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Prevalence and multiplex PCR for enterotoxin genes of *Staphylococcus aureus* isolates from *subclinical mastitis* and kareish cheese

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Abstract
Aim*: Staphylococcus aureus* was categorized as a contagious pathogen incriminated in subclinical mastitis as well as in dairy products. From public health view, this organism causing food poisoning outbreaks via contamination of food products with its toxins. This study highlight the prevalence of *Staphylococcus aureus* among subclinical mastitic cases as well as cheese samples and the dominant enterotoxigenic genes.
Methodology: examination of 100 samples (50 from subclinical mastitis, and 50 kareish cheese) from Sadat city, Menoufia province.
Results & Interpretation: bacteriological culturing on selective and specific medium revealed that 26.7% and 30% in subclinical mastitis and kareish cheese respectively were due to *S. aureus*. Furthermore, multiplex PCR proved to be efficient technique for detection of different enterotoxins genes. The sea, seb and see genes were the most prevalent genes among the tested *S. aureus* isolates. Although, no detection of sec and sed genes were observed. In conclusion, it can be concluded that molecular characterization of *S. aureus* and its enterotoxins genes will be beneficial in designing control and preventive measures of *S. aureus* infection in human and animals.

Keywords: enterotoxins; food, PCR, *S. aureus*, subclincal mastitis

Introduction
Staphylococcus aureus was responsible for wide range of illness in human and animals. In the dairy industry, it causes about 50% of intramammary infection [1, 2]. The losses due to *S. aureus* subclinical mastitis include higher losses in milk production, increase culling rate, veterinary and treatment cost [3]. This organism was frequently inhabitant in the skin of udder microbiota [4] under special circumstances, invade and colonized into udder tissues of dairy animals leading to inflammation and may produce serious form of mastitis [5]. Regarding food poisoning outbreaks, enterotoxins of S aureus considered the most famous detecting in foods poisoning in human [6]. Thus assured via consumption of contaminated food products and milk products by *S. aureus* toxins [7]. In generally, early study of [8] reported that 14% to 40% of all human food poisoning outbreaks were associated with staphylococcal enterotoxins, that manifested at first by sudden onset of fever, vomiting, nausea, abdominal cramps and diarrhea [9, 10]. The existence of some virulence factors as enzymes, toxins, surface antigens, and capsule have an important role in S aureus pathogenicity as in intramammary infection, it depends on the induction of immunosuppression [11] through stimulating of the proinflammatory cytokines and T-cell that evoked the inflammatory features. One of these factors is enterotoxins which proved to be more tolerance to pasteurization and higher temperature [12]. The enterotoxins constitute and reported as an important virulence agent involved in food poisoning and toxic shock syndrome [13]. The most commonly identified genes were (SEA, SEB, SEC, SED, and SEE) [14]. In Brazilian study the higher frequency and detection of both see and seb genes in clinical and subclinical mastitis [15]. However, other studies [16, 17] have been described a new genes such as (SEG, SHE, SEI, SEJ, SEK, SEL, SEM, SEN, and SEO). Therefore this study aimed to estimate the prevalence and applying of multiplex PCR for identifying of enterotoxin genes from *S. aureus* isolates from subclinical mastitis and kareish cheese.

Material and Methods

Sampling and processing of collected specimens

Milk samples were collected aseptically in a 5 ml sterile plastic tubes (50 samples). Then samples examined for subclinical mastitis by CMT that done according to [18]. Mastitic milk samples were centrifuged and sediment were streaked on specific medium. Another 50 kareish cheese, were randomly collected from supermarkets in Sadat City. Each sample about 10 gm was collected in a sterile plastic bag. All collected samples were transported in cool ice boxes and transported into the laboratory for further investigation.

Isolation and identification of *S. aureus*

The processed samples from mastitic milk and kareish cheese were streaked into Baird-Parker agar (Oxoid Ltd.) and 10% sheep-blood agar. Plates were incubated for 24–48 h at 37°C. Then, examination of the suspected colonies of *S. aureus* was done through Gram staining as according [19]. The biochemical and virulence properties as catalase, coagulase as determined by [20], haemolysis activity on blood agar and Deoxyribonuclease (DNase) testing onto DNase agar [21].

Multiplex PCR for detection of enterotoxin genes of *S. aureus* isolates

DNA extraction: The extraction of DNA from samples was performed using the QIA amp DNA Mini kit (Qiagen Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in table (1)

For multiplex PCR of enterotoxins, Primers were applied in a 50- µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 16 µl of water, and 7 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer. For gel analysis, 40 µl of the multiplex PCR products were loaded in each gel slot. Gelpilot 100 bp DNA ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Sea	GGTTATCAATGTGCGGGTGG	102	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[22]
	CGGCACTTTTTTCTCTTCGG							
Seb	GTATGGTGGTGTAACTGAGC	164						
	CCAAATAGTGACGAGTTAGG							
Sec	AGATGAAGTAGTTGATGTGTATGG	451						
	CACACTTTTAGAATCAACCG							
Sed	CCAATAATAGGAGAAAATAAAAG	278						
	ATTGGTATTTTTTTTCGTTC							
See	AGGTTTTTTCACAGGTCATCC	209						
	CTTTTTTTTCTTCGGTCAATC							
Target gene	**Primers sequences**	**Amplified segment (bp)**	**Primary Denaturation**	**Amplification (35 cycles)**			**Final extension**	**Reference**
				Secondary denaturation	**Annealing**	**Extension**		
Sea	GGTTATCAATGTGCGGGTGG	102	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[22]
	CGGCACTTTTTTCTCTTCGG							
Seb	GTATGGTGGTGTAACTGAGC	164						
	CCAAATAGTGACGAGTTAGG							
Sec	AGATGAAGTAGTTGATGTGTATGG	451						
	CACACTTTTAGAATCAACCG							
Sed	CCAATAATAGGAGAAAATAAAAG	278						
	ATTGGTATTTTTTTTCGTTC							
See	AGGTTTTTTCACAGGTCATCC	209						
	CTTTTTTTTCTTCGGTCAATC							

Results

Table 2: Distribution and prevalence of enterotoxin genes among *S. aureus* isolates from subclinical mastitis milk and kareish cheese

Source of samples	total nu of samples	Positive Staphylococci species	*Staphylococcus aureus*		Negative	
	100	35	Number	%	Number	%
Sub clinical mastitis	50	15	4	26.7%	11	73.35
Cheese	50	20	6	30%	14	70%

Table 3: Distribution and prevalence of enterotoxin genes among *S. aureus* isolates from subclinical mastitis milk and kareish cheese.

Sample	Results				
	Sea	***Seb***	***Sec***	***Sed***	***See***
1	-	+	-	-	-
2	+	-	-	-	+
3	+	+	-	-	+
4	-	-	-	-	-
5	+	-	-	-	+
6	+	-	-	-	+
7	+	+	-	-	+
8	-	+	-	-	-
9	-	+	-	-	-
10	+	-	-	-	+

The results of enterotoxin genes in *S. aureus* isolates from subclinical mastitis milk and kareish cheese illustrated in table (3) revealed that sea and see enterotoxin genes were the most prevalent among 10 *S. aureus* isolates with 60% followed by seb (50%), while no detection for sec and sed enterotoxin genes in any tested samples. Samples from 1-4 isolated from subclinical mastitis while 5-10 from kareish cheese. In subclinical mastitis all the detected enterotoxin genes (sea, seb and see) with the same percentage 20%. In contrast, the sea and see enterotoxin detected in 40% followed by seb enterotoxin 30% in kareish cheese.

3. Multiplex PCR for detection of enterotoxins genes in *S. aureus* isolates from subclinical mastitis and kareish cheese.

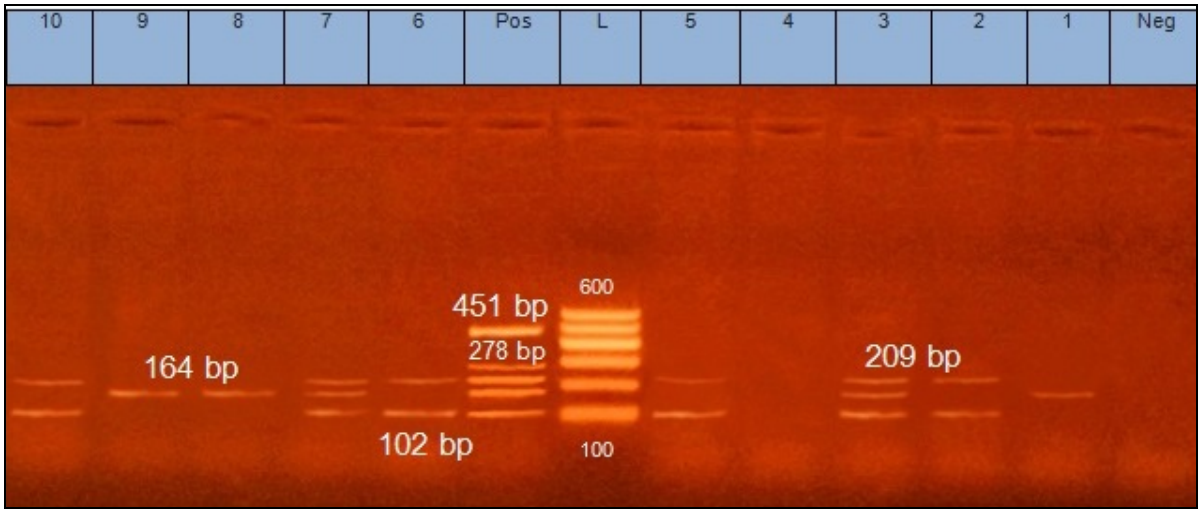

Fig 1: Multiplex PCR for 10 *S. aureus* isolates (1-4 isolated from subclinical mastitis; 5-10 from kareish cheese), the sea genes were positive in 6 samples (2, 3, 5, 6, 7 and 10) at 102 bp; the seb genes detected in 5 samples (1, 3, 7, 8, 9) at 164 bp; the see detected in 6 samples (2, 3, 5, 6, 7, 10) at 209 bp, with no detection for sec and sed genes at 451 bp and 278 bp respectively

Discussion

Mastitis constitutes the major economic and productive disease in dairy cows all over the world. Mastitic milk can possess a serious hazard to human consumers due to higher bacterial count or toxins [23, 24]. The incidence of subclinical mastitis form has become more prevalent 15-40 times than clinical form [23] so, this form of mastitis may act as continues source of new infection to mammary glands [25] that often ended by serious form of clinical mastitis [26]. One of the adverse effect on the milk quality due to subclinical mastitis is the decreasing of shelf life period of raw milk [27]. Many studies proved that *Staphylococcus aureus* is a major bacteria existed in bovine mastitis [28]. This study revealed that the prevalence rate of *S. aureus* from subclinical mastitis was 26.7%. Nearly similar findings in Egypt reported by [29, 30] they estimated *S. aureus* prevalence were 28.2% and 29.16% respectively. Additionally, [31] recorded (29%) in Bangladesh. On the other hand, higher prevalence (55.58%) was reported in the study of [32]. However, [33] mentioned that approximately 30%- 40% of all mastitis cases caused by *S. aureus*. In contrast, the prevalence rate in kareish cheese was 30% in our study. These nearly in contact with [34] who detected *S. aureus* isolates in 27% from 200 milk and cheese samples in Iran. Although, a little lower prevalence rate 25% had been recovered from 100 Iranian white and feta cheese samples by [35]. The higher prevalence 75% and 46% has been obtained by [36, 37] respectively. The lower prevalence (10%, 9.5%, and 7.7%) were observed in different countries in the studies of [38, 39, 40] in Iran, turkey and European countries respectively. The observed variation in prevalence of *S. aureus* especially in milk products may be reflect the level of sanitary measures in milk manufacture or due to the differences in technological methods in cheese manufacture [35]. Regarding staphylococcus food poisoning that considered a major etiology of gastroenteritis in human [41]. This depend on the ability of *Staphylococcus aureus* to produce toxins or virulence factors that facilitated the disease occurrence [42]. The S. auerus enterotoxins (SE), particular SEA-SEE were the most classical discovered genes in cattle, sheep and goats milk [1, 43] Showed that the host environment played a role in the adaptation of S. auerus in target host through production of SE. Modern molecular techniques as multiplex PCR has successfully adapted for detection of enterotoxins in *S. aureus* isolates from subclinical mastitis milk and Karich-cheese was

done in this work as showed in fig 1 and table (3). It's clear that sea and see enterotoxin genes were the most prevalent among the tested *S. aureus* isolates followed by seb, while no detection for sec and sed enterotoxin genes. In subclinical mastitis enterotoxin genes (sea, seb and see) were detected in 20%. In contrast, the sea and see enterotoxin detected in 40% followed by seb enterotoxin 30% in Karich-cheese. These were parallel to results of [44] who revealed the sea gene was the most prevalent gene, followed by seb, sec, sed and see. Furthermore, [45] found that sea gene was the prevalent SEs (36.7%) followed by seb (17.4%), see (16.5%), sec-1 (11.01%), and sed (6.4%). Although, disconnected with [46] reported lower prevalence (1.6%) of sea gene. The absence of sec gene in our study can be explained that the sec gene has three subtypes (sec1, sec2, and sec3) that classified according to antigenic properties or the diversity in the sequencing of enterotoxin C [47]. There was no detection of sed gene in our findings. This almost observed in the result of [48] and [44] found that sed was the lowest dominant enterotoxins genes in very low percentage 0.5%*

Conclusion

Our results concluded that *Staphylococcus aureus* was identified as one of the contagious cause of intramammary infection in the dairy farm as well as food poisoning outbreak worldwide. The pathogenicity of this organism relies on some virulence agents as enterotoxins that exert a toxic effect on host cell or in food products. Several enterotoxins genes, particularly, (sea, seb, sec, sed, and see) genes were the most identified from *S. aureus* isolates of milk origin. These genes appear to have a critical role in the pathogenicity of *S. aureus* in subclinical mastitis and food poisoning cases. Further studies about the using of these genes in genotyping of *S. aureus* isolates of milk origin are needed in future.

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