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Molecular detection of *Staphylococcus aureus* enterotoxin genes isolated from mastitic milk and humans in El-Behira, Egypt

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Abstract

Background and Aim: Milk is a chief source of many nutrients. However, we must also bear in mind that it is a potential source for many cases of food poisoning. This study was conducted to investigate the prevalence of cow mastitis and evaluate the presence of enterotoxins and antibiotic resistance patterns in *Staphylococcus aureus* isolated from milk and contact humans in El-Behira Province, Egypt.

Materials and Methods: A total of 680 milk samples from 170 cows and 86 human samples consisting of 43 hand swabs and 43 nasal swabs were analyzed. The milk samples were subjected to the California mastitis test.

Results: The general occurrence was 23.1% (157/680) where 48 quarters had clinical mastitis and 109 had subclinical mastitis. Subsequently, *S. aureus* was isolated in Baird-Parker agar where typical and atypical colonies were selected and submitted to coagulase and complementary tests. Out of 48 samples of mastitic milk studied, 16 (33.3%) showed contamination by *S. aureus* whereas 109 samples of subclinical mastitis showed contamination in only 18 (16.5%). On the opposite hand, of the 86 human samples, 33 revealed *S. aureus* contamination, corresponding to 38.37% of the samples. Furthermore, multiplex polymerase chain reaction targeting *nuc* and the staphylococcal enterotoxin-encoding genes *sea, seb, sec, sed,* and *see* were performed after culture, revealing that 88.2% (30/34) of milk samples and 93.9% (31/33) of human samples were variably positive to those genes.

Conclusion: The use of *nuc* gene based PCR is an accurate and rapid method for *S aureus* isolates detection. A high prevalence of multiple drug-resistant isolates of *S. aureus* recovered from both human and milk represents further evidence for possible veterinary hazards as well as public hazards, especially to those that consume milk from this region.

Keywords: antibiotic sensitivity, dairy production, enterotoxins, multidrug resistance, multiplex polymerase chain reaction, *nuc* gene, *Staphylococcus aureus* enterotoxin genes, *Staphylococcus aureus.*

Introduction

Mastitis has a significant impact on dairy production, affecting animal health, welfare and milk quality, and causing major economic losses to the dairy industry [1]. *Staphylococcus aureus* is one of the major bacterial pathogens causing bovine mastitis [2,3]. Subclinical and clinical mammary gland infection can produce large numbers of *S. aureus*, which are believed to be extremely contagious, and are easily spread through milking [4,5].

S. aureus is incriminated in most food toxicity outbreaks. Its pathogenicity is due to the production of staphylococcal enterotoxins (*SEs*) [6]. Several varieties of *SEs* can be distinguished serologically. Most staphylococcal food intoxication outbreaks are associated with classical *SE, sea-see*; however, *sea* is the most widely implicated [7,8]. Several molecular techniques are used for the rapid, accurate detection of *S. aureus* isolates, including multiplex polymerase chain reaction (PCR) [9,10].

S. aureus exist in the throats, nasal passages, hair, and skin of nearly 50% of healthy individuals. Food handlers are, therefore, assumed to be a potential source of contamination for food, either directly or indirectly [11,12]. Food handlers act as vehicles for human infection and may, therefore, pose a public health threat [13,14]. The signs of staphylococcal food poisoning, such as abdominal cramps, nausea, vomiting, and diarrhea, occur 2-4 h following the ingestion of contaminated foods, and vary in severity depending on health status of the individual and the amount of *SEs* ingested [15,16].

The use of *nuc* gene-based PCR which encode thermonuclease is a very common use and can identify

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all isolates of *S aureus* from intramammary infection faster than conventional culturing methods. There is a clear need for strict hygienic measures, to reduce the risk of bacterial contamination [17-19].

The use of antimicrobial drugs in humans and animals is poorly controlled, leading to the development of drug resistance. Multidrug-resistant bacteria, including *S. aureus*, are, therefore, frequently isolated from both humans and animals [20,21].

This study was conducted to investigate the prevalence of mastitis and evaluate the presence of enterotoxins and antibiotic resistance patterns in *S. aureus* isolated from milk and contact humans in El-Behira Province, Egypt.

Materials and Methods

Ethical approval and informed consent

All procedures performed in this study, including collection of human and milk samples from animals were done in accordance with the Egyptian ethical standards of the National Research Committee. All human subjects gave their consent for the collection of samples, with the agreement that any identifying details of the individuals should not be published.

Study area and period

This study was conducted in five dairy farms in El-Behera Governorate, which is a Governorates of Egypt with the capital city of Damanhur, located in the West Delta. The study was conducted from January to July 2020.

Samples and sample preparation

Samples were collected at random from El-Behira Province. The samples were 680 milk samples from 170 cows, and 86 human samples, including 43 hand swabs and 43 nasal swabs, from apparently healthy individuals in contact with the cows. The samples were transferred immediately to the laboratory under full aseptic conditions and tested for mastitis using the California mastitis test (CMT) followed by bacteriological isolation and identification of *S. aureus*. The histories of the animals and the results of clinical examination for the presence of clinical or subclinical mastitis were recorded.

CMT

The CMT is a screening test for mastitis [22]. The CMT results can be 0 (negative, trace); 1 (weak positive); 2 (distinct positive); or 3 (strong positive). A test score of 0 was interpreted as a negative result, while scores of 1 or 2 were considered subclinical mastitis, and a score of 3 indicated clinical mastitis. Herds with 25% or more cows with a CMT score of >1 were defined as positive for mastitis. The 680 milk samples collected from 170 cows were tested using being the CMT and aseptically gathered into sterile plastic tubes (10-15 mL/sample). The positive samples were transported in an icebox to the laboratory.

Pre-enrichment

All samples were diluted with buffered peptone water at a proportion of 1:9.

Isolation and identification of S. aureus

All samples were cultured on mannitol salt agar (HiMedia, India) and then incubated at 37°C for 24 h. Colonies suspected to be positive were recultured on Baird-Parker medium (HiMedia) with egg yolk tellurite emulsion (HiMedia) and incubated for 24-48 h at 37°C. For further identification of their cultural, morphological, staining, and other biochemical characteristics, one colony was selected and streaked on mannitol salt agar and incubated at 37°C for 24-48 h [23].

Morphological characteristics

The smear contained Gram-positive, spherical cells arranged in irregular clusters after staining with Gram stain.

Tube coagulase test

Five typical colonies were incubated in a tube holding brain heart infusion broth (HiMedia) at a temperature of 37°C for 24 h. Then, 0.3 mL was transported to a sterile tube containing 0.5 mL of rabbit plasma (HiMedia) and incubated at a temperature of 37°C for 6 h. The reaction was scored as positive if the resulting clot was immobile when the tube was tilted.

Catalase test

The presence of catalase was tested using 3% hydrogen peroxide (H_2O_2). Liberation of oxygen bubbles was taken as indicating a positive reaction.

PCR of the *nuc* gene was used to confirm the classification of the cells as *S. aureus*. Entire isolates were then preserved in brain heart infusion broth with 15.0% glycerol at −80°C.

DNA extraction and purification

The chromosomal DNA of *S. aureus* was extracted by the boiling method as previously described Aldous *et al*. [24], with some alterations. Baird-Parker agar colonies were subcultured onto nutrient agar plates and incubated overnight 37°C. Pure colonies were then transferred to 5 mL of nutrient broth and incubated with aeration at 37°C, employing a shaker machine set at 120 rpm. One milliliter of each culture was then transferred into a 1.5 mL Eppendorf tube, centrifuged at 13,000 rpm for 10 min, and the cell pellets were collected. The cell pellets were cleaned with distilled water and recentrifuged. Then, 200 µL of deionized water was dissolved and mixed by manual shaking. Each Eppendorf tube was maintained at 100°C for 10 min followed by cold shock for 10 min on ice. The tubes were centrifuged again at 10,000 rpm for 10 min, and the supernatant (100-150 µL) was transferred into a fresh Eppendorf tube. The DNA purity and concentration were assessed using optical density with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA)

at various wavelengths [25] and stored at −80°C until further use.

Multiplex PCR conditions

PCR was used to detect the *nuc* gene and the *SEs sea, seb, sec, sed*, and *see* the specific primers described in Table-1. The PCR mixture contained 1.5 mM MgCl2, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton®X-100, 200 mM of each deoxynucleotide triphosphate, 0.2 mM of the individual primers, and 0.625 U Taq polymerase (all the reagents from Promega Corp., Madison, Wisconsin, USA). The amplification was accomplished using an automatic thermocycler T-1 (Biometra). The positive control was *S. aureus* reference strain (ATCC 29213; *sea, seb, sec, sed,* and *see*).

The PCR cycles consisted of pre-heating at 95°C for 10 m, denaturation at 94°C for 5 m, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min. The amplification was performed for 35 cycles with a final extension step at 72°C for 3.5 min.

The PCR products were analyzed using electrophoresis in 1.5% agarose gel containing 0.5 mg of ethidium bromide per mL. Gels were visualized and photographed with Image Master VDS (Pharmacia Biotech). The sizes of the amplification products were estimated by comparison with a 100 bp DNA step ladder (Promega Corp.).

The antibiogram pattern of the isolated *S. aureus* to some antimicrobial agents was constructed using the disk diffusion method, as described by Clinical and Laboratory Standards Institute [26].

Results and Discussion

S. aureus is a widespread member of the natural microflora of the human nasal passages and skin [27]. It is considered to be a possible pathogen, since it may deleteriously affect both human and animal health by causing severe necrotic lesions, abscesses, food poisoning, and bacteremia [28].

In this study, 680 milk samples from 170 cows were analyzed (Table-2). CMT was initially performed on all milk samples, and the overall cow-level prevalence of mastitis was 39.4% (67/170). The overall occurrence in quarter of the udder was 23.1% (157/680), with 48 quarters having clinical mastitis and 109 having subclinical mastitis.

The results presented in Table-3 show that out of 48 samples of mastitic milk studied, 16 (33.3%) were positive for *S. aureus*, whereas 109 samples with subclinical mastitis had only 18 (16.5%) positive for *S. aureus*, producing an overall incidence of 21.6%. Our results are very similar to those of Kirkan *et al*. [29] who confirmed that *S. aureus* was the major pathogen causing bovine mastitis, being identified from 23.1% of the inspected farms, and from 28.3% of the milk samples studied in Turkey. Zeinhom *et al*. and Mansour *et al*. [30,31] reported that 12% and 16.3% of tested milk samples were *S. aureus* positive, respectively. Jahan *et al*. [32] reported that the prevalence of *S. aureus* was 25.53%, and Pexara *et al*. [33] reported that the overall occurrence of *S. aureus* in milk was 24.3%, and Ameen *et al*. [34] found a prevalence of 30%. Many studies have detected a higher occurrence of *S. aureus* such as 62.0% [35], 36.3% [36], and 37.32% [37]. The variation in frequencies of *S. aureus* in milk may be because of improper or suboptimal hygiene or poor farm management [38].

Of 86 human samples, 33 (38.37%) (44.2% of hand swabs and 32.6% of nasal swabs) were *S. aureus* positive (Table-4). This result agreed with those of Khalifa *et al*. [39] who found a prevalence of *S. aureus* of 43.33% from milkers' hands and 36.66% from their

Table-1: Primer sequences, anticipated product size, and sets of multiplex PCR used.

Gene	Primer sequence	Size (bp)	Reference
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	**279**	[35]
sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	
seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164	
sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	491	
sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	495	
see	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	430	

PCR=Polymerase chain reaction

Table-2: Detection of mastitis in cows and quarters using CMT.

Type of sample	Cow (n=170)		Quarters (n=680) that is (170*4)	
	No.	%	No.	%
Occurrence of mastitis	67	39.4	157	23.1

CMT=California mastitis test

Table-3: Prevalence of *Staphylococcus aureus* from clinical and subclinical mastitic milk samples according to ordinary culturing methods

Type of milk (mastitis)	Frequency of *Staphylococcus aureus*	
	No.	%
Clinical mastiti (n=48)	16	33.3
Subclinical mastitis (n=109)	18	16.5
Total (n=157)	34	21.6

Table-4: Prevalence of *Staphylococcus aureus* from contact human samples according to ordinary culturing methods.

Type of sample	Frequency of *Staphylococcus aureus*	
	No.	%
Hand swab (n=43)	19	44.2
Nasal swab (n=43)	14	32.6
Total (n=86)	33	38.37

noses. El-Shenawy *et al*. [40] recovered *S. aureus* from 45% of Egyptian dairy workers. The nasal prevalence *S. aureus* was 33% [41]. The results of this experiment are quite different from those reported by Rasha [42] who confirmed that the isolation rate from hand swabs was 60%. *S. aureus* exists in the environment, and humans can transfer it to food, serving as a source of contamination [12].

PCR assay is a rapid and extremely sensitive procedure, and is a very useful tool for the recognition of *SE* genes in clinical *S. aureus* isolates [43,44]. Multiplex PCR using *nuc* and *SE*-encoding genes was performed after culture (Table-5 and Figure-1) and found that 88.2% (30/34) of milk samples and 93.9% (31/33) of human samples contained *S. aureus*. Our results are compatible with those of Javid *et al*. and Elbehiry *et al*. [19,45]. Those authors discussed the role of the *nuc* gene as a potential gold standard marker gene in the detection of *S. aureus*. Another study was conducted in Egypt and confirmed the existence of the *nuc* gene in all *S. aureus* strains [45,46]. This contrasts with the results published by Abdeen *et al.* [47] who found that approximately 70% of *S. aureus* isolates carried the *nuc* gene.

The noses and hands of food handlers carry enterotoxigenic *S. aureus* and so are considered the chief cause of food contamination. The classical *SE* gene is distributed differently in different countries, due to differences in the habits of food consumption in each region. The PCR products of the recovered *S. aureus* confirmed a relationship between food contaminated with *S. aureus* and the handlers of food [39].

Table-6 shows the enterotoxin profile genes found in *S. aureus* isolates from bovine mastitis, in which *sea* was the most common enterotoxin, which agreed with previous findings [7,48-50].

The results presented in Table-7 show that *sea* was the most widespread *SE* gene in the skin [40] and noses of Egyptian food handlers El-Shenawy *et al*. [51], either from carriers or patients [43]. Wongboot *et al*. [43] suggested that the differences in the geographic distribution of *S. aureus SEs* genes may be explained if *SEs* are located on mobile genetic elements that may be exchanged between bacteria of the same or different species.

Sec is the most widespread *SE* in Italian dairy products [52], while *sed* is the most predominant *SE* in dairy products [53]. Udo [54] reported that *seb* is the most detected *SE* on the hands of food handlers. Soriano *et al*. [55] reported that *seb* and *sed* were the predominant *SEs* in food handlers, while Collery *et al*. [56] reported that *seb* is the *SE* most shared among nasal isolates. Chang *et al*. and Gholamzad *et al*. [57,58] disagreed with our findings in that they reported that *seb* was the most common *S. aureus* enterotoxin gene.

The determination of combined *SEs*, such as *sea-seb* and *seb-sed* in both milk and human isolates, indicates the zoonotic importance of such toxins. This finding agrees with that of Hoque *et al*. [35] who detected *sea+seb*. Khalifa *et al*. [39] and Hasan and Hoshyar [50] reported a combined *sea+sec SE*.

Globally, the misuse and unsupervised use of antimicrobial agents in the treatment of animal and human infections has contributed to the emergence of antimicrobial resistance [20,37].

Our results concerning the sensitivity of *S. aureus* to different antibiotics show multiple drug resistance, providing further evidence of a potential public and veterinary health hazard (Table-8). Animals can also transmit resistant strains to humans [4,59,60].

We found high levels of resistance to gentamycin and ciprofloxacin in milk and human isolates (Table-9), which agreed with the findings of Hoque *et al*., Zeinhom *et al*., Ameen *et al*., Reta *et al*. and Klimešová *et al*. [30,35,61,62]. These authors also detected high resistance to gentamycin in animal

Table-5: Identification of *Staphylococcus aureus* from milk and human using PCR in comparison with culture methods.

Source	Culture method			PCR		
	No. of samples examined	**No. of positive**	**%**	**No. of samples examined**	**No. of positive**	**%**
Milk	157	34	21.7	34	30	88.2
Human	86	33	38.37	33	31	93.9

PCR=Polymerase chain reaction

Table-6: Enterotoxin profile genes found in *Staphylococcus aureus* isolates from bovine mastitis.

Enterotoxin gene	Number (%) of *Staphylococcus aureus* isolates		
	Clinical mastitis milk (n=13)	**Subclinical mastitis milk (n=21)**	**Total (n=34)**
Sea	3	7	10
Seb	0	3	3
Sec	1	2	3
Sed	1	1	2
See	0	2	2
Sea-seb	0	1	1
Seb-sed	1	0	1
Total	6 (46.2)	16 (76.2)	22 (64.7)

Table-7: Enterotoxin profile genes found in *Staphylococcus aureus* isolates from human.

Enterotoxin gene	Number (%) of *Staphylococcus aureus* isolates		
	Hand swab (n=19)	Nasal swab (n=14)	Total (n=33)
Sea	5	3	8
Seb	2	1	3
Sec	1	2	3
Sed	0	1	1
See	1	0	1
Sea-seb	2	1	3
Seb-sed	1	1	2
Total	12 (63.2)	9 (64.3)	21 (63.6)

Table-8: Multidrug resistance (MDR) observed among *Staphylococcus aureus* isolates from milk and human samples.

Sample type	Number of antimicrobials				
	1	2	3	4	5
	No. (%) of MDROs				
Milk samples n=34	0 (0.0)	10 (23.5)	15 (32.4)	5 (14.7)	4 (11.8)
Human samples n=33	4 (12.1)	9 (27.3)	6 (18.2)	14 (42.4)	0 (0.0)
Total (n=67)	4 (5. 97)	19 (28.36)	21 (31.34)	19 (28.36)	4 (5.97)

Table-9: Resistance of *Staphylococcus aureus* isolates from milk and human to different antibacterial agents.

Antimicrobial	Disk concentration	Zone of resistance	Milk isolates n (34)		Human isolates n (33)	
			No.	%	No.	%
Penicillin	10 units	≤28	13	38	12	36.4
Gentamicin	10 µg	≤12	6	17.6	8	24.2
Azithromycin	15 µg	≤13	0	0	1	3
Ciprofloxacin	5 µg	≤15	9	26.5	10	30.3
Levofloxacin	5 µg	≤15	3	8.8	2	6
Clindamycin	2 µg	≤14	0	0	0	0
Erythromycin	15 µg	≤13	4	11.8	6	18.2
Doxycycline	30 µg	≤12	5	14.7	4	12.1
Norfloxacin	10 µg	≤12	1	2.9	0	0

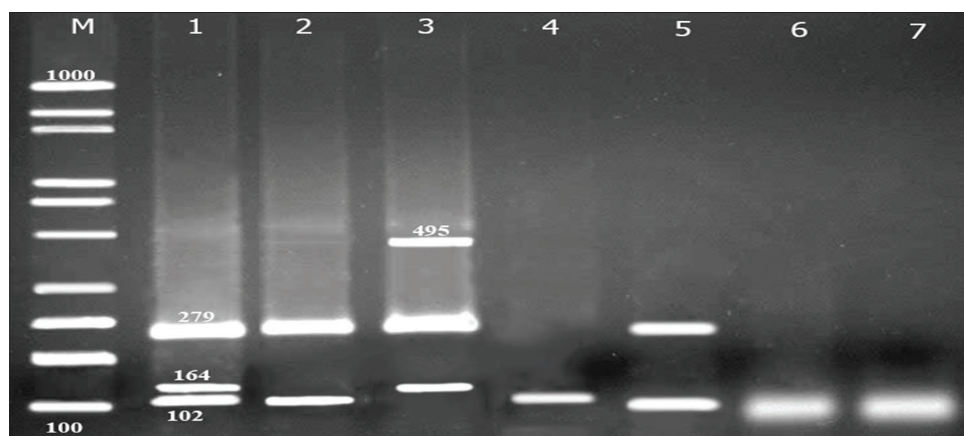

Figure-1: Gel electrophoresis of multiplex polymerase chain reaction to *nuc* gene and five *Staphylococcus aureus* enterotoxin genes (*sea*, *seb*, *sec*, *sed*, and *see*).

isolates and to gentamycin and ciprofloxacin in human isolates. Higher sensitivity was recorded to gentamicin in human isolates in Iran [44], while, El Faramaway *et al*. [63] in Egypt found increased resistance in *S. aureus* isolates from clinical mastitis to penicillin and gentamicin. Pexara *et al*. [33] indicated that a high percentage of resistance to clindamycin was detected among *S. aureus* isolates.

Conclusion

The data presented here show that pathogenic *S. aureus* was isolated from milk and human samples at various numbers, probably because of poor hygiene. Multiplex PCR targeting the *nuc* gene and *SEs* can lead to rapid and accurate detection of *S. aureus* in milk. *S. aureus* carries multiple virulence genes which are of clinical importance in *S. aureus*. A high prevalence

of multiple drug-resistant *S. aureus* isolates provides further evidence for potential public and veterinary health hazards.

Authors' Contributions

MSD and SAZ: Conceptualization. NAI, MAS, SAZ and EMAT: Methodology. MSD, YFE, NAI, SAZ, and MAS: Validation. SAZ, NAI, and GAP: Formal analysis. MSD, NAI, YFE, SAZ, and MAS: Investigation. YFE, SAZ, and MAS: Resources. MSD, SAZ, and NAI: Data curation. NAI, YFE, and MAS: Writing – original draft preparation. MSD, SAZ, and NAI: Writing – review and editing. MSD and YFE: Visualization. SAZ, MAS, and YFE: Supervision. SAZ, MAS, and YFE: Project administration. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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