



Molecular Characterization of Virulence Genes of *Staphylococcus aureus* Isolated from Camel Clinical Mastitis in Egypt

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ABSTRACT

Background: *Staphylococcus aureus* (*S. aureus*) is well-recognized worldwide as one of the main etiological agents of mastitis in dairy animals. The current investigation was undertaken to determine the prevalence and virulence factors of *S. aureus* isolated from camels with clinical mastitis in Egypt.

Methods: A total of 200 milk samples were collected from camels suffering from clinical mastitis and they were examined for *S. aureus*. Both conventional culture-based methods as well as a molecular-based approach were used to identify the bacterium. *S. aureus* isolates were further examined by polymerase chain reaction (PCR) assay for the presence of seven virulence genes namely, clumping factor A (*clfA*), alpha-hemolysin (*hla*), beta-hemolysin (*hly*), toxic shock syndrome toxin-1 (*tsst-1*) staphylococcal enterotoxin A (*sea*), intercellular adhesion A (*icaA*) and intercellular adhesion D (*icaD*).

Result: Out of the 200 samples screened, 60 (30%) were confirmed as *S. aureus*. All *S. aureus* isolates (100%) were found positive for *clfA*, *hly*, *icaD* and *tsst-1* genes. The *icaA* gene was identified in 2 (10%) isolates, *sea* gene was detected in 3 (15%) isolates whereas, none of the isolates was found positive for the *hla* gene. The findings of our study reveal a notable prevalence of *S. aureus* harbouring virulence genes, indicating a crucial role for these factors in the pathogenesis of camel mastitis.

Key words: Camel, Mastitis, *S. aureus*, Virulence genes.

INTRODUCTION

The dromedary camel (*Camelus dromedarius*) is the most predominant and widely distributed livestock species that is extensively found and adapted to hot and dry environments, particularly in Arab countries (Abdallah and Faye, 2012).

Similar to the milk of other dairy animals, camel milk has all necessary nutrients with valuable nutritional properties; as it contains a higher mineral content, lower sugar and cholesterol levels and contains approximately two to three times more vitamin C compared to milk of cow (Barłowska *et al.*, 2011). As a result, camel milk has the potential to fulfill a substantial portion of the daily nutritional requirements (Abdurahman, 2006).

Mastitis is widely recognized worldwide as a serious disease problem affecting all dairy animals including camels, with a significant economic impact in animal husbandry (Sinha *et al.*, 2018). Almost all camel rearing countries, regardless of their economic status, have documented various occurrence of camel mastitis, including Pakistan (Aqib *et al.*, 2017), Algeria (Hadeef *et al.*, 2018), Ethiopia (Balemi *et al.*, 2021; Demlie *et al.*, 2023), Somalia (Mohamud *et al.*, 2020), Sudan (Hussein and Saad, 2017), Kenya (Mwangi *et al.*, 2022), Saudi Arabia (Aljahani *et al.*, 2020), Egypt (Diab *et al.*, 2021), Emirates (Tigani-Asil *et al.*, 2020) and Iraq (Sheet *et al.*, 2021).

Numerous infectious agents capable of causing mastitis have been documented globally. Nevertheless, bacterial infections are broadly acknowledged as the main

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cause of mastitis in domestic animals (Bradley, 2002). *Staphylococcus aureus* (*S. aureus*) has been identified as one of the most significant bacterial pathogens accountable to cause mastitis in all dairy animals as well as camels (Vatalia *et al.*, 2021). Successful intramammary infection of *S. aureus* depends on the production of a variety of virulence determinants that enhance the processes of adhesion, colonization and invasion of the bacterium into the mammary tissue, concurrently enabling evasion of the host's immune response. (Monistero *et al.*, 2018). Currently, more than 40 virulence-associated genes have been recognized in various *S. aureus* strains (Stotts *et al.*,

2005). Therefore, their identification and characterization can greatly improve our understanding the epidemiology and pathogenesis of staphylococcal mastitis (Zecconi *et al.*, 2006).

The family of Bacterial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) includes a variety of adhesins, namely Clumping Factors A and B (*clfA* and *clfB*) and Fibronectin-Binding Proteins A and B (*fnbA* and *fnbB*). These adhesins play a crucial role in promoting the attachment of bacterial surface proteins to host tissue and various extracellular matrix components like fibrinogen, collagen and fibronectin proteins (Foster *et al.*, 2014). This binding is known to promote the initial stage of *S. aureus* infection (Zaatout *et al.*, 2019).

Furthermore, *S. aureus* can produce a variety of cytolytic toxins, such as hemolysins and Panton-Valentine leukocidin (PVL). Hemolysins are harmful toxins that have the potential to damage various types of host cells, such as erythrocytes, epithelial, monocyte, endothelial and macrophage cells (Berube and Bubeck Wardenburg, 2013). These toxins provoke a cytolytic effect through the creation of β -barrel pores within the plasma membrane, leading to the release of cellular contents and eventual lysis of the target cell (Kaneko and Kamio, 2004). Four distinct varieties of hemolysins have been recognized in all *S. aureus* isolates namely, α , β , γ and δ toxins; of these, α and β play a significant role in the pathogenicity of *S. aureus* strains responsible for mastitis (Ariyanti *et al.*, 2011).

S. aureus is widely recognized as one of the main causes of foodborne illness worldwide, owing to its remarkable capacity to generate a diverse array of heat-stable enterotoxins. These enterotoxins are short, proteolytic enzymes that remain stable at high temperature, retaining their biological and immunological activities in milk even following pasteurization and exposure to gastrointestinal enzymes (Hennekinne *et al.*, 2012). To date, approximately 23 serologically distinct enterotoxins belonging to *S. aureus* have been identified (Denayer *et al.*, 2017). Among these enterotoxins, five major antigenic types known as, *sea*, *seb*, *sec*, *sed* and *see* are well characterized and represent typical enterotoxins (Nia *et al.*, 2016).

In addition to enterotoxins, toxic shock syndrome toxin-1 (*tsst-1*) is another distantly related protein, produced by *S. aureus* and involved in toxic shock syndrome in humans. These toxins display superantigenic activity as they interact with antigen presenting cells and T lymphocytes, leading to cellular proliferation and increased cytokine expression (Omoe *et al.*, 2003).

Furthermore, slime formation is believed to be a virulence factor that increases the ability of pathogens to adhere to host tissues and diminish the immune response (Atkin *et al.*, 2014). The slime formed by *S. aureus* is mainly consists of exopolysaccharide poly-N-acetyl- β -1,6-glucosamine (PNAG), that is released by enzymes that are encoded in the intercellular adhesion (*ica*) gene. The *ica*

locus, a component of the “accessory genes” genome, comprises four main genes, namely, *icaA*, *icaB*, *icaC* and *icaD* genes, of which *icaA* and *icaD* play a crucial role in slime production (Arciola *et al.*, 2001).

To the best of our knowledge, the detailed characterization of *S. aureus* virulence genes causing camel mastitis, has only been scarcely examined in Egypt and other developing countries. Therefore, the current study is aimed to determine the occurrence of *S. aureus* in camels with clinical mastitis and, to assess the virulence genes of the isolates using a conventional polymerase chain reaction (PCR) assay.

MATERIALS AND METHODS

Ethical statement

All research procedures were executed in accordance with the guidelines and regulations set by the Research Ethics Committee of the College of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt. The ethical approval number is KFS 2017/3.

Collection of milk samples

Prior to sample collection, appropriate measures were taken to guarantee udder hygiene in she-camels. This involved washing the udders with water, drying them with a clean cloth and sanitizing teat apices with cotton swabs soaked in 70% alcohol. The milkers' hands were also thoroughly washed with soap and water and were subsequently rinsed with 70% alcohol. After discarding the first few streamss of milk, around 10 ml of milk were aseptically collected from each quarter into sterile labelled containers, which were then stored in an icebox and immediately transported to the Bacteriology Laboratory at the Faculty of Veterinary Medicine, Kafrelsheikh University, for bacteriological examination.

Study area

Between January 2021 and August 2021, a total of 200 milk samples were collected from she-camel suffering from clinical mastitis within different localities in Marsa Matrouh governorate. This region is situated in the northernmost part of Egypt and and known to hold one of the densest camel populations in the country.

Isolation and identification procedures of *S. aureus*

Bacteriological examinations of *S. aureus* were performed following the standard methods that were previously described (Quinn *et al.*, 2002). In brief, a loopful (10 μ l) of each milk sample was aseptically streaked onto the surface of 5% defibrinated sheep blood agar and tryptic soy agar media (Oxoid Limited, Hampshire, England). The plates were then incubated aerobically at 37°C for 24-48 h. The grown colonies on previous media were subsequently sub-cultured on Mannitol Salt Agar (MSA) (Oxoid Limited, Hampshire, England) and Baird-Parker agar (BPA) (Oxoid Limited, Hampshire, England) that was supplemented with

an egg yolk-tellurite emulsion (Oxoid, UK) as selective media. These agar plates were then incubated at 37°C for 24-48 h. A portion of a typical and well-isolated colony was subcultured on the corresponding medium in order to obtain a pure culture for further bacteriological identification. The typical staphylococcal isolates were initially identified based on their colonial morphology, pigment production, hemolytic pattern on blood agar and Gram staining. Then, suspected *Staphylococcus* colonies were characterized for biochemical characteristics including catalase, oxidase, sugar fermentation and coagulase activities. Afterwards, single colonies from all presumptive pure cultures of *S. aureus* isolates were picked up and sub-cultured onto nutrient agar slants and preserved at 4°C for further use.

Genomic DNA Extraction and molecular confirmation of the presumptive *S. aureus* isolates

All presumptive *S. aureus* isolates were subjected to additional molecular confirmation by amplifying the *S. aureus*-specific *nuc* gene (encoding a thermonuclease) using PCR method outlined in a previously published protocol (Gao *et al.*, 2011). For this purpose, the isolates were freshly cultured in brain heart infusion broth (Oxoid Limited, Hampshire, England) and incubated overnight at 37°C.

Following this, genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's guidelines. The extracted DNA was stored at -20°C until further analysis.

The PCR for *nuc* gene were carried under the following cycling condition: initial denaturation at 94°C for 5 min, 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 40 sec and extension at 72°C for 40 sec, followed by final extension at 72°C for 10 min.

Molecular detection of *S. aureus* virulence genes

A total of 20 molecularly confirmed *S. aureus* isolates were examined by a uniplex PCR assay for the presence of seven virulence genes that have been involved in *S. aureus* pathogenicity. The selected genes, namely, *clfA* (clumping factor A), *hla* (alpha-hemolysin), *hly* (beta-hemolysin), *tsst-1* (toxic shock syndrome toxin-1), *sea* (staphylococcal enterotoxin A), *icaA* (intercellular adhesion A) and *icaD* (intercellular adhesion D) were analyzed.

The target genes, primer sequences, amplified fragments of PCR products and relevant references have been concisely outlined in Table 1. The reaction mixture of PCR for *nuc* gene and the virulence factors genes

Table 1: Target genes, oligonucleotide primer sequences and anticipated product size used to amplify virulence factors of *Staphylococcus aureus* isolates.

Virulence factors	Target gene	Primer sequence (5'-3')	Amplicon size (in bp)	References
Thermonuclease	<i>nuc</i>	ATATGTATGGCAATCGTTTCAAT GTAAATGCACTTGCTTCAGGAC	395	(Gao <i>et al.</i> , 2011)
Clumping factor A	<i>clfA</i>	GCAAAATCCAGCACAAACAGGAAACGA CTTGATCTCCAGCCATAATTGGTGG	638	(Mason <i>et al.</i> , 2001)
Hemolysins	<i>hla</i>	GAAGTCTGGTGAACACCTGA TGAATCCTGTCGCTAATGCC	704	(Fei <i>et al.</i> , 2011)
	<i>hly</i>	CAATAGTGCCAAAGCCGAAT TCCAGCACCAACGAGAAT	496	
Intracellular adhesion	<i>icaA</i>	CCT AAC TAA CGA AAG GTA G AAG ATA TAG CGA TAA GTG C	1315	(Ciftci <i>et al.</i> , 2009)
	<i>icaD</i>	AAA CGTAAG AGA GGT GG GGC AAT ATG ATC AAGATA	381	
Enterotoxin (A)	<i>sea</i>	GGTTATCAATGTGCGGGTGG CGGCACTTTTTCTCTTCGG	102	(Mehrotra <i>et al.</i> , 2000)
Toxic shock syndrome Toxin-1	<i>tsst-1</i>	ACCCCTGTTCCCTTATCATC TTTTCAGTATTGTAACGCC	326	

Table 2: Cyclic conditions for PCR amplification of *Staphylococcus aureus* virulence genes.

Target Genes	Initial denaturation	Amplification (35 cycles)			Final extension
		Denaturation	Annealing	Extension	
<i>clfA</i>	94°C/5 min.	94°C/30 sec.	55°C/40 sec.	72°C/45 sec.	72°C/10 min.
<i>hla</i>	94°C/5 min.	94°C/30 sec.	53°C/40 sec.	72°C/45 sec.	72°C/10 min.
<i>hly</i>	94°C/5 min.	94°C/30 sec.	53°C/40 sec.	72°C/45 sec.	72°C/10 min.
<i>icaA</i>	94°C/5 min.	94°C/30 sec.	49°C/60 sec.	72°C/60 sec.	72°C/10 min.
<i>icaD</i>	94°C/5 min.	94°C/30 sec.	49°C/ 40 sec.	72°C/40 sec.	72°C/10 min.
<i>sea</i>	94°C/5 min.	94°C/30 sec.	57°C/ 30 sec.	72°C/30 sec.	72°C/7 min.
<i>tsst-1</i>	94°C/5 min.	94°C/30 sec.	50°C/40 sec.	72°C/40 sec.	72°C/10 min.

consisted of 12.5 µl of Emerald Amp GT PCR mastermix (Takara RR310A, Japan), 1 µl of 20 pmol of each forward and reverse primer, 5 µl of bacterial DNA template and 5.5 µl of nuclease free water. The target genes were amplified through uniplex reactions, using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster, CA).

Several PCR protocols were employed to detect the target genes of the *S. aureus* isolates. The thermal cycling conditions for each reaction are delineated in Table 2. Following the PCR amplification, the PCR products were subjected to electrophoresis on 1.5% agarose gel (Sigma-Aldrich, Co., St. Louis, MO, USA) at 120 volts for 60 min and

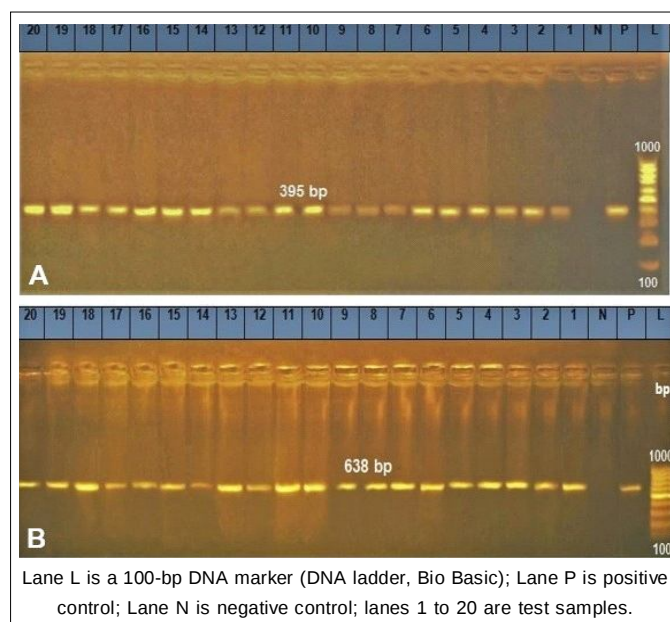


Fig 1: Agarose gel electrophoresis of the PCR products of *nuc* gene at approximately 395 bp (A) and clumping A (*clfA*) gene at approximately 638 bp (B) of representative *S. aureus* isolates.

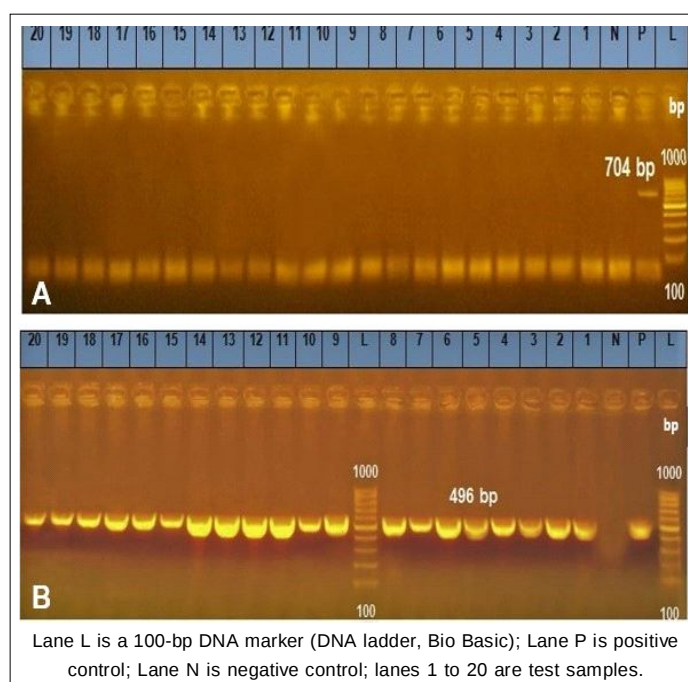


Fig 2: Agarose gel electrophoresis of the PCR products of alpha-hemolysin (*hla*) gene at approximately 704 bp (A) and beta-hemolysin (*hlyB*) gene at approximately 496 bp (B) of representative *S. aureus* isolates.

then stained with 0.5 µg/ml ethidium bromide (Sigma-Aldrich, Co., St. Louis, USA). A 100-bp DNA ladder (Fermentas, USA) was used as a molecular weight marker in each gel. Finally, the PCR products were then visualized and captured using a UV transilluminator (Bio-Rad Laboratories Inc., Hercules, CA, United States).

RESULTS AND DISCUSSION

Overall prevalence of *S. aureus*

S. aureus-related mastitis has been recognized as one of the most significant udder infections in all dairy animals, including camels (Qayyum *et al.*, 2016). In this study, a total of 94 phenotypically positive *S. aureus* isolates were identified, of which 60 yielded an amplicon for the *nuc* gene (395 bp) by PCR and were confirmed as *S. aureus* (Fig 1A). Thus, based on the detection of *nuc* gene in the tested isolates, the overall prevalence of *S. aureus* in this study was 30%. Similar findings have been documented in previous studies, including Algeria (35.62%) (Saidi *et al.*, 2021) and China (24%) (Liu *et al.*, 2022). In contrast, previous investigations conducted in other countries, including Kenya (Kirwa *et al.*, 2021) and Pakistan (Aqib *et al.*, 2017), have indicated a higher occurrence of *S. aureus* mastitis in camels, with prevalence rates of 83.6% and 74.6%, respectively. Conversely, lower prevalence rates have also been reported in other countries such as Ethiopia (17.2%) (Balemi *et al.*, 2021) and Iraq (10.2%) (Al-Rammahi *et al.*, 2018). The observed variations in the prevalence rates of *S. aureus* mastitis in she-camel may be attributed

to distinct geographical locations and diverse herd management practices.

Molecular detection of *S. aureus* virulence genes

Since *S. aureus* is a well-known pathogen associated with mastitis in various animal species, including camels, therefore it is crucial to identify and characterize the virulence factors of this pathogens. In this study, a total of 20 out of 60 *S. aureus* isolates were randomly selected and examined for target virulence genes. The findings of current study indicate that all examined *S. aureus* isolates were found positive for *clfA* gene as depicted in Fig 1B, supporting previous speculation of researchers that this gene could play a crucial role in the initial attachment of *S. aureus* to the epithelial cells lining the teat canal (Da Costa *et al.*, 2014). In China, Zhang *et al.* (2020) achieved similar findings, where the adhesion gene *clfA* being the most prevalent (89.29%) among *S. aureus* isolates from bovine mastitis. Conversely, a significantly lower prevalence rate of *clfA* gene (25%) was observed in *S. aureus* isolated from bovine subclinical mastitis in central Ethiopia (Tegegne *et al.*, 2021).

Staphylococcal hemolysins are recognized as significant virulence factors that are responsible for bacterial invasion and evasion of the immune system. The results of the current investigation revealed that none of the *S. aureus* isolates identified harboured the *hla* gene, whereas the *hlyB* gene was detected in all examined isolates (Fig 2). These findings align with those of a previous investigation conducted by Larsen *et al.* (2002),

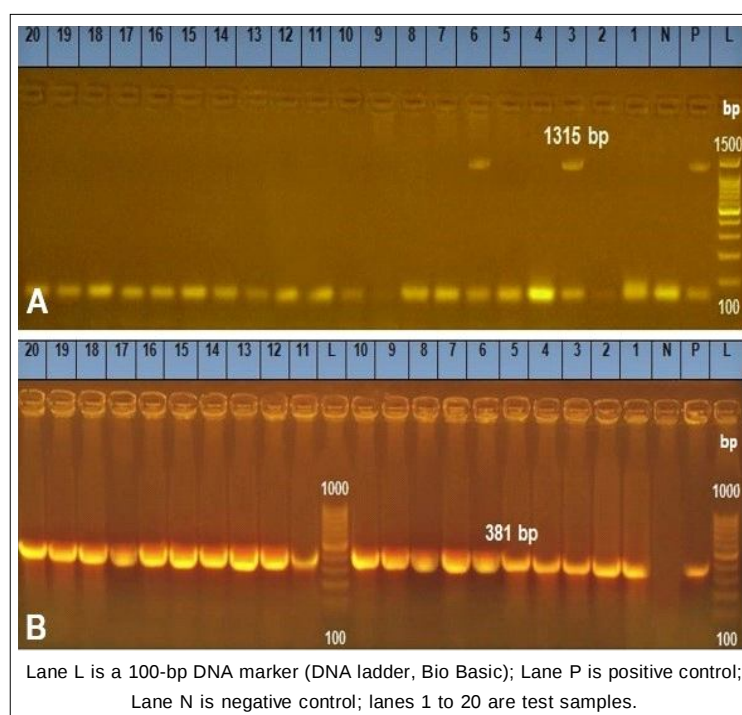


Fig 3: Agarose gel electrophoresis of the PCR products of Intracellular adhesion A (*icaA*) gene at approximately 1315 bp (A) and Intracellular adhesion D (*icaD*) gene at approximately 381 bp (B) of representative *S. aureus* isolates.

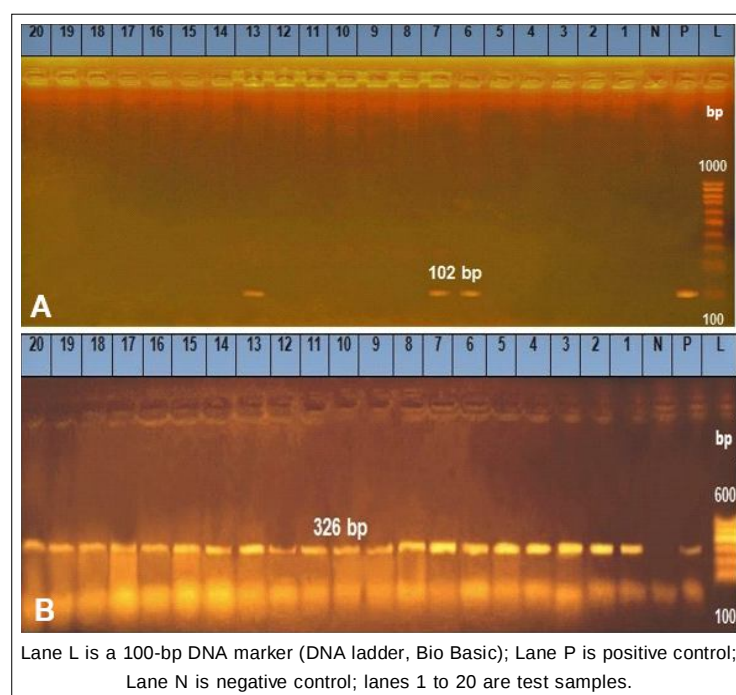


Fig 4: Agarose gel electrophoresis of the PCR products of enterotoxin A (*sea*) gene at approximately 102 bp (A) and toxic shock syndrome-1 (*tsst-1*) gene at approximately 326 bp (B) of representative *S. aureus* isolates.

wherein higher proportions (97%) of the *hlyB* gene were detected in *S. aureus* isolates. In contrast to our findings, Salasia *et al.* (2004) reported a significant occurrence (100%) of *hlyA* gene in *S. aureus* isolated from cases of bovine clinical mastitis. Furthermore, Yadav *et al.* (2015) documented a higher prevalence of *hlyA* (93.75%) and *hlyB* genes (81.25%) in bovine isolates. The divergences in the frequencies of *hlyA* and *hlyB* genes detected in previous and present investigations could potentially be attributed to variations in the animal populations examined, the methodologies employed and evolution of *S. aureus* strains.

In the present study, it was observed that all tested *S. aureus* isolates were found positive for *icaD* gene (100%) (Fig 3A), whereas, only 2 (10%) isolates were detected positive for *icaA* gene (Fig 3B). Our findings support the hypothesis reported by Namvar *et al.* (2013) who suggested that *S. aureus* would not be able to form biofilm unless isolates exhibit positivity for the *icaD* gene. Consistently, Chaudhari *et al.* (2020) also observed that none of the isolates tested were positive for the *icaA* gene whereas, 18 out of the total isolates (34.61%), were found to be positive for the *icaD* gene in *S. aureus* isolated from bovine mastitis. Additionally, Camila *et al.* (2017) demonstrated that none of the 32 *S. aureus* strains isolated from buffalo milk, milking machines and milker's hands tested positive for *icaA*, while only 7 were positive for *icaD* gene. This finding differs from that of Tegegne *et al.* (2021), where the *icaD* gene was not identified in any of the analyzed *S. aureus* strains isolated from bovine subclinical mastitis.

Mastitis, which is caused by *S. aureus*, may poses a significant threat to human health, due to the potential of

pathogen transmission as well as the presence of enterotoxins in milk or dairy products. According to our findings, among the 20 isolates that were examined, only 3 (15%) isolates of them harboured the *sea* gene (Fig 4A). Our results are consistent with the studies conducted by Grispoldi *et al.* (2019) and Monistero *et al.* (2020) which indicated that the prevalence of *sea* gene was found in 35.29% and 65.60% of *S. aureus* isolates, respectively. In contrast, Fursova *et al.* (2020), Tegegne *et al.* (2021), as well as Neelam *et al.* (2022), did not detect the *sea* gene in their respective studies.

All *S. aureus* isolates that were examined in this study exhibited positivity for the *tsst-1* gene (Fig 4B). This is in contrast to more recent investigations conducted in China (Zhang *et al.*, 2020) and Ethiopia (Tegegne *et al.*, 2021), which reported that none of the *S. aureus* isolates from bovine mastitis exhibited positivity for the *tsst-1* gene. However, another study reports that 30% of the clinical isolates containing the *tsst-1* gene (Zhang *et al.*, 2018). The different prevalence rates of virulence genes among *S. aureus* that are associated with mastitis and that have been documented in earlier studies in various localities may be attributed to the evolution of local *S. aureus* strains, sources of the samples and the geographical distribution.

CONCLUSION

The findings of this investigation have revealed the existence of some virulence genes in *S. aureus* particularly *clfA*, *hlyB*, *icaD* and *tsst-1* which were observed to be more prevalent in comparison to other genes such as *icaA* and *sea*. The high frequency of virulence factor-producing strains

observed in this study suggests a substantial contribution of these factors in the pathogenesis of camel mastitis. Further examination of other virulence genes in a large study population is warranted for epidemiological analysis.

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Disclaimers

The views and conclusions expressed in this article are exclusively attributable to the authors and do not necessarily reflect the perspectives of their respective affiliated institutions. The authors bear the responsibility for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All research procedures were executed in accordance with the guidelines and regulations set by the Research Ethics Committee of the College of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt.

Author contributions

SMH and HAF involved in the conception, design as well as evaluation of the study. MMA, HAF and IEE was contributed in sampling, preparing for experiment and conducted the classical bacteriological and molecular techniques. IEE collaborated with SMH in data analysis and interpretation. IEE, HAF and MMA wrote and revised the manuscript for important intellectual content. The manuscript was approved for publication by all authors.

Conflict of interest

None of the authors have any conflict of interest to declare.

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