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Frequency of Enterotoxins and Associated Biofilm Formation Genes among *Staphylococcus aureus* Isolated from Mastitic Cow Milk in Egypt

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Abstract: *Staphylococcus aureus* is among the bacteria closely associated with clinical bovine mastitis. It is isolated from mastitis animals mainly possessing virulence factors. This research was intended to defining the frequency of some virulence factors associated genes among *S. aureus* isolates. From a period start from June to August 2019, a total of eighty-five raw milk samples were collected from cows showing clinical mastitis from different farms in and around Giza government. The presence of the classical enterotoxins (*sea, seb, sec, sed, see*) and four biofilm-related (*icaA, icaD, bap* and *fnbA*) genes were investigated in all isolates by using PCR. All isolates were negative for *Sea, Sec* and *See*. Three of 15 isolates were positive for *sed* (20%) and two isolates were positive for *seb* gene (13.3 %). This study revealed the presence of *fnAb* gene in all isolates (100%). Furthermore, *icaD* gene was in 11 isolates (73.3%) and *icaA* gene was present in three isolates (20%). Finally, all isolates were negative for *bap* gene. Conclusion: Further studies on distribution of different *Staphylococcus aureus* isolates and genes answerable for their pathogenicity of clinical mastitis is necessary.

Key words: *Staphylococcus Aureus* · Enterotoxins · Mastitis · Biofilm · Virulence Factors

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the highest significant causing factors of infectious clinical and subclinical mastitis in dairy herds and in different mammals widespread [1].

Bovine mastitis is a disease that can be classified as clinical or subclinical, that causes universal large economic losses. Mastitis cows are appeared with intensive symptoms (engorged of udder, abnormal milk secretion, with reduced quantity of milk), but may be without signs or detectable changes in milk properties [2, 3].

The isolated *S. aureus* from cows with bovine mastitis produces some of toxins, as toxic shock syndrome toxin and enterotoxin that can lead to food poisoning [4]. Besides fully identification *S. aureus* from bovine mastitis, it is important to distinguish between isolation characters to understand additional information about the division of special genotypes, in addition to recognize the distribution of genotypes due to their effect on human and animal healthiness [5].

Pathogenicity and defenses of *S. aureus* strains are referring to some virulence factors and toxins genes. These factors are diverse in their importance according to the phase of the infection of mastitis, where each strain produces some factors of *S. aureus*. These factors are relaying on disease intensity and the treatment sensitivity [6].

The significant variety of virulence factors among those pathogen focus on staphylococcal bacterial enterotoxins (SE) and adhesion gene [7]. Some strains have the ability to produce the enterotoxins inside food causing the staphylococcal food-poisoning (SFP) [8]. In the last years, various types of SEs were discovered and staphylococcal-like (SEl) proteins were identified [9].

Formation of biofilm is a significant characteristic of *S. aureus* that preserves bacteria against the antimicrobial factors, leading to persistent mastitis in infected animals. A biofilm consists of the extracellular matrix and water. Matrix is created by the intercellular adhesion of polysaccharides, which at the site of *ica* gene [10].

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Biofilm-associated protein (Bap) that is also important in the formation of biofilm [10]. During formation of biofilm, bap and polysaccharides are important in the intercellular adhesion step [11].

The aim of the present research was to identify and characterize genotypes of *S. aureus* strains isolated from cow milk with mastitis. By assessing the incidence of genes associated with the creation enterotoxins and biofilm- related formation genes such as *icaA, icaD, bap* and *fnbA*.

MATERIALS AND METHODS

Isolation and Identification of *Staphylococcus aureus*: A total of eighty-five samples of raw milk were collected from cows presenting with clinical mastitis from different farms in and around Giza city at the period from June to August 2019. Milk samples were collected in clean sterilized tubes and kept in an icebox during transportation to the laboratory of the Microbiology and Immunology department at National Research Centre, Giza, Egypt, where the research was carried out. Milk samples were collected after cleaning the cow's teats, then discarding some milk streams, cleaning the teats with moistened cotton balls of 70% alcohol. The mastitis test was detected using California mastitis test and positive samples were transported to the lab on ice to isolate and characterize bacteria.

Isolation of *S. aureus* was carried out according to method described by Singh and Prakash [12], with slight modification. 90 ml sterile peptone water was added to 10 ml of each milk sample, mixed and incubated at 37°C for 24 hrs. On Baird Parker agar (BP) media, a loop full of enriched milk homogenate was streaked then incubated at 37°C for 48 hours. The appearance of specific black colonies enclosed by a white halo were specified to *S. aureus* bacteria. The pure colonies were picked and streaked on Nutrient agar (Oxoid) and then were incubated at 37°C for 24 hours.

S. aureus confirmatory biochemical tests were performed using catalase test, coagulase test, acetoin production, DNase test, oxidase and D-mannitol fermentation. Isolates that were positive in all these tests except oxidase test which was negative were classified as *Staphylococcus aureus* [13, 14]. The confirmed *S. aureus* isolates were kept and stored in BHI with 40% [v/v%] glycerol at -80°C.

DNA Extraction: DNA extraction was implemented according to QIAamp DNA mini kit instructions (QIAamp DNA Mini Kit, Catalogue no.51304) with some modifications than that described by the manufacturer's recommendations. Concisely, 200 µl of the sample and 200 µl of lysis buffer was added, the mixture was incubated at 56°C for 10 min. Then, 200 µl of (96%) ethanol was also added to the lysate. Next, the sample was centrifuged. Nucleic acid was eluted with 100 µl of elution buffer which was supplied with the kit.

PCR Amplification: Multiplex and Uniplex PCR were carried out to detect the enterotoxins and biofilm formation genes respectively. The following primers were used from Metabion (Germany) shown in (Table 1) and their PCR conditions was presented in (Table 2).

In preparation of enterotoxins multiplex PCR Master Mix, primers were employed in a 50 µl reaction containing 25 µl of Emerald Amp GT PCR Master Mix (2X) (Thermo Scientific), 1µl of each primer (20pmol concentration), 9 µl of PCR grade water and 6 µl of template DNA.

However, in preparation of uniplex PCR Master Mix, primers were employed in a 25µl reaction containing 12.5µl of Emerald Amp GT PCR Master Mix (2X) (Thermo Scientific), 1µl of each primer (20pmol concentration), 4.5µl of PCR grade water and 6 µl of template DNA.

Negative control samples were utilizing simultaneously with each test reaction by replacing the template DNA with sterilized water in the PCR mixture. Twenty µl of each uniplex PCR product and 40µl of each multiplex PCR product were loaded to the gel. Each amplification product was separated by electrophoresis in a 1.5% agarose gel (Applichem, Germany, GmbH) in TBE buffer according to that described by Sambrook *et al*., [18] with slight modification. The reaction was performed in an applied bio system 2720 thermal cycler.

For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Gel pilot 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) with size range: 100-600 bp, Generuler 100 bp plus ladder (Fermentas, Thermo Scientific, Germany) with size range: 100-1500 bp and Genedirex 100 bp DNA ladder H3 RTU, Cat. No. DM003-R500 with size range: 100-1000 bp. all those different size ranges ladders were used to determine the fragment sizes. Ethidium bromide staining (0.5μg/ml) allowed the visualization of DNA fragments with the system of gel documentation and the data was analyzed and evaluated

Table 1: Oligonucleotide sequence of the used primers

Gene	Primer sequence (5'-3')	Size of amplified product	Reference
Sea	GGTTATCAATGTGCGGGTGG	102 bp	[15]
	CGGCACTTTTTTCTCTTCGG		
Sed	CCAATAATAGGAGAAAATAAAAG	278 bp	
	ATTGGTATTTTTTTTCGTTC		
See	AGGTTTTTTCACAGGTCATCC	209 bp	
	CTTTTTTTTCTTCGGTCAATC		
Seb	GTATGGTGGTGTAACTGAGC	164 bp	
	CCAAATAGTGACGAGTTAGG		
Sec	AGATGAAGTAGTTGATGTGTATGG	451 bp	
	CACACTTTTAGAATCAACCG		
icaA	CCT AAC TAA CGA AAG GTA G	1315 bp	[16]
	AAG ATA TAG CGA TAA GTG C		
icaD	AAA CGT AAG AGA GGT GG	381 bp	
	GGC AAT ATG ATC AAG ATA		
Bap	CCC TAT ATC GAA GGT GTA GAA TTG	971 bp	[11]
	GCT GTT GAA GTT AAT ACT GTA CCT GC		
fnbA	CAT AAA TTG GGA GCA GCA TCA	127 bp	[17]
	ATC AGC AGC TGA ATT CCC ATT		

Table 2: Temperature and time conditions of the primers used in PCR amplification

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Enterotoxins	94°C	94°C	57°C	72°C		72°C
	5 min.	30 sec.	40 sec.	45 sec.	35	10 min.
icaA	94°C	94°C	49°C	72°C		72°C
	5 min.	30 sec.	1 min.	1 min.	35	12 min.
icaD	94°C	94°C	49°C	72°C		72°C
	5 min.	30 sec.	40 sec.	40 sec.	35	7 min.
Bap	94°C	94°C	62°C	72°C		72°C
	5 min.	30 sec.	40 sec	50 sec	35	10 min.
fnbA	94°C	94°C	50°C	72°C		72°C
	5 min.	30 sec.	30 sec	30 sec	35	7 min.

through computer software. Bands determination was possible with a 100-bp DNA ladder [Gel Pilot 100 bp ladder (cat. no. 239035 and cat.no. 239045) supplied from QIAGEN, USA].

RESULTS

Isolation and Identification of *S. aureus*: Result analysis revealed that, out of total 85 samples of raw milk isolated from mastitis cows 15 isolates (17.65 %) was *S. aureus* according to morphological, cultural characteristics and biochemical tests.

Detection of Classical Enterotoxin and Biofilm Related Genes of *S. aureus*: The presence of enterotoxin (*sea, seb, sec, sed* and *see*) and four biofilm-related genes (*icaA, icaD, bap* and *fnbA*) was investigated in all isolates by using PCR. All isolates were negative for *sea, sec* and *see*. Three of 15 isolates were positive for *sed* (20%) and two isolates resulted positive for *seb* gene (13.3%).

The study revealed the presence of *fnbA* gene in all isolates (100%), *icaD* gene in 11 isolates (73.3%), *icaA* gene was present in three isolates (20%) and all isolates were negative for *bap* gene as reported in (Table 3) and shown in (Fig. 1-5).

DISCUSSION

Milk is generally sterile in cow and buffalo udder, provided they do not have mastitis (udder infection). If they have mastitis, a large number of generally Gram positive bacteria such as *Streptococcus* and *Staphylococcus spp*. It may be present in the milk as soon as it leaves the udder [19].

In our study, phenotypic and genotypic methods were used to describe isolates of *S. aureus* from mastitis bovine milk samples. Results analysis showed that among the 85 samples of raw milk isolated from mastitis cows, 15 isolates (17.65%) were *S. aureus*. The Prevalence results of our study was higher than other studies of

Table 3: Frequency of Classical Enterotoxin and Biofilm Related Genes in *S. aureus* isolates from cow's milk with clinical mastitis.

Sample	*Sea*	*Seb*	*Sec*	*Sed*	*See*	*icaA*	*icaD*	*bap*	*fnbA*
1	-	-	-	-	-	+	+	-	+
2	-	+	-	-	-	+	+	-	+
3	-	-	-	-	-	+	+	-	+
4	-	-	-	+	-	-	+	-	+
5	-	-	-	+	-	-	+	-	+
6	-	-	-	+	-	-	+	-	+
7	-	-	-	-	-	-	-	-	+
8	-	-	-	-	-	-	-	-	+
9	-	-	-	-	-	-	+	-	+
10	-	+	-	-	-	-	+	-	+
11	-	-	-	-	-	-	+	-	+
12	-	-	-	-	-	-	+	-	+
13	-	-	-	-	-	-	+	-	+
14	-	-	-	-	-	-	-	-	+
15	-	-	-	-	-	-	-	-	+

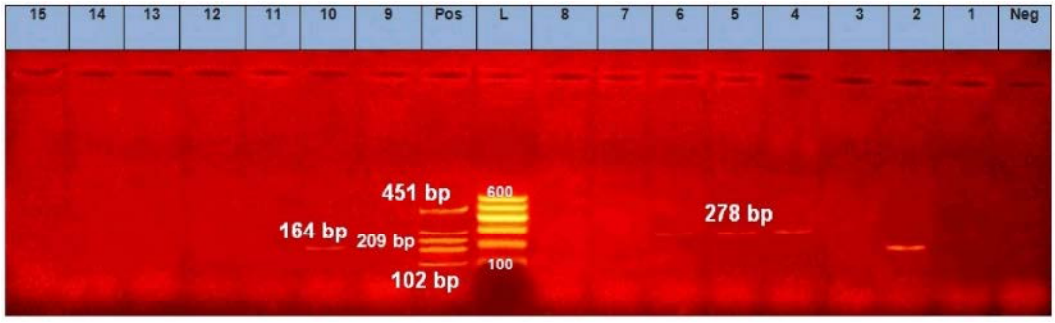

Fig. 1: PCR results for amplification of enterotoxin genes among *S. aureus* isolates. lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. 239035), lane Pos: positive control, lane Neg: negative control, lanes 1,3,7,8,9,11,12,13,14 and 15: negative for enterotoxins and lane 2and 10: positive for *seb* at 164 bp, lanes 4,5 and 6: positive for *sed* at 278 bp.

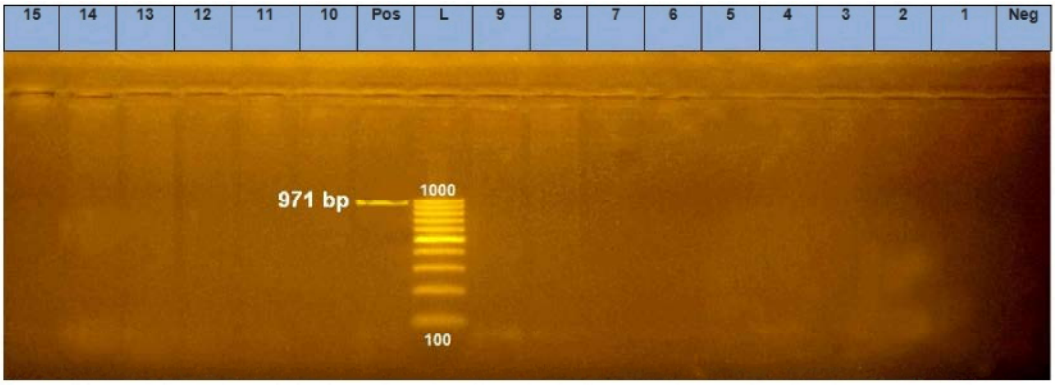

Fig. 2: PCR results for amplification of *bap* gene at 971bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. SM0243), lane Pos: positive control, lane Neg: negative control and all isolates were negative for *bap* gene.

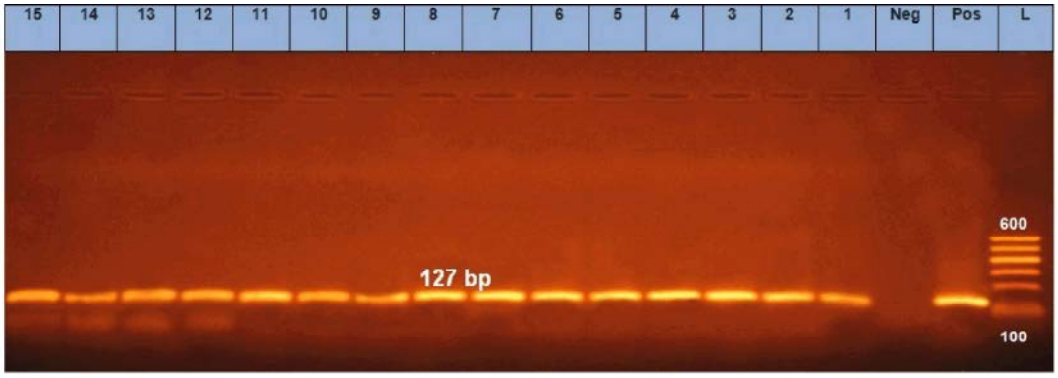

Fig. 3: PCR results for amplification of *fnbA* gene at 127 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. 239035), lane Pos: positive control, lane Neg: negative control and all isolates were positive for *fnbA* gene.

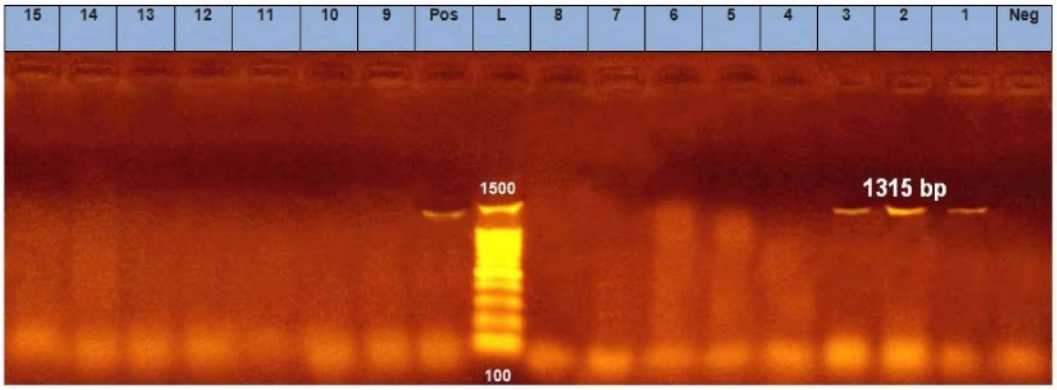

Fig. 4: Pilot 100 bp ladder cat. no. 239045), lane Pos: positive control, lane Neg: negative control, lanes 1,2 and 3: positive for *icaA* gene and lanes 4,5,6,7,8,9,10,11,12,13,14 and 15: negative for *icaA* gene.

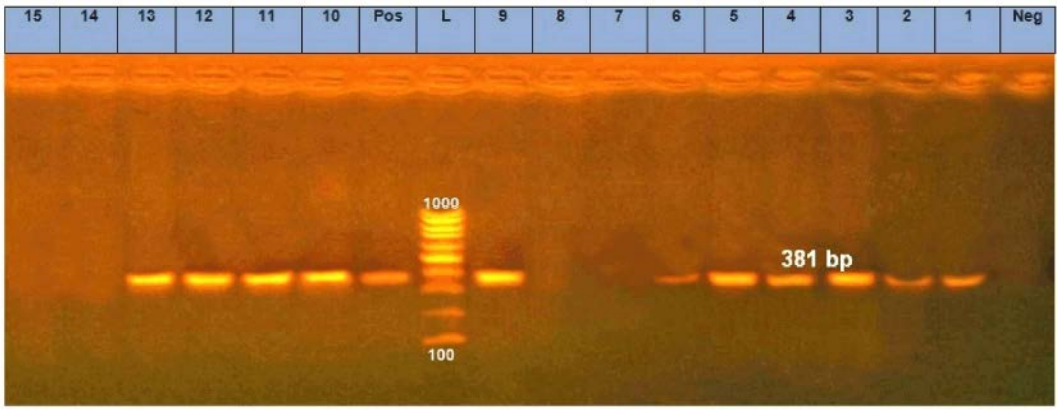

Fig. 5: PCR results for amplification of *icaD* gene at 381 bp among *S. aureus* isolates. Lane L: 100-bp DNA ladder (Gel Pilot 100 bp ladder cat. no. SM0243), lane Pos: positive control, lane Neg: negative control, lanes 1,2,3,4,5,6, 9,10,11,12 and 13: positive for *icaD* gene and lanes 7,8,14 and 15: negative for *icaD* gene.

Shah *et al*. [20] 8.3%, Ekici *et al*. [21] 9.50%, Normano *et al*. [22] 12.80%, Singh and Prakash [12] 10.34%, Kumar and Prasad [23] 6.6%, Addis *et al*. [24] 10 %.

S. aureus causes vital economic losses and represents a potential public health risk because of its ability to produce enterotoxins [25]. Several studies have confirmed the presence of SEs genes among *S. aureus* cultured from raw milk and their food products [26-29]. Once enterotoxins were already produced, these activities will maintain their biological activity even after heat treatment [30].

In the present study, the presence of five classical toxins (*sea*, *seb*, *sec*, *sed*, *see*) and four biofilm-related *icaA, icaD, bap* and *fnbA* genes was investigated in all isolates by using PCR. All isolates were negative for *Sea, Sec and See*. Three of 15 isolates were positive for *sed* (20%), two isolates resulted positive for *Seb* gene (13.4%) and all isolates were negative for *bap* gene as shown in (Table 3).

It was reported that all isolates were negative for *sea, seb, see* and *seq*. One possible explanation may be the use of one pair of primers that may not have known all allelic variants of these genes [31]. The results confirm the results of previous studies claim that *seb*, *see* and *seq* could not be detected in *staphylococcal* isolates from cow's milk [32,33]. However, toxicological tests have recently become available and their epidemiological impact must be monitored [34].

The various types of enterotoxin genes of *S. aureus* isolates can be attributed in many studies to differences in the geographical area [35], the difference in the primers used, the types of samples, the source of the samples and the environments [36]. In this regard, future improvement and combining molecular and immunoassays are needed in order to be able to assess the presence of toxin genes and the spread of toxin itself [34].

The presence of biofilm genes is associated with the pathogenic bacteria because it can contribute to evading host immune defenses and the difficulty of eliminating bacteria. Some authors have indicated that *Staphylococcus* biofilm is one of the main causes of chronic mastitis [7]. The ability to form biofilms helps the persistence of *S. aureus* in infection and subclinical and clinical cases of bovine mastitis [37].

We investigated that the presence of *fnbA* gene in all isolates (100%), *icaD* gene in 11 isolates (73.5%), *icaA* gene was present in three isolates (20%) and isolates were negative for *bap* gene as reported in (Table 3). The most prevalent gene was the *ica* adhesion gene, the ratio of isolates that forming *icaD* was 36.84% [31]. All the *S. aureus* isolates had the ability to form a biofilm-forming membrane and 41.66% of isolates were positive for adhesion genes (*icaA, icaD, fbnA* and *clfA*) [38].

Studies have shown that *S. aureus* strains did not have the ability to form biofilm unless they produced *icaD* gene [39]. The *ica* adhesion genes are one of the most important virulence factors associated with the pathogenesis of mastitis [40]. In addition, the expression of icaA and icaD in vitro is of concern because bacterial biofilms can cause a persistent infection [41]. The enterotoxin genes detection rate was not associated with the biofilm formation ability of the *S. aureus* isolates [42].

CONCLUSIONS

S aureus is one of the clinical and subclinical causative agents of mastitis in dairy herds around the world. Our analysis survey was to determine the prevalence of *S. aureus* in raw cow's milk with clinical mastitis from completely different farms in and around Giza city.

There is a high prevalence of *S. aureus* with biofilm production factors (*fnbA* and *icaA* genes) that will contribute to *S. aureus* without interruption in dairy farms leading to severe infection and hidden food poisoning.

Our results can facilitate understanding the relationship between virulence factors and clinical mastitis caused by different strains of *S. aureus*. However, additional investigations are necessary regarding the diversity of *S. aureus* isolates and the genes responsible for causing mastitis.

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