P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com
Research Article
https://doi.org/10.47278/journal.ijvs/2022.176

Prevalence, Virulence Genes and Antibiogram Susceptibility Pattern of *Staphylococcus aureus* and *Streptococcus agalactiae* Isolated from Mastitic Ewes

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Article History: 22-624 Received: 21-May-22 Revised: 22-Jun-22 Accepted: 29-Jun-22

ABSTRACT

Clinical mastitis is a common disease found in dairy ewes worldwide that results in great economic losses. This study was designed to investigate the prevalence rate of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from clinically mastitic ewes, determine the virulence determinant genes, and identify antimicrobial sensitivity assay of the isolated bacteria. A total of 37 milk samples were collected from 27 ewes diagnosed with clinical mastitis. These revealed 12 isolates of *S. aureus* (32.4%), followed by 7 isolates of *S. agalactiae* (18.9%). Real-time polymerase chain reaction was used to identify eight strains of *S. aureus* as possessing the thermonuclease (*nuc*) gene, and five isolates of *S. agalactiae* were positive for the presence of the cell surface protein (*cfb*) gene. Eight isolates of *S. aureus* and five isolates of *S. agalactiae* isolated from dairy ewes with clinical mastitis in El-Sharkia, Egypt, possessed several virulence determinants. The antibiotic sensitivity pattern was performed and a large proportion of isolates of *S. aureus* were found to be sensitive to Vancomycin, Kanamycin, Ciprofloxacin, and Gentamycin, whereas large proportions were highly resistant to Penicillin G and Tetracycline. *S. agalactiae* isolates revealed full sensitivity to Vancomycin, Ciprofloxacin, and Oxacillin, but they were highly resistant to Kanamycin and Penicillin G. The virulence gene should be considered when developing prevention and treatment program for clinical mastitis in ewes in regional areas of isolation. It is necessary that the antibiogram findings of drugs with the highest sensitivity be used to replace the currently used antibiotics with those that are more effective.

Key words: Mastitis, Sheep, *S. agalactiae, S. aureus,* Virulence genes.

INTRODUCTION

Ovine mastitis is an inflammation of the mammary gland tissue resulting from bacterial infection. Small ruminant flocks suffering with mastitis result in significant economic losses due to reduced milk quality and yield, cheese may contain pathogenic bacteria, discarded milk after treatment, culling of infected animals and increased treatment costs and mortality (Fthenakis 2019; Martí-De Olives et al. 2020).

Mastitis is classified as both clinical and subclinical. Clinical mastitis shows signs of inflammation in the udders of infected animals. In addition, there are visible abnormalities present in the milk, including clots, abnormal color, and blood (Fthenakis 2019; Du et al. 2022). On the contrary, subclinical mastitis does not show visible signs in the udder or milk but is commonly diagnosed through elevated somatic cell count as indicated by the California Mastitis Test (CMT) (Hussain et al. 2013; Tvarozkova et al. 2019; Mohsin et al. 2022).

Various causative bacterial agents are involved in the pathogenesis of ovine mastitis. Bacterial pathogens causing mastitis are classified as either contagious or environmental forms. The contagious forms of *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (*S. agalactiae*), known as Group B *Streptococcus* (GBS), are the most common etiological agents responsible for causing mastitis in sheep in which an infected udder is the reservoir of these agents and is spread between animals during milking (Contreras et al. 2007; Ruegg 2017; Ji et al. 2020).

Cite This Article as: Abd-Elfatah EB, Fawzi EM, Elsheikh HA and Shehata AA, 2023. Prevalence, virulence genes and antibiogram susceptibility pattern of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from mastitic ewes. International Journal of Veterinary Science 12(2): 152-160. https://doi.org/10.47278/journal.ijvs/2022.176

The *nuc* gene is known as a *S. aureus* species-specific gene that hydrolyzes DNA and RNA in host cells, causing tissue damage and aiding in the dissemination of the *Staphylococci*. It also facilitates its escape from host immune systems when captured by neutrophil extracellular traps (NETs). In addition, for decades, the *nuc* gene has been regarded as the gold standard for identification of *S. aureus* (Torres et al. 2019). Some Polymerase Chain Reaction (PCR)-based tests previously targeted the *cfb* gene of GBS, which encodes the Christie–Atkins–Munch-Peterson (CAMP) factor and seemed to be more sensitive than the CAMP test. Real-time PCR is a reliable and rapid method for detection of pathogenic bacteria as it can be completed in one day, thus rapidly identifying causative agents in milk that is crucial to the economical control and management of udder health (Guo et al. 2019; Asfour et al. 2022).

S. aureus and *S. agalactiae* both produce large numbers of potential virulence factors that enable the microorganisms to survive in the host and contribute to host damage. Furthermore, these virulence factors may be associated with adaptation and clinical manifestations in different host species. Therefore, it is necessary to detect these factors to control and reduce disease dissemination in dairy herds (Madigan et al. 2018).

S. aureus strains produce many virulence factors, and among these factors, staphylococcal protein A (*SpA*) gene is a membrane bound exoprotein of bacterial cell walls that inhibits phagocytosis by leukocytes (Wada et al. 2010; Khan et al. 2013; Kobayashi and DeLeo 2013; Vasileiou et al. 2019). The intercellular adhesion protein A gene (*icaA*) is more specifically a gene responsible for a biofilm formation that plays an important role in the pathogenesis of staphylococcal infections generally and mastitis specifically (Schönborn et al. 2017; Andrade et al. 2021). Furthermore, methicillin-resistant *S. aureus* (MRSA) strains can encode an isolated factor required for the expression of the methicillin resistance A (*femA*) gene by Khodadadi et al. (2016), and the *hlg* gene is a staphylococcal gamma hemolysin that has the ability to lyse erythrocytes and can increase the virulence of the infection (Kumar et al. 2009).

The virulence factors of *S. agalactiae* give it the ability to colonize and survive in its host, among them important genes are the Cα surface proteins (*bca* gene), Cβ (*bac* gene), and *rib* (*rib* gene). These genes are related to ability of the microorganism to the immune system evasion (Sharma et al. 2013). In addition, the cyl operon encoding a β-hemolysin (*hylB)* gene encodes hyaluronate lyase, which allows the bacteria to spread from the initial site of infection (Wang et al. 2014; Han et al. 2022).

The isolation of bacteria through microbiological examination is considered the gold standard and enables the identification of the most effective drugs to prevent and limit the spread of infection. In this case, isolated bacteria were subjected to antimicrobial sensitivity assay by the disk diffusion method utilizing a broad spectrum of antibiotic disks against both *S. aureus* and *S. agalactiae* (Singh et al. 2018).

Antimicrobial resistance is developed among causative bacterial agents as a result of extensive and haphazard use of antimicrobials. Most of the people also don't follow the European Commission's guidelines to conduct sensitive tests prior to treatment to reduce the development of resistant strains of bacteria as the multidrug-resistant bacteria can provoke a significant threat to animal and human health (Vliegher et al. 2018; Dhingra et al. 2020).

In Egypt, small ruminants are reared for wool and meat production, and generally, most shepherds do not use measures to protect udders from mastitis infection before and during the lactation period. In addition, it is generally believed that the milk produced by each ewe is sufficient for the growth and development of their newborns. Therefore, this study was designed to detect the prevalence of *S. aureus* and *S. agalactiae* isolated from clinically mastitic sheep's milk in El-Sharkia Governorate, Egypt. Its further aim was the identification of specific virulence genes associated with pathogenicity of isolated microorganisms and the study of antibiotic sensitivity pattern of specific isolated bacterial strains.

MATERIALS AND METHODS

Animals

This study was conducted with 27 dairy ewes, which were admitted by their owners from different localities near the Zagazig Veterinary Clinic from April 2021 to October 2021. These animals suffered from clinical mastitis in one or both quarters at different levels of intensity, and the history provided by the owners clarified that most of these animals lived in pastures and fed on low nutrient rations. The non-tack animals required attention, including intramammary infusion of long-acting antibiotics, especially during the dry period. The investigation of physical examinations of the mammary glands by palpation to detect pain, asymmetry and fibrosis of the quarters, and inspection of the milk to look for visible abnormalities in its coloration and for the presence of flakes, clots, and watery secretions to screen for the presence of clinical mastitis was performed according to Kelly (1984).

Sampling and Isolates Identification

To reduce contamination during sample collection, 37 mastitic milk samples were collected from one or both halves of the infected sheep involved in the study. The teat ends were scrubbed with 70% ethyl alcohol, and the near teat samples were collected first, followed by the far ones. About 10ml of milk was collected into a sterile test tube after discarding the first three milking streams. Samples were placed in an ice box and transported to the Zagazig University, Veterinary Animal Medicine Laboratory, to be inoculated on standard bacteriological media as was employed by Markey et al. (2013). Briefly, a loopful of sample milk from each infected quarter was streaked on blood agar enriched with 7% sterile sheep blood agar and incubated aerobically at 37°C for 24-48h. The plates were identified by conventional methods, including gross colony morphology, Gram's stain, and hemolytic characteristics, and were tested for catalase and tube coagulase using rabbit plasma according to Markey et al. (2013). *S. aureus* and *S. agalactiae* colonies were selected and subcultured on Mannitol (HiMedia) and Edward media (Oxoid), respectively, and incubated aerobically at 37°C for 24–48h.

Molecular Identification of *S. aureus* and *S. agalactiae* by SYBR Green Real-Time PCR

The colonies of isolate were also confirmed using real-time-PCR on *nuc* and *cfb* genes of *S. aureus* and *S. agalactiae*, and oligonucleotide primers were performed as shown in Table 1.

SYBR Green real-time PCR was applied according to Martinon and Wilkinson (2011). DNA samples were amplified using 12.5µL of 2x QuantiFast SYBR Green PCR Master Mix (FastStart™ PCR Master, 04710436001, Roche), 2µL of primers (Chromogen Company, South Korea), 5µL of DNA template, and 25µL of molecular biology grade water. The following cycling settings were used for real-time-PCR: one cycle of 95°C for 5min, 40 cycles of repeated denaturation at 95°C for 15s, and annealing and extension at 62°C for 1min. The fluorescence intensity of SYBR Green and the melting curve analysis were evaluated, and a threshold cycle (Ct) under 35 and a specific melting temperature (Tm) indicated a positive result.

Molecular Identification of Virulence Genes

DNA of *S. aureus* and *S. agalactiae* extracted from pure bacterial colonies were applied to molecular identification of virulence genes using a QIA amp DNA Mini Kit, catalog no. 51304, according to the manufacturer's instructions. According to the Emerald Amp GT PCR Master Mix (Takara), Cat # RR310A, the total 25µL reaction mixture contained 12.5µL Emerald Amp GT PCR Master Mix (2x premix), 1.0µL (20pmol) of forward primer, 1.0µL (20pmol) of reverse primer (Primers from Chromogen Company, South Korea), 4.5µL PCR grade water, and 6µL template DNA. Oligonucleotide primers used in the study were sourced from Metabion (Germany) and are listed in Table 2. Amplification reaction of *S. aureus* and *S. agalactiae* genes are shown in Table 3.

Agar gel was prepared from 1.5% agarose gel (AppliChem GmbH, Germany) dissolved in 100ml TBE buffer at room temperature. It was then heated to dissolve all granules with agitation and allowed to cool to 70°C, after which 0.5µg/ml Ethidium bromide (Sigma - Aldrich) was added and carefully mixed. The gel was loaded with 20µL of PCR product sample, negative control, and positive control. A 1–5 volts /cm power supply was applied, and after 30 min, the run was halted, and the gel was transported to an ultraviolet cabinet and photographed using a gel documentation system (Alpha Innotech, Biometra) (Sambrook et al. 1989).

Antibiotic Sensitivity Test

The test was employed by the disk diffusion method according to Winn et al. (2006). Prepared stock solutions were obtained by dissolving a few colonies in a tube containing 5ml of sterile 0.85% physiological saline until the turbidity was adjusted to match a 0.5 McFarland standard tube (1.5×10^8 CFU) using adequate light. A swab of bacterial culture was streaked on the dried surface of a Mueller-Hinton plate. Fourteen different antimicrobial disks (Oxoid), shown in Table 5, were firmly pressed onto the inoculated plate and into the agar to ensure complete contact with the agar, and then the plates were incubated at 37°C for 24–48h. According to the Clinical Laboratory Standards Institute (CLSI 2015), the degree of antimicrobial sensitivity against isolates was determined by measuring the visible clear zone of inhibition that hindered the growth of bacterial isolates.

Statistical Analysis

Data were expressed as counts and percentages. For all of the analyses, exact binomial tests for two categories, and chi-square tests for three categories, were run to test differences in proportions of each of the studied factors (diseased quarters, age, lactation stage, number of suckling lambs, teat lesions, and milk condition). $P<0.05$ was considered statistically significant. All analyses were performed with R version 4.1.1.

RESULTS

Clinical Findings

The clinical examination of 21 lactating ewes showed that one or both halves of the udder suffered from redness and swelling, in addition to abnormalities in milk secretion such as clotted milk and/or milk tinged with pus and blood. Six ewes suffered from atrophy and fibrosis of one or both halves of the udder.

Prevalence and Risk Factors Associated with Clinical Mastitis

The history provided by the owners and clinical findings were used to evaluate the relationship between the potential risk factors (age, lactation stages, teat injuries, number of suckling lambs, and milk condition) and the prevalence of clinical mastitis in ewes as illustrated in Table 4. Exact binomial test and chi-squared fit results revealed that the proportions of mastitis were not the same among age categories, P=0.018 (significant). These increases in the proportion of infection were associated with increased age, as the highest percentage of clinical mastitis was found in ewes aged 4 years and above (55.6%), followed by ewes during the mid-age period (2-4y) (33.3%). A lower percentage of infection was recorded during the early-age period (1-2y) (11.1%). The proportion of mastitis also differed according to the number of suckling lambs, P=0.005, and the proportion of infection was much higher when there were two lambs suckling (77.8%), compared to only one lamb (22.2%). Mastitis showed a higher proportion (88.9%) in animals with teat lesions than those without (11.1%), P=0.00004 (highly significant). Ewes milk with flakes or clots recorded the highest prevalence of mastitis (70.4%), compared to bloody milk (18.5%), or watery milk (11.1%), P=0.0002. Lastly, the proportion of mastitis did not differ based on the stages of lactation or presence of diseased quarters ($P>0.05$).

Bacteriological Examination of Milk Samples

Out of 37 collected milk samples, 27 positive samples were identified when first examined bacteriologically on blood agar media. Subcultures of growth colonies on Mannitol and Edward media revealed that the 12 *Staphylococcus spp.* isolates (32.4%) grew as pale light yellow colonies and the 7 *Streptococcus spp.* isolates (18.9%) grew as small transparent bluish-gray colonies.

Table 1: Oligonucleotide primers used in Rt- PCR assay

Isolates	Genes	Primers	Reference
S. aureus	*nuc*	F: CACCTGAAACAAAGCATCCTAAA R: CGCTAAGCCACGTCCATATT	Li et al. (2015)
S. agalactiae	*cfb*	F: GGG AAC AGA TTA TGA AAA ACC G R: AAG GCT TCT ACA CGA CTA CCA A	Diaz et al. (2013)

Table 2: Primers sequences of virulence genes

Isolates	Genes	Sequence	Amplified product	Reference
Coagulase positive *S. aureus* (CPS)	*femA*	F: AAAAAAGCACATAACAAGCG R: GATAAAGAAGAAACCAGCAG	132 bp	Mehrotra et al. (2000)
	spa	F: TCA ACA AAG AAC AAC AAA ATG C R: GCT TTC GGT GCT TGA GAT TC	226 bp	Wada et al. (2010)
	hlg	F: GCCAATCCGTTATTAGAAAATGC R: CCATAGACGTAGCAACGGAT	937 bp	Kumar et al. (2009)
	icaA	F: CCT AAC TAA CGA AAG GTA G R: AAG ATA TAG CGA TAA GTG C	1315 bp	Ciftci et al. (2009)
S. agalactiae	*bca*	F: CAGGAAGGGGAAACAACAGTAC R: GTATCCTTTGATCCATCTGGATACG	535 bp	Manning et al. (2006)
	bac	F: TGTAAAGGACGATAGTGTGAAGAC R: CATTTGTGATTCCCTTTTGC	530 bp	Bobadilla et al. (2021)
	hylB	F: TTATCATCCAGCGCCTCCTAG R: GTGGTGATAACTGACTTCTTGGGA	245 bp	
	rib	F: CAGGAAGTGCTGTTACGTTAAAC R: CGTCCCATTTAGGGTCTTCC	369 bp	

Table 3: Cycling condition of *S. aureus* and *S. agalactiae* virulence genes

Isolates	Target genes	Primary Denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
S. aureus	*FemA*	94˚C/5min	94˚C/30s	50˚C/30s	72˚C/30s	35	72˚C/7min
	Spa	94˚C/5min	94ºC/30s	55˚C/ 30s	72˚C/30s	35	72˚C/7min
	hlg	94˚C/10min	94˚C/1min	55˚C/1min	72˚C/1min	30	72˚C/10min
	ica A	94ºC/5min	94ºC/45s	68˚C/45s	72ºC /90s	30	72ºC/10min
S. agalactiae	*bca*	95°C/5min	95ºC/35s	68°C/40s	72°C/33s	30	72°C/2min
	bac	94°C/2min	94ºC/30s	50°C/1min	72°C/1min	30	72°C/2min
	hyl B	94°C/2min	94ºC/30s	50°C/1min	72°C/1min	30	72°C/2min
	rib	94°C/2min	94ºC/30s	50°C/1min	72°C/1min	30	72°C/2min

Table 4: Overall number of examined ewes on the basis of diseased quarters, age, lactation stages, number of suckling lambs, and teat lesions

Studied factors	Prevalence of mastitis		P-value
	No	%	
Diseased quarters			
One half	17	63	0.177^{NS}
Two halves	10	37	
Age (Years)			
1-2	3	11.1	0.018*
2-4	9	33.3	
≥4	15	55.6	
Lactating stages			
Early (< 3 m)	14	51.9	0.09^{NS}
Mid (3-5 m)	5	18.5	
Late (>5 m)	8	29.6	
No of suckling lambs			
One	6	22.2	0.005***
Two or more	21	77.8	
Teat lesion			
With teat lesion	24	88.9	0.00004***
Without teat lesion	3	11.1	
Milk condition			
Flakes or clots	19	70.4	0.0002***
Blood	5	18.5	
Watery	3	11.1	

Detection of *S. aureus* and *S. agalactiae* Specific Genes by SYBR Green real-time-PCR

The detection of *nuc* and CAMP factor (*cfb*) genes of *S. aureus* and *S. agalactiae*, respectively, was carried out using SYBR Green real-time-PCR. The results showed eight positive pathogenic *S. aureus* against the *nuc* gene, which showed an amplification curve at C_T (threshold cycle) with values of 27, 28, and 30. The results showed five positive *S. agalactiae* isolates against the *cfb* gene, which showed an amplification curve at C_T values of 19, 22, and 24. Finally, the negative one had no amplification (Fig. 1).

Distribution of Virulence factors of *S. aureus* and *S. agalactiae*

Eight positive isolates (*nuc* gene positive) of *S. aureus*, and five positive strains of *S. agalactiae* isolates (*cfb* gene positive), were exposed to conventional PCR by using specific primers (Table 2), and with reaction conditions as shown in Table 3, to predict the pathogenesis and to better understand the mechanism associated with adhesion, invasiveness, tissue damage, and immune evasion of isolated bacteria. Four virulence genes were chosen for each species, with *femA*, *spa*, *hlg*, and *IcaA* representing *S. aureus* (Fig. 2), which was represented by a single amplicon of approximately 132bp (62.5%), 226bp (50%), 937bp (75%), and 1315bp (75%), respectively, whereas all isolates of *S. agalactiae* have *bca* gene, *bac* gene, and the *hylB* gene with a single amplicon amplification of approximately 535, 530, and 245bp, respectively. In addition, *rib* gene was photographed at 369bp and detected in only 60% of the examined specimens (Fig. 3).

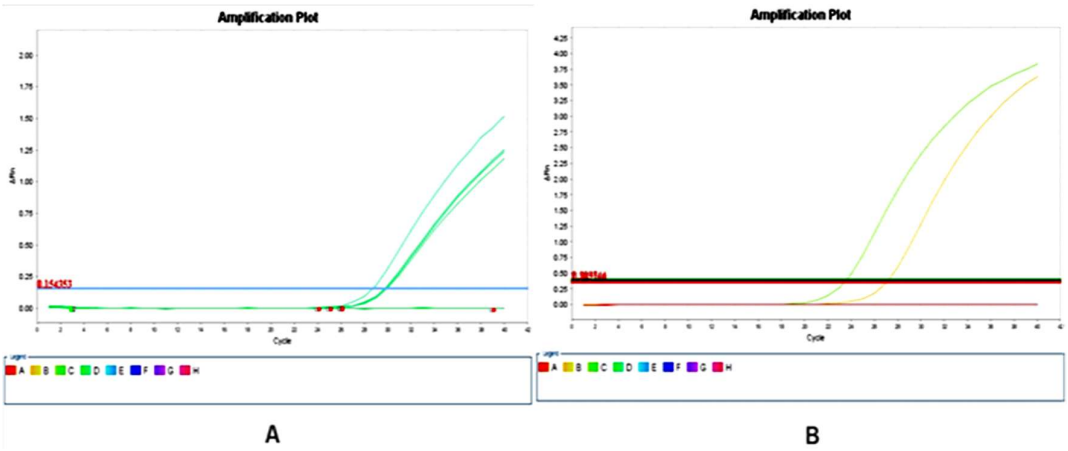

Fig. 1: Syber Green RT- PCR using *nuc* gene (A) and *cfb* gene (b).

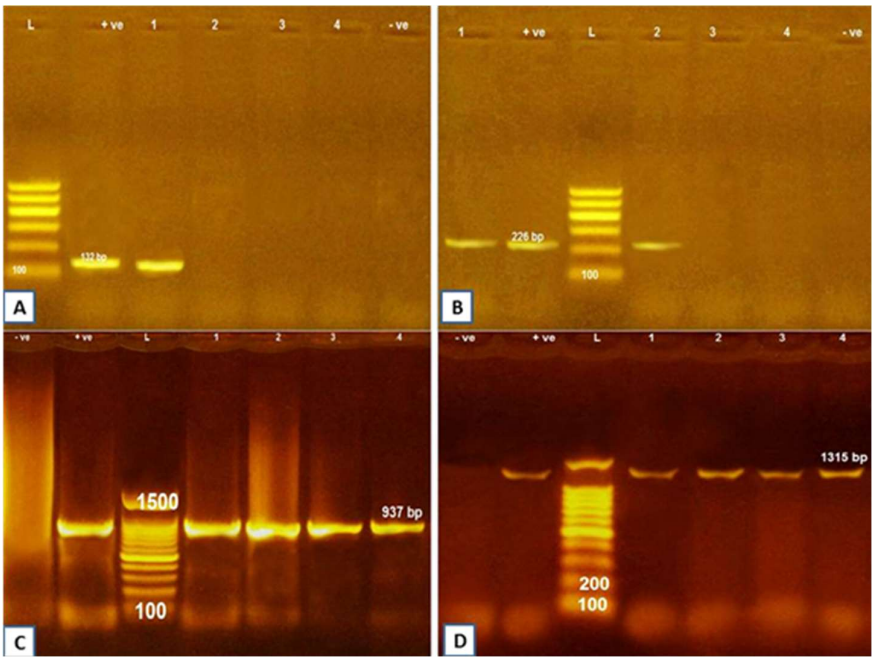

Fig. 2: Amplification of the different *S. aureus* target genes. Amplified band of *femA* gene at 132 bp (A), Amplified band of *spa* gene at 226 bp (B), amplified band at 937 bp of the *hlg* gene (C), amplified band at 1315 bp of the *ica A* gene(D), L: DNA ladder.

Table 5: Overall antimicrobial sensitivity of *S. aureus* and *S. agalactiae* from dairy ewes with clinical mastitis

Antibiotic disc	Symbol	Disk potency (µg/mL)	No of *S. aureus* %		No of *S. agalactiae* %	
			S	R	S	R
Cefepime	FEP	30	6 (75)	2(25)	2(40)	3 (60)
Cefotaxime	CTX	30	6(75)	2(25)	1(20)	4 (80)
Vancomycin	VA	30	8 (100)	0 (0)	5 (100)	0(0)
Ciprofloxacin	CIP	5	6 (75)	2(25)	5(100)	0(0)
Tetracycline	TE	30	3 (37.5)	5(62.5)	3(60)	2(40)
Gentamycin	CN	10	6(75)	2(25)	4(80)	1(20)
Streptomycin	S	10	6(25)	2(25)	2(40)	3(65)
Kanamycin	K	30	8(100)	0(0)	1(20)	4(80)
Penicillin G	P	10	2(25)	6(75)	1(20)	4(80)
Doxycycline	DO	30	6(75)	2(25)	4(80)	1(20)
Chloramphenicol	C	30	7(87.5)	1(12.5)	3(60)	2(40)
Erythromycin	E	15	4(50)	4(50)	3(60)	2(40)
Oxacillin	OX	1	5(62.5)	3(37.5)	5(100)	0(0)
Sulfamethoxazole + Trimethoprim	SXT	25	2(25)	6(75)	3(60)	2(40)

S, sensitive; R, resistant.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility patterns of eight isolates of *S. aureus*, and five isolates of *S. agalactiae*, to 14 antimicrobial drugs are shown in Table 5. Different percentages of susceptibility and resistance against some antimicrobial drugs were recorded. Results showed that *S. aureus* isolates were highly resistant to Penicillin G (75%), Sulfamethoxazole + Trimethoprim (75%), and

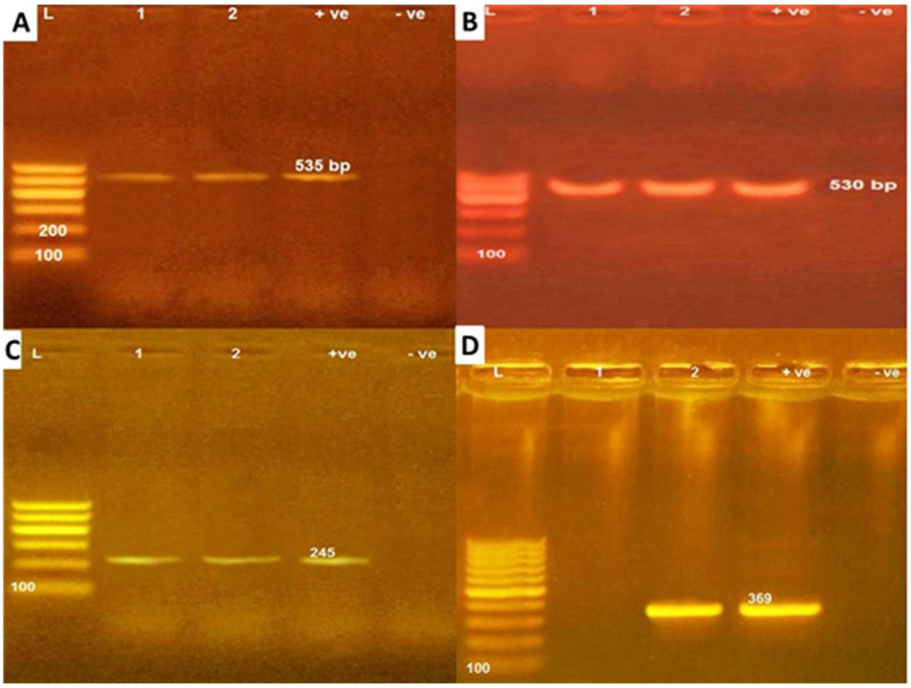

Fig. 3: Amplification of the different *S. agalactiae* target genes. Amplified band of *bca* gene at 535 bp (A), amplified band of *bac* gene at 530 bp (B), amplified band at 245 bp of the *hyl B* gene (C), amplified band at 369 bp of the *rib* gene (D), L: DNA ladder.

Tetracycline (62.5%) but were highly susceptible to Vancomycin, Kanamycin, Chloramphenicol, Cefepime, Cefotaxime, Ciprofloxacin, and Gentamycin, with different degrees ranging from 75 to 100%. Antimicrobial susceptibility for *S. agalactiae* isolates (n=5) revealed that all tested isolates of *S. agalactiae* were sensitive to Vancomycin, Ciprofloxacin, and Oxacillin (100% each) and Gentamycin, Tetracycline, Doxycycline, and Chloramphenicol appeared to have a moderate effect on *S. agalactiae*, whereas 80% (4/5) of *S. agalactiae* isolates were resistant to Kanamycin and Penicillin G.

DISCUSSION

Milk provides high water activity and nutritional value and serves as a powerful media for growth and multiplication of microorganisms whenever suitable conditions are available. Clinically infected dairy ewes admitted to the Zagazig Veterinary Clinic were infected with clinical mastitis in various forms (acute and chronic) on the udder halves associated with or without systemic reaction, in addition to various abnormalities in the condition of the milk. These findings align with Constable et al. (2017).

In this study, it was clear that unilateral mastitis was the most common finding in clinical mastitis in sheep, and these results were in line with previous studies (El-Shymaa 2018). In Table 4, it is shown that older ewes (≥4 years) were found to be more susceptible to clinical mastitis with an incidence of 55.6%. The stage of lactation is associated with the occurrence of clinical intramammary infections (IMIs) as most mastitic cases were recorded close to the lambing period, as compared with those occurring later (Table 4). This may be related to a decrease in immunity during the pre-parturient period that increases the risk of udder infection with mastitis. These results are in alignment with other assays (Mork et al. 2007). The number of suckling lambs per dairy ewe was positively related to increased mastitis prevalence relative to those suckling a single lamb (77.8% versus 22.2%, respectively), and this reflects that the ewes with multiple lambs had more numerous teat bites and subsequently developed more teat lesions that predispose the ewes to bacterial colonization in the mammary ducts. These results were also in harmony with results recorded in other reports (El-Shymaa 2018).

Small ruminant IMIs are mainly of bacterial origin by contagious pathogens (*S. aureus* and *S. agalactiae*) or environmental pathogens (*E. coli*, *S. uberis*, *S. dysagalactia*, and CNS) (Constable et al. 2017). From 37 collected milk samples from sheep with clinical mastitis, 12 isolates (32.4%) were positive for *Staphylococcus spp.*, and 7 strains (18.9%) were positive for *Streptococcus spp.* on specific media. The results of this study were in accordance with similar findings in Norway by Mork et al. (2007) and in Egypt by El-Shymaa (2018) who recorded that *Staphylococcus* is the most important pathogenic agents of clinical mastitis in ewes. The discrepancies in the occurrence of *S. aureus* mastitis between study areas could be explained by differences in climatic circumstances, herd management, and breed diversity. Manual contact of the mammary gland by herdsmen, contaminated bedding material from infected lambs, and lambs nursing from ewes other than their dam can all spread *S. aureus*. Mastitis caused by *S. agalactiae* is also a highly contagious infectious bacterial agent that can spread quickly from one lactating animal to another (Barkema et al. 2009).

Although culture-based methods of detection of bacterial pathogens are inexpensive and reliable, these methods are laborious and time-consuming, so assays based on PCR could overcome the drawbacks of culture-based methods due to their specificity and sensitivity. In this study, SYBR Green real-time-PCR assay was used to detect *nuc* and *cfb* genes specific to *S. aureus* and *S. agalactiae* isolates, respectively, using specific primers. Ceniti et al. (2017) detected the *Staphylococci nuc* gene of animal origin other than *S. aureus*. Furthermore, the SYBR

Green real-time-PCR assay indicated that eight isolates (66.7%) encode the *nuc* gene of *S. aureus*-specific region of the *nuc*, which can degrade both DNA and RNA, and its enzymatic activity can resist 100°C for at least one h; this result was in line with the findings of other investigations (Sallam et al. 2015), which reported that PCR amplification for *nuc* gene is an accurate, rapid, and safe method to screen for *S. aureus* isolates.

Five isolates (71.4%) out of seven strains of *S. agalactiae* demonstrated positively to the *cfb* gene, known as a cell surface protein, that produces a traditional Christie–Atkins–Munch–Peterson (CAMP) phenomenon, which is a typical half-moon forming hemolytic zones on plates of blood agar. This result was stated by El-Behiry et al. (2015), who confirmed a broad frequency of *cfb* gene possessing *S. agalactiae*.

The presence or absence of virulence in pathogenic agents is a critical factor that determines the course of the disease. Although several virulence-associated genes in *S. aureus* are known, this study focused only on four important genes (*femA*, *spa*, *hlg*, *icaA*). Amplification of these genes revealed that all four genes were present among *S. aureus* isolates. The PCR assay in this study showed that 62.5% of *S. aureus* isolates had the *femA* gene. Khodadadi et al. (2016) recorded that *femA* gene was found in all resistant and susceptible *S. aureus* isolates and confirmed that this *S. aureus*-specific gene is an essential factor for methicillin resistance. The amplification of the *spa* gene in *S. aureus* at 226bp in 50% of CPS isolates (Fig. 2) illustrates that the *spa* gene can be used in *S. aureus* typing. While the *IcaA* gene was present in 75% of CPS isolates, Azara et al. (2017) reported that this gene is one of the virulence factors in the pathogenicity of ovine mastitis via biofilm formation, which can limit antimicrobial agent's effectiveness.

After *S. aureus*, *S. agalactiae* is regarded as one of the most contagious mastitis pathogens, that causing serious problems in dairy herds and having a number of virulence factors that support *S. agalactiae* colonization in various tissues of both human and animals as well as assisting the bacteria's survival by limiting or evading host immune responses (Carvalho-Castro et al. 2017).

Studying the different virulence genes is a way to identify potential methods for the prevention and control of mastitis. In this study, four virulence genes *bca*, *bac*, *hylB*, and *rib* were screened in the five isolated strains of *S. agalactiae*. All *bca*, *bac*, and *hylB* genes were found in isolated strains with a percentage of 100%, while *rib* gene was found in three isolates. These findings indicate that these four genes likely play important roles in the pathogenesis of udder inflammation in dairy sheep in Egypt. Abd El-Razik et al. (2021) in Egypt found that bac and *hylB* genes were found in all isolated *S. agalactiae*, whereas El-Behiry et al. (2015) reported the presence of *hylB* gene in most of the isolated strain (81.93%), and only 9.3% of strain had the *bca* gene from dairy cattle. The zoonotic importance of group B streptococci can be proofed by Bobadilla et al. (2021) who detected *bca*, *bac*, *hylB*, and *rib* genes where isolated from pregnant women in Argentina.

The antibiotic sensitivity test in this study applied on *S. aureus* and *S. agalactiae* isolated from clinical mastitis against 14 antimicrobial disks revealed that *S. aureus* isolates were highly sensitive to Vancomycin, Kanamycin, Chloramphenicol, Cefepime, Cefotaxime, Ciprofloxacin, and Gentamycin and confirm similar findings that were recorded by Singh et al. (2018) and Ariffin et al. (2019). *S. aureus* isolates were found to be highly resistant to Penicillin, Tetracyclines, and Sulfamethoxazole + Trimethoprim with degrees ranging from 62.5 to 75%. These results mostly agreed with the study of Chu et al. (2012), who reported that *Staphylococcus spp.* isolated from mastitis in goats showed resistance to Penicillin (50%) and Tetracycline (100%), in contrast to Ariffin et al. (2019), who reported that *S. aureus* from mastitic milk had low Tetracycline resistance rates (11%). While *S. agalactiae* was shown to be sensitive to Vancomycin, Ciprofloxacin, and Oxacillin; Gentamycin, Tetracycline, Doxycycline, and Chloramphenicol had only moderate effects. On other hand, *S. agalactiae* isolate was resistant to Penicillin (80%). While Mir et al. (2014) and Singh et al. (2018) indicated that *S. agalactiae* is highly sensitivity to Ofloxacin followed by Ciprofloxacin, Gentamicin and lower sensitive to Tetracycline, Erythromycin and Vancomycin.

Multiple drug resistance among bacteria is a worldwide problem related to prolonged use of antimicrobial agents without testing of antibiotic susceptibilities of causative bacteria, and this may increase the risk of antibiotic resistance genes being transmitted to the gut microbiota of humans. Therefore, antimicrobial sensitivity tests help to determine the resistance and susceptibility of bacteria toward a proper drug and thus help in mastitis treatment (Mahlangu et al. 2018).

Conclusion

A large proportion of bacteria revealed to cause clinical mastitis in ewes was *S. aureus* (32.4%), followed by *S. agalactiae* (18.9%). Out of four virulence factor concerns associated with *S. aureus*, *hlg* and *IcaA* genes were most frequent, followed by *femA* and to a lesser extent *spa* gene. When evaluating *S. agalactiae*, 100% of strains contained *bca*, *bac*, and *hylB* and less frequently the *rib* gene. This suggests that the protein that codes these virulence genes must be included in locally produced vaccines. Most of the strains of *S. aureus* are susceptible to the most widely used antibiotics according to the antibiogram sensitivity assay conducted, but excluding in treatment the use of Penicillin G, Sulfamethoxazole + Trimethoprim, and Tetracycline, while the strains of *S. agalactiae* were highly susceptible to treatment with Vancomycin, Ciprofloxacin, and Oxacillin. There is limited data available about clinical mastitis of ewes, so further investigation is warranted. Routine inspection with bacterial isolation, the determination of the relationships of various virulence genes, and the better understanding of antibiotic susceptibility patterns are essential in controlling mastitis and achieving effective treatment and the future production of location-specific vaccines containing essential epitopes.

Acknowledgments

The authors thank the owner of the cases admitted to Zagazig Veterinary Clinic for their facility and collection of samples. The authors also thank the workers in the Department of Animal Medicine, Zagazig University,

Egypt, for their help in sample collection. The authors did not receive funding for doing this work.

Authors contributions

EBA and AAS contributed to the concept planning and the study's execution. EMF and HAE performed the analysis of data, and the interpretation. EBA and EMF prepared the initial draft of the manuscript. All authors had complete access to all the data in the study, took responsibility for data reliability and analysis accuracy, and approved the final submission.

Competing Interests

The authors do not have conflict of interest to declare.

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