Detection of enterotoxigenic *Staphylococcus aureus* in raw milk and cream using multiplex PCR

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Abstract: This study was conducted to determine the prevalence of enterotoxigenic *S. aureus* in raw milk and cream in Sharkia governorate, Egypt. A total of 90 samples 45 of each raw milk and cream were randomly collected from dairy shops. These samples were investigated bacteriologically to detect occurrence of enterotoxigenic *S. aureus*. Overall, 21 *S. aureus* isolates were identified from the examined samples with an incidence of 23.3%. The highest isolation rate was observed in cream samples (28.9%) followed by raw milk samples (17.8%).The ability to synthesize classical *staphylococcal* enterotoxin (SEA-E) was determined using multiplex PCR, in 9 of 21 samples. Results revealed that 5 (55.6%) enterotoxigenic *S. aureus* isolates were carrying sea followed by 2(22.2%) isolates positive for sec and only one isolate was positive for sed. Both sea and sec genes present in only one isolate. Differences in SE type prevalence compared with the present study likely reflect the distinct origin of the isolates. It can be considered that food handlers are the most usual contamination source leading to food poisoning. [Abd El-fatah, E.N. and Tahoun, A.B.M. **Detection of enterotoxigenic *Staphyococcous aureus* in raw milk and cream using multiplex PCR**. *J Am Sci* 2013; 9(12):961-968]. (ISSN: 1545-1003).
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1. Introduction

S. aureus is one of the most common agents causing food poisoning ***(Le Loir et al., 2003)***. It is involved in intramammary infections in bovine causing economic losses and milk safety problems ***(Taverna et al., 2007)***. It produce a number of protein, toxins and extracellular virulence factors that one of the most important of them is enterotoxin that cause food poisoning ***(Orwin et al., 2003)***. *Staphylococcal* enterotoxins (SEs) have been divided into five major serological types (SEA, SEB, SEC, SED, and SEE) on the basis of their antigenic properties ***(Letertre et al., 2003)***. During the 1990s new SEs (SEG, SEH, SEI and SEJ) were reported, and their genes described ***(Munson et al., 1998 & Zschock et al., 2005)***.

Data resulting from partial or complete genome sequence analyses have led to the description of further "new" se genes: sek, sel, sem, sen, seo, sep, seq, ser and seu ***(Fitzgerald et al., 2001; Jarraud et al., 2001; Orwin et al., 2001; Omoe et al., 2002; Orwin et al., 2003; Letetre et al., 2003 & Omoe et al., 2005)***.

The role these new SEs play in food poisoning has not yet been clarified ***(Vernozy- Rozand et al., 2004 & Boerema et al., 2006)***. Some are reported to lack emetic activity while others still have to be tested. Consequently it has been proposed to designate them "*staphylococcal* entero -toxin-like" (SEl) ***(Lina et al., 2004)***.

Staphylococcal enterotoxins are low molecular weight proteins (MW 26.900-29.600 KD). These are encoded by genes embedded in mobile genetic elements such as phages, (not in plasmids) and pathogenicity islands ***(Martin et al., 2004)***. Heat resistance is one of their most important physical and chemical properties, their biological activity remains unchanged even after thermal processing of food ***(Martin et al., 2004 & Chapaval et al., 2006)***. For the above mentioned reason, these toxins can cause epidemic gastroenteritis. Actually, SEB is the most important enterotoxin that causes gastroenteritis.

The toxins enter from the alimentary tract into the blood circulation, stimulates the vomiting center of the involuntary nervous system, causing nausea, vomiting, abdominal cramps and diarrhea ***(Rosec and Gigaud, 2002 & Letertre et al., 2003)***. Most enterotoxin serotypes are heat stable and may resist inactivation by gastrointestinal proteases like pepsin. The B and C serotypes are cleaved by digestive enzymes in the cysteine loop site, but this cleavage is not effective against their toxicity and antigenic properties ***(Paciorek et al., 2007 & Rossalxn, 1990)***. Although *S. aureus* is not difficult to cultivate and easily identified, there is still need for rapid and sensitive DNA –based assay specific for detecting *S. aureus* ***(Saei et al., 2010)***.

The polymerase chain reaction (PCR), which is a technique for the in vitro amplification of specific segments of DNA, offers a rapid, sensitive and specific identification method for the genes responsible for toxins produced by *S. aureus* ***(Mehrotra et al., 2000 & Anvari et al., 2008)***.

Detection of SE-genes by PCR allows the determination of potentially enterotoxigenic *S. aureus* irrespective of whether the strain produces the toxin or not the inability to detect the enterotoxin by

immunological methods may occur due either to low-level production of enterotoxin or to mutation in the coding region or in a regulatory region. For this reason, PCR may be considered more sensitive than methods that determine SE-production as immunological methods ***Zschock et al., 2000 & (Holeckova et al., 2002).***

This study was conducted to determine the prevalence of enterotoxin genes in isolates of *S. aureus* recovered from raw milk and cream samples.

2. Material and Methods

2.1. Collection and preparation of samples *(APHA, 1992)*:

A total of 90 samples 45 of each raw milk and cream were randomly collected from dairy shops in Sharkia governorate, Egypt. Samples were then transferred to the laboratory under refrigeration and stored at temperatures between 0 and 4 °C until being analyzed.

2.2. Isolation and Identification of isolates:

The isolation of *S. aureus* stains was carried out using the standard method. Briefly, preparing decimal dilution from aseptically collected milk samples ***(APHA, 2004)*** from the previously prepared decimal dilutions of the examined samples, 0.1 ml. was spread onto the dry surface of Baired-Parker agar medium plates using a sterile bent glass rod and incubated at 37±1 ºC for 48±2 hours. Colonies (circular, smooth, convex, moist, grey to jet black, shiny, greater than one mm. in diameter with or without narrow white margin and surrounded by clear halo-zone extending into the opaque medium) in plates having 20-200 colonies were counted and recorded ***BAM, online (2009).*** Pure separate suspected colonies were picked up from Baired-Parker agar plates and cultured on slope agar and incubated at 37ºC for 24 hours. Purified isolates were subjected to further microscopical and biochemical identification according to ***Cowman and Steel (1974) & BAM, online (2009).***

2.3. a. DNA extraction methods

DNA was extracted according to ***Cremonesi et al. (2006)***. From overnight grown bacterial culture, incubated in BHI broth at 37 °C and containing approximately 1-3 billion cells About 1.5 ml of the culture was centrifuged at 6000 rpm for 2 minutes and supernatant was discarded. Approximately, an average of 10µg of DNA was obtained from 108cfu/ml.

2.3. b. Primer design

For the PCR assays, all of the primers pair sequences used in the current study, as well as their target genes and predicted amplicon size are listed in Table(1).

2.3. c. PCR protocols

First, to test the efficiency of two extraction procedures for *S. aureus* detection, the DNA obtained from different source was amplified by means of the PCR reaction described in ***Cremonesi et al. (2006)***.Then, the sea, seb, sec, sed, see, enterotoxin genes were amplified by amultiplex PCR; primers and reaction conditions have been described in ***Cremonesi et al. (2005)***.

2.3.d. Gel electrophoresis of amplified products

The PCR products were analyzed by electrophoresis in a 1.5% agarose gel. Electrophoresis was performed for 30 minutes at 120 V, while staining the amplicons with ethidium bromide for 15 minutes.

3. Results

Prevalence of *staphylococcus* Species from the Examined Samples:

As shown in Table (2), out of 90 examined samples, 84 isolates were identified as staphylococcus species (93.3 %), The highest rate of staphylococcus species isolation was observed in cream samples (100%) followed by raw milk 86.7%. 21 *S. aureus* isolates were identified from the examined samples with an incidence of 23.3%. The highest isolation rate was observed in cream samples (28.9%) followed by raw milk samples (17.8%).

Detection of Enterotoxigenic *S. aureus* isolates:

Using multiplex PCR, out of 21 *S. aureus* isolated from the examined samples, 9 could produce enterotoxins as shown in table (3) Results revealed that out of 3 enterotoxigenic *S. aureus* isolates of raw milk samples, 2 isolates were positive for sea gene, 1 isolate was positive for sec gene. Out of 6 enterotoxigenic *S. aureus* isolates of cream samples, 3 isolate was positive for sea gene, 1 isolate was positive for sec gene, 1 isolate was positive for sed and 1 isolate was positive for both sea and sec genes.

4. Discussion

The presence of *S.aureus* in raw milk generally comes from cows with mastitis, handlers or deficient hygiene. When found in milk, high levels of contamination can be reached quickly under favorable conditions. Its presence in foods can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause toxic food infections ***(Quintana and Carneiro, 2006).***

In the present study total of 90 samples 45of each raw milk and cream were randomly collected from dairy shops in Sharkia governorate, Egypt. These samples were investigated bacteriologically to detect occurrence of enterotoxigenic *S. aureus* among the examined samples. Routine diagnostic and identification procedure for detection of staphylococcus isolates were applied.

Table(1): Oligonucleotide primers sequences used for PCR amplification of enterotoxins (SEs) genes *(Chiang et al., 2005 & Bendahou et al., 2009)*.

Target gene	Primer name	Nucleotide sequence (5→3)	(bp)
Sea	SEA1 SEA2	AAAGTGCCGATCAATTTATGCCTA GTAATTAACCGAAGGTTCTGTAGA	219
Seb	SEB1 SEB2	TCG CAT CAA ACT GAC AAA CGA CACTTTTTCTTT GTCGTAAGATAA	410
Sec	SEC1 SEC2	AACATTAGTGATAAAAAAGTG AAA TTGTAAGTTCCCATT ATC AAA GTG	234
Sed	SED1 SED2	GCAGATAAAAATCCAATAATA GGA TACTAAAGAAAC TTC TTT TTG TAC	331
See	SEE1 SEE2	TTA CAA AGA AAT GCT TTA AGC TAA ACC AAA TTT TCC GTG	456

Table(2): Prevalence of enterotoxigenic *S. aureus* isolated from the collected samples.

Samples	*staphylococcus* species		*S. aureus*		Enterotoxigenic *S.aureus*	
	No.	% *	No.	% *	No.	% * *
Raw milk No.=45	39	86.7	8	17.8	3	37.5
Cream No.=45	45	100	13	28.9	6	46.2
Total No.=90	84	93.3	21	23.3	9	42.9

*Percentage was calculated to the number of the examined samples.
**Percentage was calculated to the number of *S. aureus* isolates.

Table(3): Toxin gene profiles of enterotoxigenic *S. aureus* isolates recovered from examined samples evaluated by multiplex PCR.

Toxin gene	No. of Enterotoxigenic *S. aureus* strains					
	Raw milk (3 strains)		Cream (6 strains)		Total (9 strains)	
	+ve gene	-ve gene	+ve gene	-ve gene	+ve gene	-ve gene
Sea	2 (66.7%)	1 (33.3%)	3 (50%)	3 (50%)	5 (55.6%)	4 (44.4%)
Seb	0 (0%)	3 (100%)	0 (0%)	6 (100%)	0 (0%)	9 (100%)
Sec	1(33.3%)	2 (66.7%)	1 (16.7%)	5 (83.3%)	2 (22.2%)	7 (77.8%)
Sed	0 (0%)	3 (100%)	1 (16.7%)	5 (83.3%)	1 (11.1%)	8 (88.9%)
See	0 (0%)	3 (100%)	0 (0%)	6 (100%)	0 (0%)	9 (100%)
Sea-sec	0 (0%)	3 (100%)	1 (16.7%)	5 (83.3%)	1 (11.1%)	8 (88.9%)

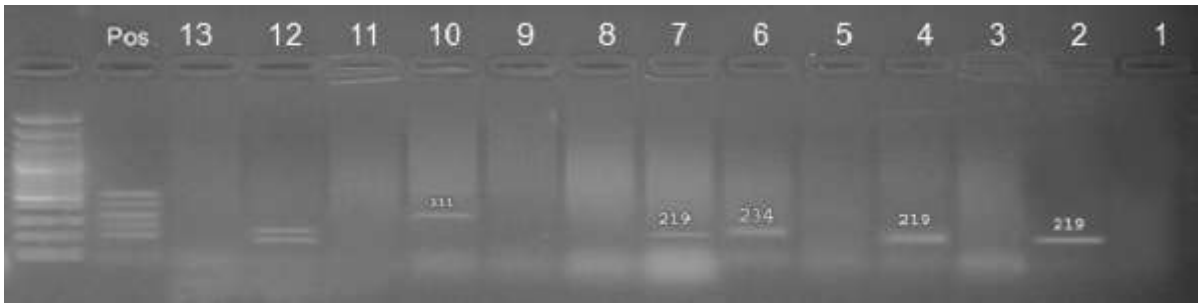

Fig.(1): Agarose gel electrophoresis showing the results of multiplex PCR for detection of enterotoxin genes among the *S. aureus* isolates of cream samples.
Lane M: 100 bp ladder DNA molecular weight marker, Lane Pos.: positive control for *see, seb, sed, sec* and *sea* genes Lane 2,4&7: positive *sea S. aureus* isolated from cream sample, Lane 6: positive *sec S. aureus* isolated from cream sample,, Lane 10: positive *sed S. aureus* isolated from cream sample, Lane 12: positive *sec* and *sea S. aureus* isolated from cream sample, Lanes1,3,5,8,9,11&13: no amplification

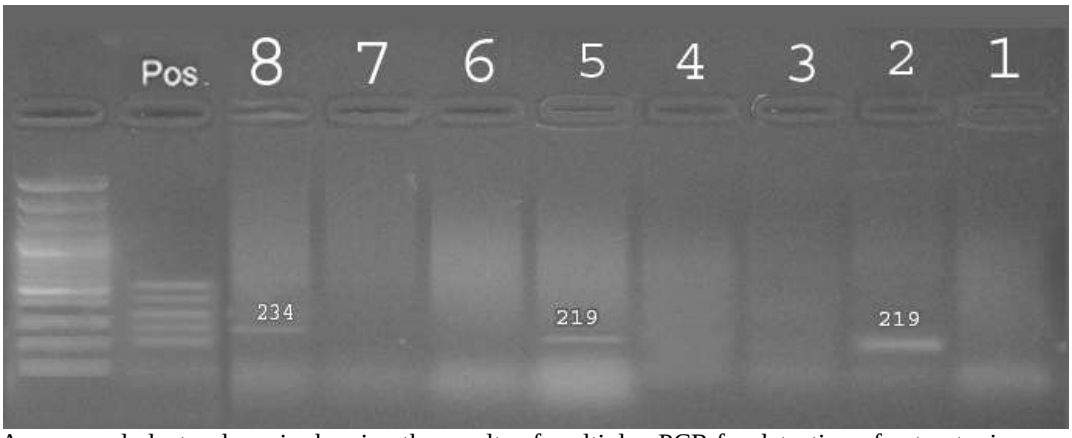

Fig.(2): Agarose gel electrophoresis showing the results of multiplex PCR for detection of enterotoxin genes among the *S. aureus* isolates of raw milk samples.
Lane M: 100 bp ladder DNA molecular weight marker, Lane Pos.: positive control for *see, seb, sed, sec* and *sea* genes Lane 2&5: positive *sea S. aureus* isolated from raw milk sample, Lane 8: positive *sec S. aureus* isolated from raw milk sample, Lanes1,3,4,6&7: no amplification.

Table (2) illustrated that out of 90 examined samples, 84 isolates were identified as staphylococcus species (93.3 %), The highest rate of staphylococcus species isolation was observed in cream samples (100%) followed by raw milk 86.7%.

21 *S. aureus* isolates were identified from the examined samples with an incidence of 23.3%. The highest isolation rate was observed in cream samples (28.9%) followed by raw milk samples (17.8%). These results of raw milk were agreed with that of ***Rahimi and Alian (2013)*** as they isolate *S.aureus* from raw milk by percentage of 17.5%.on the other hand higher results were reported by ***Khudor et al. (2012)*** where *S.aureus* isolated from raw milk by percentage of 28.5% also ***OIliveira et al.(2011) and Rall et al.(2008)*** isolated *S. aureus* from raw milk by percentage of 68% and 70.4% respectively.

Fooladi *et al.*(2010) and Rahimi (2013) isolated only 18%and 5.6% *S.aureus* from cream samples respectively, giving lower results than our study.

The determination of *staphylococcal* enterotoxin type has a long history of successful use in epidemiological studies in both clinical and environmental microbiology studies. As our knowledge of the molecular genetic structure of these organisms increases, it becomes increasingly more difficult to test for all of the known phenotypes, with genotype analysis often providing the only way the diversity of different subspecies types can be identified. Oligonucleotide primers for specific detection of enterotoxin genes sea, seb, sec, sed, and see have previously been reported ***(Johnson et al., 1991; Tsen and Chen 1992 & Tsen et al., 1994)***, these were used in individual PCR assays, thus requiring several PCRs for each sample to screen for the presence of all of the enterotoxin genes. ***Monday and Bohach (1999)*** have recently described a multiplex PCR assay for the detection of all of the *staphylococcal* enterotoxin genes, but again this assay uses separate primer pairs for each toxin gene to be detected.

Generally, five classical *staphylococci* enterotoxin (SE) SEA to SEE were recognized. It was shown that about 95% of staphylococcal food-poisoning outbreaks were caused by strains carrying the classical SE and the remaining 5% of coagulase positive species; *S. hyicus* and *S. intermedius* outbreaks were associated with other identified ***(Wang et al., 2012)***

Using multiplex PCR, out of 21 *S. aureus* isolated from the examined samples, 9 (42.9%) could produce enterotoxins as shown in table(2) which found in raw milk by percentage of 37.5% and in cream by percentage of 46.2%. the results in raw milk are similar to that of ***El-Jakee et al.,(2013)*** who found enterotoxigenic strains by percentage of 35.7%.others found lower results *as* ***Veronica et al.(2011) & Rahimi et al.(2012)*** who found enterotoxigenic strains by percentages of 11.7% and 20.8% respectively. higher results could be detected by ***Rahimi and Alian (2013)*** who found enterotoxigenic strains by 57.1%.

The results of cream are higher than that founded by ***Fooladi et al.(2010)*** who isolated enterotoxigenic strains only by percentage of 27.7%, while ***Rahimi (2013)*** found that all of the *S. aureus* isolated from cream samples were enterotoxigenic.

Results in table (3) revealed that out of 3 (37.5%) enterotoxigenic *S. aureus* isolates of milk samples, 2(66.7%) isolates were positive for sea gene, 1(33.3%) isolate was positive for sec gene.

Others found sea but with lower percentage than our study as ***Adwan et al.(2005); Rall et al.(2008); Rahimi et al. (2012) &ElJakee et al.(2013);*** 7.1%,41%, 12.7% and 40% respectively. While ***Veronica et al.(2011) & Khudor et al.(2012)*** didn't find sea at all.

For sec results, ***Veronica et al.(2011) & Rahimi et al.(2012)***found it but with lower percentages 7.4% and 8.3% respectively. others found it also by low percetages as ***Adwan et al.(2005); Rall et al.(2008); Khudor et al.(2012) & El-Jakee et al.(2013)*** 14.3%,20.5%,18.5%, 20% respectively.

Rahimi and Alian (2013) didn't find sec at all. while ***Sharma et al.(2000); Tsegmed (2006) & Khudor et al.(2012)*** found only sec by percentage of 11.1%, 19% and 18.5% respectively and none of these isolates harbou-ring other SEs genes..

In our study in raw milk we didn't find any genes coding for more than one enterotoxin in one sample while ***Veronica et al.(2011)*** found a combination between sea-sed-see by percentage of 1.1% and ***El-Jakee et al.(2013)*** found combination between seb-sed by percentage of 20%.

Rall et al.(2008)found that 64.1% exhibited only one enterotoxin gene, 23.1% carried gene coding for 2enterotoxins, 5.1% were positive for 3 genes and genotypes encoding 4genes were detected in 7.7%.

In table (3) Out of 6 (46.2%) enterotoxigenic *S. aureus* isolates of cream samples, 3 (50%) isolates were positive for sea gene, 1 (16.7%) isolate was positive for sec gene, 1(16.7%) isolate was positive for sed and 1 (16.7%) isolate was positive for both sea and sec genes.

Fooladi et al.(2010) found sea but by higher percentages 60%, while ***Rahimi (2013)*** couldn't find any one of the classical enterotoxins but he found combination between new se genes as seg-sei and sei-seh.

On the other hand, we found that enterotoxin A was the most commonly produced toxin. Moreover, enterotoxin A is most often implicated in cases of staphylococcal food poisoning ***(Shale et al.,2005).*** The dominance of *S. aureus* enterotoxin A isolates in our present study has been also reported by other researchers for *S. aureus* recovered from food samples ***(Tsen et al., 1998 & Bendahou et al., 2009)***

Other investigators showed that Sec was the most frequent type in the *S.aureus* isolated from milk and milk products of the bovine and ovine. ***(Kenny et al., 1993; Kuroishi et al., 2003; Echcpd, 2003 & Tkacikova, 2003).*** The highest frequency of Sec in bovine *S. aureus* isolates from may be occurred because *Staphylococcal* isolates from different animal species produce host specific SECs***.(Monday and Bohach,1999 & Bhunia, 2008).*** Furthermore, the SEs could be able to indicate the origin of the *S.aureus* strains because it was observed that a higher ratio of isolates from bovine produced SEC and those from human produced mainly SEA ***(Ahari et al., 2009).***

The ability of *S. aureus* isolates to produce one or more SEs in food products is linked to *staphylococcal* food poisoning ***(Bennett, 2005).*** Enterotoxigenic strains of *S. aureus* have been reported to cause a number of diseases or food poisoning outbreaks in many countries because of ingestion of contaminated dairy products or milk with *staphylococcal* enterotoxins **(Balaban and Rasooly, 2000; Echcpd, 2003; Oliver *et al.*, 2005; Ikeda *et al.*, 2005 & ISfID, 2010).**

In the present work 56.1% of *S. aureus* isolates were negative to the five classical enterotoxin genes.This might be explained by the fact that these isolates either have not harboured any gene of enterotoxins or thy might have other types of SEs which are family of 18 serological types of heat stable enterotoxin ***(MacLauchlin et al., 2000; Ikeda et al., 2005; Rall et al., 2008 & Bhunia, 2008).***

Our result shows that milk was less contaminated than cream and this also was detected by ***Fooladi et al. (2010)***. Differences between the results may be based on the differences in the cream production techniques, as it need more handling or storage conditions. It could be also related to the unclean conditions where the cream is produced and the personnel involved in production among these samples.

Differences in SE type prevalence compared with the present study likely reflect the distinct origin of the isolates. It can be considered that food handlers are the most usual contamination source leading to food poisoning. Nevertheless, since these toxins resist heat treatment, the present findings indicate a potential public health hazard and underscore the need to establish both effective bovine mastitis control programs and proper milk cooling methods to limit *S. aureus* presence and multiplication in bulk tank milk. ***(Veronica etal., 2011).***

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