streptococci* involved in mastitis in cattle with molecular investigation the pattern distribution of some virulence associated genes of the recovered isolates.

MATERIALS AND METHODS

Ethical approval

Handling of animals were according to the guidelines of animal ethics committee, faculty of veterinary medicine, Kafrelsheikh University, Egypt.

Sampling

A total of 150 milk samples (115 from clinical mastitic cows and 35 from apparently healthy cows) were collected from lactating cows (Friesian and Jersey breeds) at different stages of lactation during the period from January 2015 to November 2015 from different areas in El- Gharbia governorate, Egypt. The udder of each animal was examined before sampling for detection of clinical signs of mastitis such as inflammation, asymmetry, hotness, swelling or any physical changes. Each udder was washed and carefully dried with clean towel then the teats were swabbed with 70% alcohol. Before sampling, the first jets of milk were rejected, then each quarter milk sample (nearly 3ml) was collected into sterile screw capped MacCarteny bottle as recommended by Blood and Handerson (1986) and submitted to the laboratory in an ice container as soon as possible for further bacteriological and molecular examination.

Isolation and identification of *Streptococcus* species

Isolation of suspected *Streptococci* was done according to Quinn et al. (2002). Briefly, the collected milk samples were incubated aerobically at 37°C for 24 h, then centrifuged at 3000 rpm for 20 min. The cream and supernatant fluid were discarded. Methylene blue stain was used routinely to detect the suggestive bacterial causes. The sediment was streaked on the surface of 5% sheep blood agar and Edward’s media (Oxoid). The inoculated plates were incubated at 37°C for 24-48 h, and examined for bacterial growth. Suspected streptococcal colonies were sub-cultured, purified and preserved in semisolid agar for further identification which was done according to Cowan (1979); Carter and Cole (1990). Also, CAMP test was carried out according to Koneman et al. (1988). The subclinical samples were subjected to total somatic cell count using California Mastitis Test (CMT) in order to confirm the subclinical status of the collected samples.

Molecular detection of *Streptococcus* virulence associated genes

Bacterial DNA extraction was done by using QIA amp DNA mini and Blood mini kit according to the manufacturer’s instructions. A molecular identification of *Streptococcus* species was performed by using species-specific oligonucleotide primers for the genes encoding 16S rRNA for the identification of *S. agalactiae, S. dysgalactiae* and *S. uberis*. For detection of some virulence associated genes, *S. agalactiae* isolates were screened for Sip, cfb and bca. *S. dysgalactiae* isolates were screened for mig while *S. uberis* isolates were screened for cfu, oppE and hasA genes. The primer pairs used in PCR protocols were selected from published papers based on specificity, compatibility and ability to target the potential virulence genes of interest.

The nucleotide sequences and anticipated molecular sizes of PCR amplified fragments for these gene-specific oligonucleotide primer sets are outlined in table 1. PCR reaction mixtures and conditions are given in table 2. The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The PCR products were analyzed by electrophoresis using a 1.5 % agarose gel in Tris-Borate EDTA (TBE) buffer. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

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Table 1. Nucleotide sequence and product length of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* virulence gene specific primers

Streptococcus spp.	Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
S. agalactiae	16S rRNA(F)	5′ ATTGATAACGACGGTGTTACTGT ′3	487	Raemy et al. (2013)
	16S rRNA(R)	5′ CATAGTAGCGTTCTGTAATGATGTC ′3	487	Raemy et al. (2013)
	sip (F)	5′ ACTATTGACATCGACAATGGCAGC ′3	266	Nithinprabhu et al. (2010)
	sip (R)	5′ GTTACTGTCAGTGTTGTCTCA′3	266	Nithinprabhu et al. (2010)
	Cfb (F)	5′CAAAGATAATGTTCAGGGAACAGATTATG′3	320	Krishnaveni et al. (2014)
	Cfb (R)	5′ CTTTTGTTCTAATGCCTTTACGTT ′3	320	Krishnaveni et al. (2014)
	bca (F)	5′ TAACAGTTATGATACTTCACAGAC ′3	535	Manning et al. (2006)
	bca (R)	5′ ACGACTTTCTTCCGTCCACTTAG ′3	535	Manning et al. (2006)
S. dysgalactiae	16S rRNA(F)	5′ GTGCAACTGCATCACTATGAG ′3	279	Raemy et al. (2013)
	16S rRNA(R)	5′ CGTCACATGGTGGAT TTTC ′3	279	Raemy et al. (2013)
	mig (F)	5′ CGTTTTTAGTTTCGGGAGCA ′3	188	Nithinprabhu et al. (2010)
	mig (R)	5′ TGCCTTCAATTGAGTCTGCTG ′3	188	Nithinprabhu et al. (2010)
S. uberis	16S rRNA(F)	5′ TGATTCCGACTACTACGCTAGAT ′3	723	Raemy et al. (2013)
	16S rRNA(R)	5′ ATACTTTGAGTTTCACCGAGTTC ′3	723	Raemy et al. (2013)
	cfu (F)	5′ TATCCCGATTTGCAGCCTAC ′3	205	Reinoso et al. (2011)
	cfu (R)	5′ CCTGGTCAACTTGTGCAACTG ′3	205	Reinoso et al. (2011)
	oppE (F)	5′ GGCCTAACCAAAACGAAACA ′3	419	Smith et al. (2002)
	oppE (R)	5′ GGCTCTGGAATTGCTGAAAG′3	419	Smith et al. (2002)
	hasA (F)	5′ GAAAGGTCTGATGCTGAT ′3	600	Ward et al. (2001)
	hasA (R)	5′ TCATCCCCTATGCTTACAG ′3	600	Ward et al. (2001)

Table 2. PCR assay conditions and reaction mixture for molecular identification of *Streptococcus* species and detection of the virulence associated genes

Reaction	Assay (1) (Raemy et al.,2013)			Assay (2) (El-Behiry et al., 2015)			Assay (3) (Krishnaveni et al., 2014)			Assay (4) (Reinoso et al., 2011)		
	Temp.(°C)	Duration	Cycle	Temp.(°C)	Duration	Cycle	Temp.(°C)	Duration	Cycle	Temp.(°C)	Duration	Cycle
Denaturation	94	60 sec	1	94	30 sec	1	94	30 sec	1	94	30 sec	1
Annealing	58	60 sec		45	60 sec		55	30 sec		48-58	30 sec	
Extension	72	10 min	35	72	90 sec	25	72	30 sec	30	72	10 min	25
Elongation	-	-	-	-	-	-	72	10 min		-	-	
Cooling	4	Infinite	-	4	Infinite	-	4	Infinite	-	4	Infinite	-
Reaction mixture	(total volume = 25 µl): 1x HotStarTaq Master Mix + 2.5 µl of diluted lysate, and 300 µM of each primer (16S rRNA of *S. agalactiae*, *S. dysgalactiae* and *S. uberis*)			25 µl containing PCR master mix with 3 mm of $MgCl_2$ + 3 µl of template DNA and 0.5 µM of each primer (*sip*, *cfb*, *bca* genes of *S. agalactiae*)			25 µl of 10X PCR Taq Buffer A+ 1µl (20 pmol) of *mig* primers of *S. dysgalactiae* + 1 µl (100 µM) of each dNTPs and 3 µl (150 ng) of template DNA			50 µl containing 1.50 U Taq polymerase with 1.5 mM $MgCl_2$, 1 µM of each primer(*cfu*, *opp*F, *has*A genes of *S. uberis*) , 0.4 µM of each of the dNTPs and 20 ng template DNA		

Assay 1 is designed for molecular identification of *S. agalactiae, S. dysgalactiae* and *S. uberis*. Assays 2, 3 and 4 are for molecular detection of virulence associated genes of *S. agalactiae, S. dysgalactiae* and *S. uberis,* respectively

RESULTS

Incidence of *Streptococcus* species in mastitic cows

Among the 150 examined milk samples, 115 from clinical cases and 35 from apparently healthy cows were subjected to CMT for detection of subclinical mastitis, 25 were positive for CMT and 10 were negative. A total of 57 isolates of *Streptococcus* species were isolated and identified [41 from clinical mastitis (35.7%) and 16 from subclinical cases (64%)]. In clinical mastitis samples, *S. agalactiae* was the predominant species (13.9%) followed by *S. uberis* (9.6%), *S. dysgalactiae* (6.1%), *S. pyogenes* (3.5%), *S. pneumoniae* (1.7%) and *S. fecalis* (0.9%). While in subclinical mastitis, *S. agalactiae* was also the most frequent isolated species (17.1%), followed by *S. uberis*, *S. pyogenes* (8.6% each) *S. dysgalactiae* and *S. fecalis* (5.7% each) (Table 3).

Molecular detection of *Streptococcus* species virulence associated genes by PCR

As a result of the molecular screening of 27 *Streptococcus* species using species specific PCR, nine isolates for each *S. agalactiae*, *S. dysgalactiae* and *S. uberis* were identified (Figure1). PCR based screening of virulence genes revealed the presence of cfb, sip and bca in *S. agalactiae* isolates with the percentage of 88.8%, 77.7% and 33.3%, respectively (Figure 2),whereas 77.8% of *S. dysgalactiae* were carrying mig gene (Figure 3). Similarly, cfu, oppF and hasA genes were detected in *S. uberis* isolates with the percentage of 55.5%, 22.2% and 33.3%, respectively (Figure 4) (Table 5).

Table 3. The incidence of *Streptococcus* species among the examined Cow's milk samples in El- Gharbia governorate, Egypt during the period from January 2015 to November 2015

***Streptococcus* Species**	Clinical mastitis (n=115)		Subclinical mastitis (n=35)		Total (n=150)	
	No.	**%**	**No.**	**%**	**No.**	**%**
S. agalactiae	16	13.9	6	17.1	22	14.7
S. dysgalactiae	7	6.1	2	5.7	9	6
S. uberis	11	9.6	3	8.6	14	9.3
S. pyogenes	4	3.5	3	8.6	7	4.7
S. pneumonia	2	1.7	0	0	2	1.3
S. fecalis	1	0.9	2	5.7	3	2
Total	41	35.7	16	45.7	57	38

No = positive number; % was calculated according to the total number of examined animals

Table 4. The incidence of some virulence associated genes in *Streptococcus* species isolated from Friesian and Jersey breeds in El- Gharbia governorate, Egypt during the period from January 2015 to November 2015

***S. agalactiae* (n=9)**						***S. dysgalctiae* (n=9)**		***S. uberis* (n=9)**					
Sip		Cfb		bca		Mig		cfu		oppF		hasA	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
7	77.8	8	88.8	3	33.3	7	77.8	5	55.5	2	22.2	3	33.3

No = positive number; % was calculated according to the positive examined samples for each species

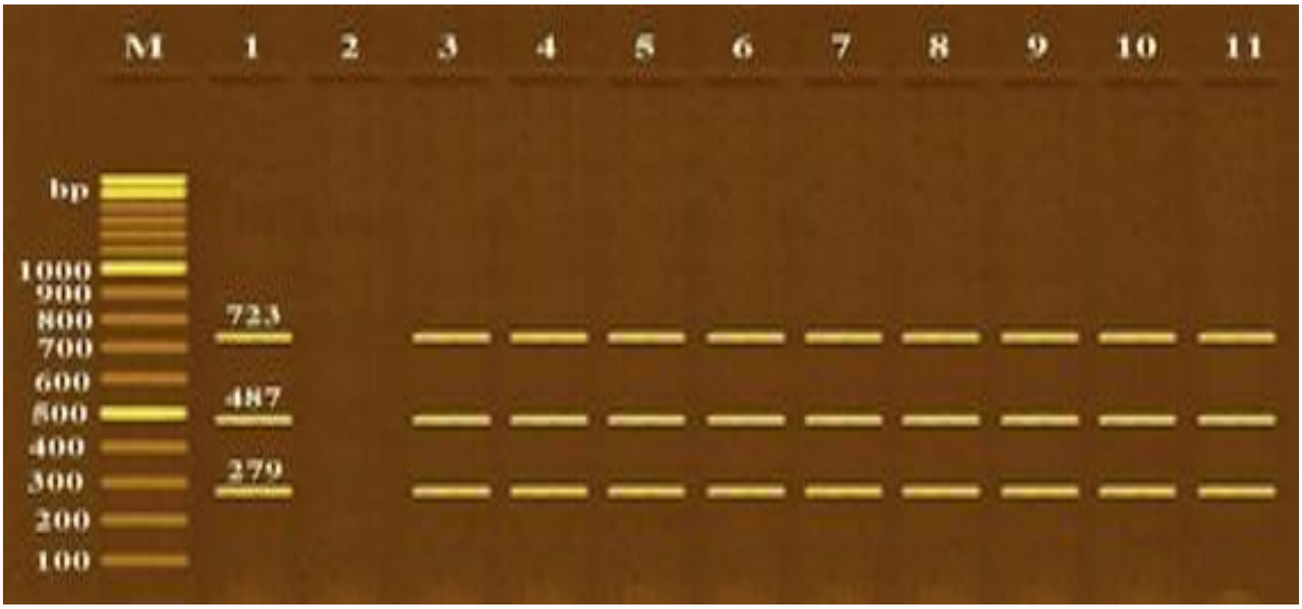

Figure 1. Agarose gel electrophoresis of multiplex PCR of GSag (487 bp) for *Streptococcus agalactiae*, GSdys (279 bp) for *Streptococcus dysgalactiae* and GSub (723 bp) for *Streptococcus uberis* as species specific genes for their identification (M=100 bp ladder, Lane 1: Control positive *Streptococcus* species for GSag, GSdys and GSub genes, Lane 2: Control negative, Lanes 3-9: positive for GSag, GSdys and GSub for *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, respectively)

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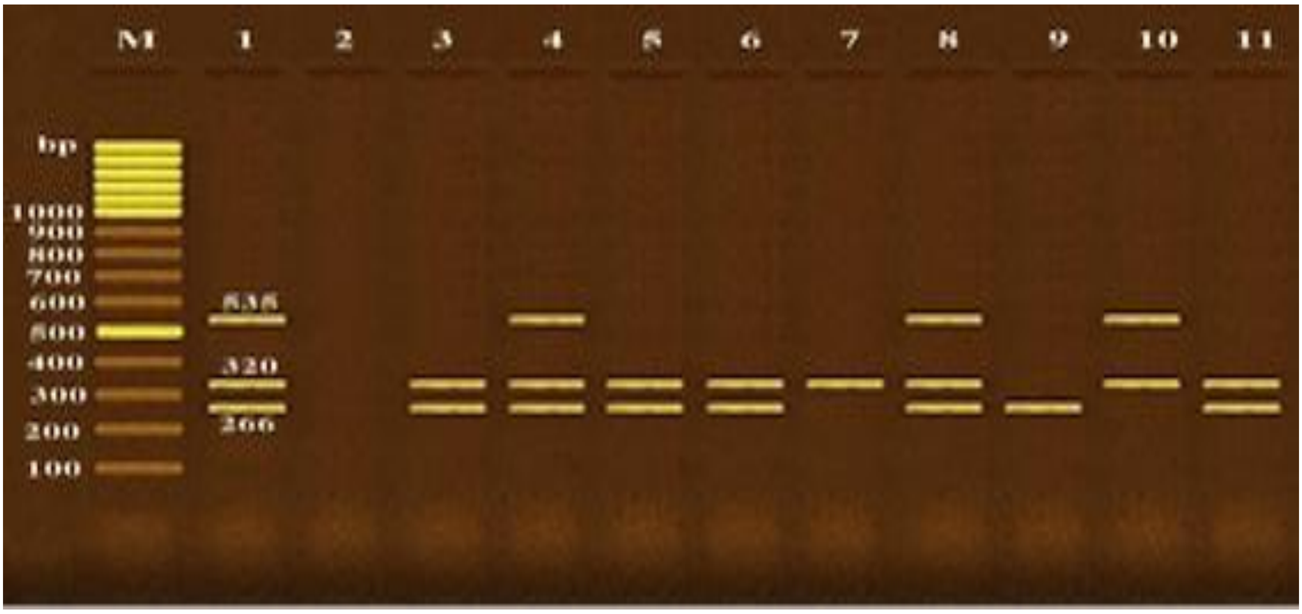

Figure 2. Agarose gel electrophoresis of multiplex PCR of *sip* (266 bp), cfb (320 bp) and bca (535 bp) as virulence genes for characterization of *Streptococcus agalactiae* strains (M= 100 bp ladder, Lane 1: Control positive *Streptococcus agalactiae* for sip, cfb and bca genes, Lane 2: Control negative, Lane 3, 5-6 and 11: Positive *Streptococcus agalactiae* for sip and cfb genes, Lanes 4 and 8: Positive *Streptococcus agalactiae* for sip, cfb and bca genes, Lane 7: Positive *Streptococcus agalactiae* for cfb gene, Lane 9: Positive *Streptococcus agalactiae* strain for sip gene and Lane 10: Positive *Streptococcus agalactiae* strain for cfb and bca genes)

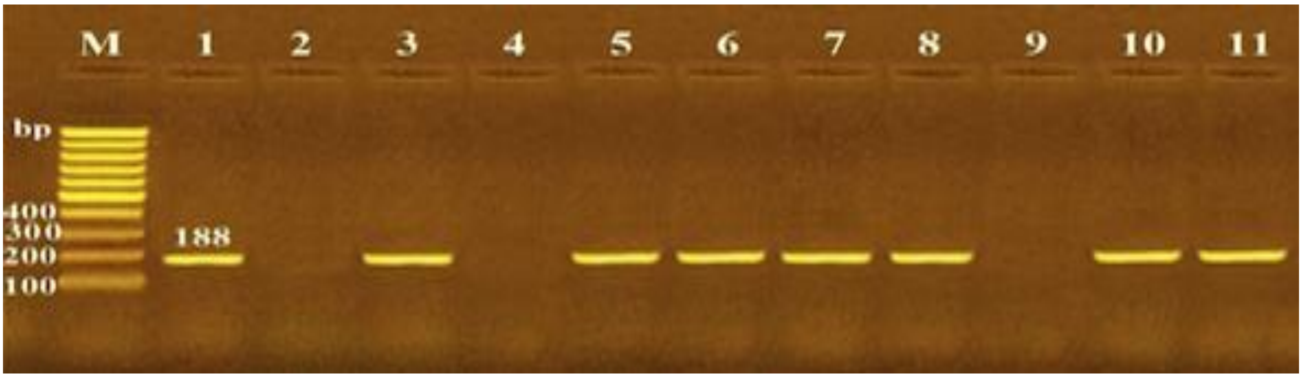

Figure 3. Agarose gel electrophoresis of PCR of mig protein gene (188 bp) virulence gene for characterization of *Streptococcus dysgalactiae* strains (M=100 bp ladder, Lane 1: Control positive *Streptococcus dysgalactiae* for mig gene, Lane 2: Control negative, Lane 3, 5-8, 10 and 11: Positive *Streptococcus dysgalactiae* strains for mig gene and Lanes 4 and 9: Negative *Streptococcus dysgalactiae* strains for mig gene)

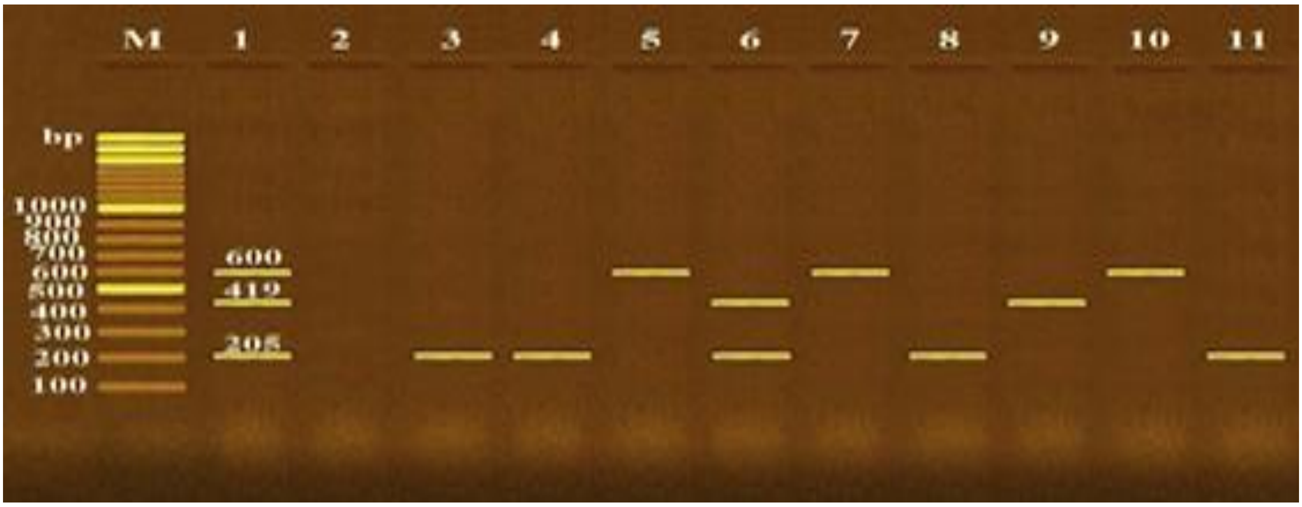

Figure 4. Agarose gel electrophoresis of multiplex PCR of cfu (205 bp), oppF (419 bp) and hasA (600 bp) as virulence genes for characterization of *Streptococcus uberis* strains (M=100 bp ladder, Lane 1: Control positive *Streptococcus uberis* for cfu, oppF and hasA genes, Lane 2: Control negative, Lane 3-4, 6, 8 and 11: Positive *Streptococcus uberis* strains for cfu gene, Lanes 6 and 9: Positive *Streptococcus uberis* strains for oppF gene and Lanes 5, 7 and 10: Positive *Streptococcus uberis* strains for *has A gene*)

DISCUSSION

Streptococcus species belong to a large group of organisms which are associated with bovine udder infections (Wyder et al., 2011). In the current study, a total of 150 milk samples were collected from cattle with mastitis (115 from clinical cases and 35 from apparently healthy cows, 25 were California mastitis test positive and represent subclinical mastitis and 10 samples were CMT negative and were discarded) and examined for presence of *Streptococci*. Fifty-seven *Streptococci* were recovered with a prevalence rate of 38%. This finding is nearly consistent with the report described by Mohanty et al. (2013). However, the present study shows much lower isolation rate than El Jakee et al. (2013) who isolated *Streptococci* with an incidence of 55%. In the present investigation, 16 *Streptococci* isolates were recovered from subclinical cases of mastitis with an incidence of 64%. Present result was in agreement with those obtained by Wahba et al. (2005). While a higher incidence of *Streptococci* in subclinical mastitis was obtained by Kia et al. (2014) with an incidence of 75%. However, other studies have reported a lower incidence of *Streptococci* from subclinical mastitis (Esron et al., 2005; Ranjan et al., 2011; chen et al., 2012; Jeykumar et al., 2013) who recovered *Streptococci* with the percentage of 9.8%, 5.7%, 15.5% and 16.1%, respectively.

With regard to clinical cases of mastitis, 41 isolates of *Streptococci* were recovered with a percentage of 35.7%. Contrary to our results, a lower incidence was recently reported by Demme and Abegaz (2015) who isolated *Streptococcus* from clinical cases at a rate of 16.7%.In our study, six different species of *Streptococci* (*S. agalactiae, S. uberis, S. dysgalactiae, S. pyogenes, S. pneumoniae* and *S. fecalis)* were isolated and identified.

Our results showed a clear overall predominance of *S. agalactiae* among *Streptococcus* species (14.7%). This finding confirms the results reported by Klimiene et al. (2005) and Kivaria and Noordhuizen (2007) who isolated *S. agalactiae* with an incidence of 15.1% and 15.4%, respectively. The high prevalence of *S. agalactiae* may indicate the poor management for the investigated cows. While higher incidences of *S. agalactiae* isolated from mastitic cows were recovered by Kuzma and Malinowski (2001), Khan and Mohammad (2005), Borkowoska et al. (2006), Momtaz et al. (2012) and El-Jakee et al. (2013) with isolation rate of 41.2%, 30%, 84.8%, 16%, 19.3%, respectively.

In our report, *S. uberis* was the second predominant *Streptococcus* species isolated from mastitic cattle with a percentage of 9.3%. A similar prevalence rate was also recently reported by El-Bagory and Zayda (2015) (9.4%). Other studies have been reported a higher incidence rate of *S. uberis* by Zadoks et al. (2003) (26%), Hussain et al. (2006) (15%), Bradley et al. (2007) (23%), Ericsson Unnerstad et al. (2009) (11%) and El Jakee et al. (2013) (15%).

A comparatively lower prevalence rate of *S. uberis* isolated from cattle with mastitis was also reported by other studies (Tenhagen et al., 2006 and Momtaz et al., 2012) with a percentage of 0.1% and 7.3 %, respectively. *S. dysgalactiae* was isolated in our study with an incidence of 6%. Other previous studied reported higher incidences such as Ericsson Unnerstad et al. (2002); Moges et al. (2011) and El Jakee et al. (2013) that isolated *S. dysgalactiae* with a prevalence rate of 15.6%, 14% and 17% respectively. Other *Streptococcus* species were isolated from the examined milk samples as *S. pyogenes, Enterococcus* species and *S. pneumoniae.* Yet these differences may be attributed to other factors rather than geographical location, such as the differences in the samples taken or type of mastitis.

S. pyogenes, a beta-hemolytic bacterium that belongs to Lancefield serogroup A, causes a wide variety of diseases in humans (Khan, 2012). The main reservoir of *S. pyogenes* includes, man, rarely cattle. *S. pyogenes* is almost exclusively associated with man, and contact with infected individuals or asymptomatic carriers is the most common source of infection (McDougall, 2005). However, previous study carried out by Khalil et al. (2014) reported that, the *S. agalactiae* and *S. pyogenes* represent the most important bacterial isolates responsible for severe losses to milk industry, in addition to the zoonotic importance of *S. pyogenes*.

Streptococcus pneumoniae colonizes the nasopharynx in mainly human at any time, and causes serious infectious diseases, such as pneumonia, septicemia, meningitis, and otitis media (Musher et al., 2005). *S. pyogenes* and *S. pneumoniae* were isolated with an incidence of 4.7% and 1.3% respectively. This is in contrast to El Jakee et al. (2013) who isolated *S. pyogen* and *S. pneumoniae* from mastitic cows in a lower percentage (2.7% and 0.7%, respectively). Their presence in the examined milk samples may be due to the bad manipulation during milking from the hand milkers. In cattle, enterococci have been associated with diarrhea in calves and bovine mastitis in dairy cattle (Rogers et al., 1992). *Enterococcus* species were recovered in our study with an incidence of 2%.

In the present study, a total of nine isolates of *S. agalactiae* were screened for some associated virulence genes, cfb, bca and sip by using multiplex PCR. Out of nine isolates, seven (77.8%) contained *sip* gene. Similarly, high percentages of *sip* gene in *S. agalactiae* have been reported by Krishnaveni et al. 2014 and El-Behiry et al. 2015, who reported *sip* gene with an incidence of 100% and 90.69%, respectively.

The cfb gene was detected in eight isolates (88.8%) of the examined *S. agalactiae*. Our results confirm the finding reported by El-Behiry et al. (2015) (93 %). However; other previous studies (Shome et al., 2012; El-Gedawy et al., 2014; Krishnaveni et al., 2014) have been reported that cfb was detected in the all obtained isolates. On the other hand, lower incidence of cfb gene was reported by Ding et al. (2016) who found cfb gene in only 50% of isolates. The cfb gene is a cell surface protein that produces a traditional CAMP phenomenon with the typical half-moon forming hemolytic zones on blood agar plates (El-Behiry et al., 2015).

The results in the present study revealed that three isolates of *S. agalactiae* (33.3%) carried the *bca* gene. The *bca* gene codes for Alpha-C protein, a surface protein that helps the bacteria to enter the host cells (Bolduc et al., 2002). Higher incidences of bca gene was reported by Duarte et al. (2004) (64.7%) and Duarte et al. (2005) (78.9%). On the other hand, lower incidences of bca gene was recorded by El-Behiry et al. (2015) (20.93%) and Ding et al. (2016) (3.7%). While Jain et al. (2012) found that none of *S. agalactiae* isolates was carrying the bca gene.

The hyaluronic acid capsule production of *S. uberis* is dependent on the has operon (hasA, hasB and hasC). In this study, hasA gene was detected in three isolates out of nine *S. uberis* (33.3%). This result was nearly agreeing with Matthews et al. (1994) who found that 44% of *S. uberis* strains were carried hasA. However, higher incidence of hasA among *S. uberis* isolates have been reported by other studies (Reinoso et al., 2011; Mirta-Lasagno et al., 2011; Almeida et al., 2013) with the percentage of 74.3, 59.4 and 100%, respectively.

Another potential virulence factor analyzed in the present study was the CAMP factor (*cfu*) which was observed in five isolates out of nine *S. uberis* strains (55.5 %). This finding is consistent with the finding described by Shome et al. (2012) (46.15%). However, other previous studies have been reported high frequency of cfu gene in *S. uberis* isolates (Shalka and Smola 1981; Hassan et al., 2000; Reinoso et al., 2011). While lower incidence was reported by Lammler et al. (1991) and Mirta-Lasagno et al. (2011) who detected cfu in a percentage of 28% and 25%, respectively among *S. uberis* isolates.

With regard to oppF gene, the data presented here showed that only two isolates of *S. uberis* were carring oppF gene with an incidence of 22.2%. OppF gene plays an important role during growth in milk (Smith et al., 2002). Higher incidences of oppF have been described by previous study (Reinoso et al. 2011) (64.1%). Moreover, Almeida et al. (2013) detected oppF in all *S. uberis* isolates.

The mig protein of *S. dysgalactiae* is involved in resisting phagocytosis by bovine neutrophils in the presence of bovine serum (Song et al., 2001). Thus, the mig protein, an M-like protein, is considered as a potential virulence factor of *S. dysgalactiae* (Krishnaveni et al., 2014). This protein could act as the sensory component of a multiple component system, whereby, binding of IgG and or IgA to mig could trigger a conformational change on this protein, resulting in the activation of secondary proteins with histidine-kinase activities that result in the modulation of gene expression of factors involved in virulence (Krishnaveni et al. 2014). The DNA sequence encoding the alpha 2-M receptor portion of the mig gene was different from other *Streptococci* and was highly specific to *S. dysgalactia*e (Jonsson et al., 1994). In the current study, Mig gene was detected in seven out of nine *S. dysgalactiae* isolates with an incidence of 77.8 %. Song et al. (2001) reported that out of 16 *S. dysgalactiae* isolates, only five strains (31%) were found to carry the *mig* α_2-M-homologous sequences linked to the IgG-binding domains.

CONCLUSION

This study revealed that *Streptococcus* species contribute to the occurrence of bovine mastitis in El- Gharbia governorate, Egypt. So, preventive measures must be taken to reduce the spread of infection. The high frequency of virulence genes in the isolates obtained in this work revealed the important role of these virulence genes in the pathogenesis of bovine mastitis. So, from our previous results we conclude that this multiplex PCR assay could be used as an alternative method in routine diagnosis for rapid, sensitive and specific simultaneous identification for *Streptococcus* spp. which play a role in the transmission and pathogenesis of bovine mastitis which is important for diseases control and epidemiological studies.

Competing interests

The authors have no competing interests to declare.

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