

Epidemiological and Genetic Studies of Enterotoxigenic *Staphylococcus aureus* Isolated from Goat and Human

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Abstract The objective of the present study was to investigate the epidemiological and genetic relationships of classical enterotoxins of *S. aureus* in goat's raw milk, meat and food handlers in Toukh city in Qalubia governorate, Egypt. A total of 100 goat, s raw milk and meat samples (50 of each) were collected from randomly distributed herds in streets for buying milk and in public markets for peddler meat. Hand and nasal swabs were collected from milkers and butchers (30 of each). All samples were subjected for bacteriological examination for isolation and identification of *S. aureus*. Isolates were underwent reversed passive latex agglutination technique for detection of enterotoxigenic *S. aureus*. A multiplex PCR assay could successfully amplify the diagnostic DNA bands of 270bp, 165bp, 69bp and 306bp of genes for staphylococcal enterotoxins A, B, C, and D respectively. PCR was applied on the serologically identified 16 (20.25%) isolates out of 79 *S. aureus* which isolated from the examined goat's food samples and human handlers by using one universal forward and reverse primers, specific for each individual toxin gene. None of the samples was positive for SEE indicating the zoonotic and genetic relationships.

Keywords: *S. aureus*, latex agglutination, multiplex PCR, enterotoxigenic gene

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1. Introduction

The goat is an important animal reservoir of enterotoxigenic staphylococci [1]. *Staphylococcus aureus* (*S. aureus*) is one of the most common agents of food poisoning outbreaks with enhanced pathogenicity due to the presence of enterotoxins [2]. Several types of staphylococcal enterotoxins have been distinguished serologically [3], namely, SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE. Several molecular typing methods have been described in order to obtain an accurate and rapid characterization of *S. aureus* isolates, such as multiplex-PCR [4] restriction fragment length polymorphism (PCR-RFLP) [5] Random amplification of polymorphic DNA (RAPD) [6], pulsed-field gel electrophoresis (PFGE) [7] and Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) [8]

S. aureus is able to grow in a wide range of temperatures (7°C to 48.5°C with an optimum of 30 to 37°C), pH (4.2 to 9.3, with an optimum of 7 to 7.5) and sodium chloride concentrations up to 15% NaCl. These characteristics enable *S. aureus* to grow in a wide variety of foods [9] The enterotoxigenic staphylococci isolated from raw goat' milk in Spain [1], Iran [10], Italian cheese [6], Italian meat [11] and goat's meat in Jordan [5]. On

heating at normal cooking temperature, the bacteria may be killed but the toxins remain active [12]. In Egyptian goats' milk samples *S. aureus* can gain access to milk either by direct excretion from udders with clinical mastitis [8] and subclinical mastitis [13] or by environmental contamination during the handling and processing of raw milk and meat [14,15].

Staphylococci are present in the nasal passages and throats and on the hair and skin of 50 percent or more of healthy individuals. Consequently, food handlers can be an important source of contamination of the food product via respiratory secretions or direct contact via hands during manufacture and handling of the food product [16]. The majority of reported Staphylococcal food poisoning outbreaks are associated with classical enterotoxins, SEA-SEE; however enterotoxin A was the most incriminated [17]. Staphylococcal food poisoning includes symptoms such as sudden onset of nausea, vomiting, abdominal cramps and diarrhea [18].

There are little information about the prevalence of enterotoxigenic strains of *S. aureus* among goat food products and their human handlers in Egypt. Thus, the aim of this study was to investigate the epidemiological and genetic characterizations of *S. aureus* in goat's milk and meat and their human skin and nasal carriage to understanding their genetic structure and determine their relationship.

2. Materials and Methods

2.1. Bacteriological Examination

2.1.1. Sampling

In the present study a total of 100 raw milk and meat samples (50 of each) were collected under aseptic condition from randomly distributed herds of goats in different streets and public markets in Toukh city, Qaluobia governorate, Egypt. About 15 ml/gm of each sample were transferred to sterile screw capped tube contain sterile nutrient broth. As well as sterile swabs were collected from apparently healthy milkers and butchers (30 of each) which was inserted in both anterior nares and other swab for palms of hands each swab transferred to a tube containing sterile brain heart infusion broth. The samples were transferred immediately in cooling ice box to the lab.

2.1.2. Isolation and Identification of *Staph. aureus* Isolates

Each milk sample was incubated for 24 h at 37°C. Each meat sample homogenized in 90 ml sterile nutrient broth for 1.5 min using sterile stomacher and then 15 ml were transferred to sterile screw capped tube and incubated at 37°C for 24 hr. Tubes containing human swabs were incubated at 37°C for 24 h. A loopfull from each sample was cultured on mannitol salt agar (Himedia, India) and incubated at 37°C for 24 hours. Suspected colonies were streaked on Baird Parker medium (Himedia, India), supplemented with egg yolk tellurite emulsion (Himedia, India) incubated for 24- 48 hours at 37°C. [19]. Characteristic appearance of circular, smooth, convex, moist, 2-3 mm in diameter, gray to black (potassium tellurite reaction) with white margin and surrounded by outer clear halo zone (egg yolk reaction) were considered to be presumptive *S. aureus*. Suspected colonies were streaked on nutrient agar slob and incubated at 37°C for 24hours for further morphological examination by Gram's stain according to [20] and biochemical identification including catalase, coagulase, the presence of DNA using DN-ase test Voges-Proskauer tests (acetone production), oxidase and D- mannitol fermentation as cited by [21].

2.2. Serotyping of *Staph.aureus* Enterotoxin

The identified isolates of *Staphylococcal aureus* were investigated serologically to verify its ability to synthesize staphylococcal enterotoxins. The strains were grown in 10 ml of brain heart infusion broth (Oxoid, UK) by shaking aerobically for 16-18 h at 37°C. After centrifugation at

9000 x g for 20 min at 4°C, the supernatant was tested for presence of staphylococcal enterotoxins A, B, C, D and E and typed using staphylococcal Enterotoxin Test Reversed Passive Latex Agglutination (SET-RPLA) as recommended by the manufacturer's protocol (Denka Seiken Co., Tokyo, Japan). Negative controls were used with all the tested samples.

2.3 Genetic Detection of *Staph.aureus* Enterotoxin

2.3.1. Extraction of DNA

Total genomic DNA was isolated using method of [4]. About 1.5 ml of the overnight growth culture of *S. aureus* strains were incubated in 5 ml of brain heart infusion broth and incubated aerobically at 37°C was centrifuged at 12,000 rpm for 2 minutes and supernatant was discarded. Genomic DNA was extracted from the bacterial pellet using an extraction kit QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA extraction and purification protocol were done according to the manufacturer's instructions.

2.3.2. Oligonucleotide Primers

The oligonucleotide primers were designed according to published DNA sequences of the *S. aureus* enterotoxin genes *sea*, *seb*, *sec*, *sed*, and *see*. One universal forward primer (SA-U) common for all enterotoxin genes and five reverse primers, each specific for one enterotoxin gene of *S. aureus*, they were manufactured in (Metabion, Germany). The sequences, corresponding sequence locations, expected products and authors of these oligoprimers are shown in (Table 1).

2.3.3. DNA Amplification by Multiplex PCR

PCR amplification was carried out in 25 µl containing 1 ng of template DNA, 25 pmol each of primers SA-U, SA-A, SA-B, SA-C/ENT-C, SA-D, and SA-E, and 12.5 µl PCR Master Mix (Jena Bioscience Co. Jena, Germany), [4]. This mixture was heated to 94°C for 5 min followed by twenty-five amplification cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 m. were performed using a thermal cycler (T- Biometra) with a final extension cycle of 5 m. at 72°C (Table 2). Following amplification, 15 µl from each PCR containing amplified products were loaded onto 1.5% agarose gel and stained with ethidium bromide in gel electrophoresis and visualized under UV rays against GeneRuler 100 bp plus DNA ladder (molecular weight marker) ready to use (Fermentas, Canada).The expected product length of the positive results were recorded in (Table 1).

Table 1. Oligonucleoloid primers of enterotoxigenic *S.aureus*

Gene	Primer Descriiion	Nucfcotkfc scquncnc	Gene location	AmpScon size (bp)	Reference
SA-U	Universal forward primer	5'-TGTATGTATGGAGGTGTAAC-3'	-	-	[4]
SA-A	Reverse primer for sea	5'-ATTAACCGAAGTTCTGT-3'	639-657	270	[41]
SA-B	Reverse primer for seb	5'-ATAGTGACGAGTTAGGTA-3'	564-582	165	[42]
SA.C	Reverse primer for sec	5'-AAGTACATTTTGTAAGnCC-3'	457-477	69	[43]
SA-D	Reverse primer for sed	5'-TTCGGGAAAATCACCOTAA-3'	676-696	306	[3]
SA-E	Reverse primer for see	5'-GCCAAAGaGTaGAG-3'	584-600	213	[44]

3. Result

Bacteriological identification of *S. aureus* in the examined samples collected from goat revealed that 29

(58%) raw milk and 9 (18%) raw goat meat samples were contaminated with *S. aureus*. While *S. aureus* isolated from 13 (43.33%) hand swab and 11 (36.66%) nasal swab of milkers were higher than those isolated from 9 (30%)

and 8 (26.66%) of butchers hand and nasal swabs respectively. Isolates were underwent (SET-RPLA) reversed passive latex agglutination technique for detection of enterotoxigenic *S. aureus*. The prevalence rate of SEs contamination in raw goat milk and meat samples were 6 (20.68%) and 1 (11.11%) respectively. Of these, two were positive for staphylococcal enterotoxin A (SEA), one for B (SEB) one for C (SEC), one for D (SED) and one SEA+SEC in milk samples and one strain produced enterotoxin A (SEA) in meat samples. Enterotoxigenic *Staphylococcus aureus* among tested milkers were positive in 4 (30.76%) and 2 (18.18%) of isolates from hand and nasal swabs respectively, while 2 (22.22%) and 1 (12.5%) isolates from butchers hand and nasal swabs respectively produced staphylococcal

enterotoxins. The serotypes of these enterotoxins were 1A (SEA), 1 B (SEB), 1C (SEC), 1D (SED) from hand swab, while two isolates produced A (SEA) and SEA+SEC from nasal swabs of milkers. All isolates of butchers produced enterotoxin A (Table 3).

Table 2. Thermo-cycling protocol of Multiplex PCR for amplification of enterotoxigenic *S. aureus*

Operation	Temp.	Time	cycles
Initial Denaturation	94°C	5m	1
Denaturation	94°C	30sec.	25
Annealing	50°C	30sec.	
Extension	72°C	2m	
Final extension	72°C	5m	1
Hold (refrigeration)	4°C	Forever/∞	

Table 3. Prevalence of enterotoxigenic *S.aureus* isolated from goat's food samples and human handlers detected by SET-RPLA

Type of samples	S. aureus		Enterotoxigenic <i>S. aureus</i> serotypes															
	No.	No. +ve	%	No. +ve	%	sea		Seb		sec		sed		see		sea+ sec		
						No.+ve	%	No.+ve	%	No.+ve	%	No.+ve	%	No.+ve	%	No.+ve	%	No.+ve
Goat																		
Raw milk	50	29	58	6	20.68	2	33.33	1	16.66	1	16.66	1	16.66	0.0	0.0	1	16.66	
Raw meat	50	9	18	1	11.11	1	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Milkers																		
Hand swab	30	13	43.33	4	30.76	1	25	1	25	1	25	1	25	0.0	0.0	0.0	0.0	
Nasal swab	30	11	36.66	2	18.18	1	50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1	50	
Butchers																		
Hand swab	30	9	30	2	22.22	2	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Nasal swab	30	8	26.66	1	12.5	1	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Total	220	79	35.90	16	20.25	8	50	2	12.5	2	12.5	2	12.5	0.0	0.0	2	12.5	

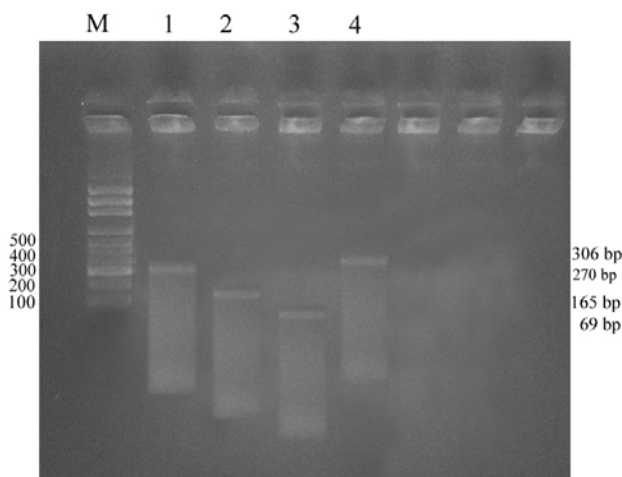


Figure 1. Agar gel electrophoreses showing the result of amplification of staphylococcal enterotoxins by multiplex PCR. M: is GeneRuler 100 plus DNA marker, lane 1: SEA, lane 2: SEB, lane 3: SEC, lane 4: SED.

The results of multiplex PCR amplification of a highly conserved region of enterotoxin genes using a universal "forward" primer (SA-U) (Table 1) in combination with toxin gene-specific reverse primers of toxin genes A to E resulted in amplification of DNA bands of 27bp, 165bp, 69bp and 306bp specific for enterotoxines A, B, C and D respectively in serologically identified 16 (20.25%) isolates out of 79 (35.90%) *S. aureus* isolated from the examined goats food samples and human handlers, none

of the samples was positive for SEE. PCR products were performed, and fragments of the expected sizes were obtained for each PCR product and that no additional or nonspecific products were generated (Figure 1).

4. Discussion

Staphylococcus aureus is one of the most common causes of food borne infections in most of the countries of the world [22]. The current data revealed Gram positive, non-spore forming cocci, arranged in grapes or irregular clusters. The results of biochemical tests proved that suspected colonies to be *Staphylococcus spp.*, catalase positive and oxidase negative. There was a good correlation between coagulase and thermo-stable nuclease reaction. Isolates showed positive coagulase reaction also produced thermo-stable nuclease. Voges Proskauer test (positive) was used to detect acetoin production and was the key test to differentiate *S. aureus* from other coagulase positive staphylococci [21,23].

Goat's raw milk and meat are considered a nutritious food because they contain several important nutrients including proteins and vitamins. Conversely, they can be a source for *Staphylococcus aureus*. Some *S. aureus* strains are able to produce staphylococcal enterotoxins (SEs) and are the causative agents of staphylococcal food poisoning [1].

In the present work it was noticed that the contamination rate of the raw milk was higher than that of

the raw meat (58% vs 18%). This was in accordance with the previous recorded data in Italy [11] in Italy.

Nearly 30-50% of the human populations are carriers of *Staphylococcus aureus* whereas its main habitat is the nasopharynx, a site where strains can persist as transitory or persistent members of the normal microbiota without causing any symptomatology [16]. In the present study *S. aureus* isolated from milkers' hands (43.33%) and nose (36.66%) were higher than those isolated from butchers' hands and nose (30% 26.66% respectively).

These results were nearly similar to colonization of *S. aureus* of Egyptian food handlers working in the milk and dairy products (45%) and of those working in meat and meat products (36%) [15]. Also nasal carriers were 36% and 31% of those working in dairy and meat products respectively [14], while were higher than those isolated from nasal swabs of (3.6%) milk distributors and (5.4%) butchers in the Omdurman [24]. Among 127 food handlers working in cafeterias in Ethiopia indicated that 16.5% of fingernail contents of the food handlers were positive for *S. aureus* [25] and lower than (57.5%) out of 200 food handlers tested positive for *S. aureus* in Botswana [26]. Unfortunately, washing hands and skin surfaces has minimal effect on reducing *S. aureus* cell numbers on humans, largely because *S. aureus* is a part of the resident flora of skin [27].

Goat is an important reservoir of enterotoxigenic Staphylococci (SE) like proteins [2]. Classical staphylococcal enterotoxins (SEA to SEE) have been reported to cause 95% of staphylococcal food poisoning. Staphylococcal enterotoxins (SE) were considered a potential biological threat because of their stability at high temperatures (100°C for 1 h) [28].

The prevalence rate of SEs contamination in raw goat milk samples were 6 (20.68%) which was slightly higher than (18.5%) [10], while was lower than 12 (55%) [6]. The occurrence of SEs contamination in raw goat milk 6 (20.68%) were higher than in raw meat 1 (11.11%). The current results seemed to be correlated with those in Italian dairy products enterotoxins (54%) higher than meat products (50%) [11].

In this work, staphylococci have grown and produced toxins in the contaminated raw milk and meat and showed the highest incidence of the *sea* enterotoxin with one strain was multi-SE Carriers SEA+SEC in milk sample. These results were in accordance with [29] who represented that SEA was the most common staphylococcus-related food poisoning whereas in contrast to the predominance of enterotoxins SEC reported in Italian dairy products [6] and SED in dairy and meat products [11].

The prevalence of enterotoxigenic *Staphylococcus aureus* among milkers were 4 (20.25%) and 2 (18.18%) of isolates from hand and nasal swabs respectively, while 2 (22.22%) and 1 (12.5%) isolates from hand and nasal swabs among butchers respectively (Table 3).

The current data is higher than enterotoxigenic *S. aureus* detected in 28 (14%) skin swab [15] and 21 (10.5%) nasal swabs [14] investigated among food handlers in three food processing plants in Egypt. The relatively high frequency of occurrence of the enterotoxigenic *S. aureus* in food handlers may be attributed to unhygienic environment and unhygienic practice of milkers randomly distributed in street for hand milking and wandering sell goat's milk.

Food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination, via manual contact or through respiratory secretions. It is noticed that the serotypes of these enterotoxins in milkers' hand swabs were 1A (SEA), 1B (SEB), 1C (SEC) and 1D (SED), while two isolates produced A (SEA) and SEA+SEC from nasal swabs of milkers. All isolates of butchers produced enterotoxin A (SEA).

It was observed that enterotoxin type A was the most prevalent type followed by types B, D, A+C and C. These observations were nearly similar to the findings investigated in skin [15] and nose [14] of Egyptian food handlers. The most frequently found enterotoxin was type A, and in contrast to those recorded in Kuwait, the majority of *S. aureus* isolates obtained from hands of food workers was type B, whereas those isolated from the nose were predominantly of types A and B [30] and enterotoxigenic *S. aureus* isolates from the nose, throat, hands and nails of food handlers in cafeterias of a Chilean restaurant were predominantly SEB and SED producers [27].

It was worth mentioning that the same serotypes of enterotoxigenic *S. aureus* isolated from food handlers previously recovered from their goat's food products (Table 3). The distribution of *S. aureus* in the goat's food environment may cause food contamination, which implies some risk of staphylococcal food poisoning in dairy and meat products, all of which support the growth of *S. aureus* [31]. The pathogenicity of food-borne *S. aureus* is associated with the ability of some strains to produce enterotoxins [32]. The source of contamination for the outbreak in Taiwan was most likely originated from a food handler [7].

The molecular genetic study was carried out to identify genes for staphylococcal enterotoxins A to E in toxigenic strains of *S. aureus* isolated from goat's food and human handlers. PCR amplified a 270bp, 165bp, 69bp and 306bp diagnostic DNA bands in all examined enterotoxins A, B, C and D respectively, none of the samples was positive for SEE (Figure 1). It was worth noting the use of multiplex PCR in the present work revealed absence of variation in amplified DNA in all toxigenic serotypes from goats and humans and indistinguishable molecular base pair indicating a zoonotic relationship.

The highest incidence of the *sea* gene from strains isolated from goat and human contact was the most prevalent type detected in 8 (50%) out of the 16 enterotoxigenic isolates followed by 2 (12.5%) for each *seb*, *sec*, *sed* and *sea+sec* gene. The amplification of the staphylococcal enterotoxin genes *sea*, *seb*, *sec* and *sed* was specific with a unique band of the predicted size present in strains producing a single and multiple toxins and there was 100% correlation between the PCR toxin typing (genotype) and toxin production as defined by the SET-RPLA (phenotype). The positive result using SET-RPLA gave a positive enterotoxin PCR assay result. These results agreed with [4] who identified the enterotoxigenic genes by the characteristic size of the PCR product (s) generated and concluded that PCR assay can detect and characterize the presence of multiple toxin genes present in one strain.

Few data are available on SE production by the presence of SE genes in Egyptian goat food isolates and

handler isolates of *S. aureus*. Here none nasal and skin isolates from milkers and butchers harbored a *see*. These results were in contrast to those recorded in Ireland on SED production where no isolate possessed the *sed* gene [33], while *seb* gene the commonest nasal carriage isolate from an Irish university student population [34].

The predominant classical SE gene varied from country to country – Ireland, *seb*; [34] Germany, *sea*, *sec*; [35] Japan, *seb*; [36]; New Zealand, *seb*; [37] Poland, *sec*; [38] and Bulgaria, *sea*; [39] This difference in toxin types depend upon the origins of staphylococcal food poisoning which differed widely among countries. This may be due to differences in the consumption and food habits in each of the countries [28]. In France, for example, the consumption of raw milk cheese is much higher than in Anglo-Saxon countries. This may explain the relative importance of milk products involved in staphylococcal food poisoning in France [40].

The absence of variation in gene identity established by the characteristic size of the PCR products of goat and human isolates indicated that there are relations between the food contamination with *S. aureus* and food handlers. Similar finding was recorded in Taiwan [7].

5. Conclusion

This study has shown that random selling of goat's food products by milkers and butchers allow enterotoxigenic *S. aureus* to be removed. The results clearly indicate relatively high frequency of occurrence of the enterotoxigenic *S. aureus* 16 (20.25%) among goat's food and their human handlers and indistinguishable diagnostic amplified base pair of enterotoxigenic gene of the *S. aureus*, which confirmed its distribution and their relationship producing public health and economic concern resulting from contamination of milk and meat due to handling, unhygienic environment and processing. The cooperation between the Public Health Authority and Veterinary Service are required to prevent the street vendors and attain consumer safety. More efforts must be applied towards the development of new system for street vendors.

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