Original Article

Enterotoxigenic coagulase positive Staphylococcus in milk and milk products, lben and jben, in northern Morocco

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Abstract

Background: The aim of this research was to determine the prevalence of enterotoxin genes (*sea-seo*) in Coagulase Positive *Staphylococcus* (CPS) isolated from unpasteurized milk and milk products. These results were compared with the results obtained by using the detection kit SET-RPLA for the specific detection of staphylococcal enterotoxins (SEA-SED).

Methodology: Eighty-one samples of milk and milk products were analyzed for the presence of *Staphylococcus* strains. Forty-six coagulase positive *Staphylococcus* isolates were tested for the production of staphylococcal enterotoxins (SEA-SED) by using the reversed passive latex agglutination method. The strains were also tested for the presence of *se* genes (*sea–seo*) by polymerase chain reaction.

Results: One or more classical enterotoxin products (SEA-SED) were observed in 39% of the strains tested, while *se* genes were detected in 56.5%. SEA and *sea* were most commonly detected. For newly discovered *se* genes among CPS isolates tested in this study, except the *seh* gene which was revealed in four isolates (8.7 %), none of the strains harbored any of the other *se* genes (*see, seg, sei, sej, sek, sel, sem, seo and sen*).

Conclusions: The finding of a pathogen such as staphylococci-producing SEs and containing *se* genes in milk and milk products in northern Morocco may indicate a problem for public health in this region. The presence of enterotoxigenic strains in food does not always necessarily mean that the toxin will be produced. For that reason, the combination of both methods (RPLA and PCR) is a guarantee for success in diagnostic analysis tests.

Introduction

Raw milk and traditional milk products such as Lben (whey "skimmed fermented milk") and Jben (Moroccan traditional cheese) are widely manufactured and consumed by the population of northern Morocco. Jben and Lben are traditionally made Moroccan dairy products, produced by farmers at home and sold through special retail trade. The production process does not follow safety standards methods. The use of raw milk, for example, can be a source of the spread of certain harmful human bacterial diseases such as tuberculosis, brucellosis, salmonellosis, and staphylococcal food poisoning [1,2]. Furthermore, enterotoxin-producing *Staphylococcus aureus* that are present in high numbers can be dangerous and harmful to human health [1]. Nearly all strains secrete a group of enzymes and cytotoxins which include four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth [3]. Some strains may produce one or more of a family of additional exoproteins, which include two forms of toxic shock syndrome toxin (TSST), the staphylococcal enterotoxins (SEA, SEB, SECn, SED, and SEE), and other enterotoxins that are less commonly found, such as G, H, I [4-8]. These toxins, which are resistant to heat treatment and to proteolytic enzymes, are quite stable over a wide range of pH [1-10]. They cause toxic shock-like syndromes, and are implicated in food poisoning and several allergic and autoimmune diseases [9]. In every outbreak, the detection of staphylococcal enterotoxin (SE) is epidemiologically essential. Data on the enterotoxigenicity of *S. aureus* strains isolated

from human beings and food in general indicate that more than 50% of strains are enterotoxigenic [10], and isolates carrying toxin genes *sea* to *see* are responsible for 95% of staphylococcal food poisoning outbreaks [11]. Growth of enterotoxigenic strains of *S. aureus* to a population of 10^6 or more cells per gram of food is generally considered necessary for production of a sufficient amount of enterotoxin to cause intoxication if the food is consumed. As little as 100 to 200 ng of staphylococcal enterotoxin (SE) can produce symptoms of intoxication [12].

In Morocco, *S. aureus* has been reported to cause 37% of food poisoning. However, care is recommended in considering these numbers, because the incidence of food-borne diseases is underestimated [13,14].

Numerous SE assays are based on the evidence of the enterotoxins directly in the food: gel diffusion tube or plate assays, a reversed passive hemagglutination method, radioimmunoassay (RIA), enzyme-linked imnnunosorbent assay (ELISA), or enzyme immunoassay (EIA) and reversed passive latex agglutination (RPLA). With these assays, it is possible to detect nanogram amounts of enterotoxins in one gram or in one milliliter of food [12,15-17]. The advantage of these methods is that enterotoxins are detected even if *S. aureus* cannot be detected due to inactivation by high temperatures used during food production processing, or preparation [17]. Some of these tests can differentiate types of staphylococcal enterotoxins from A to E and others cannot. SET RPLA could differentiate the enterotoxins A-D but its use is time-consuming. Due to the use of latex particles, no interference with endogenous enzymes has been observed [18]. The introduction of molecular techniques and the availability of DNA sequence information for all described SEs have allowed the detection of specific enterotoxin gene sequences by Polymerase Chain Reaction (PCR) [19-23]. The PCR-based approach is considered to be accurate, defined and rapid, but it can only show the presence of specific enterotoxin genes rather than the production of the SE protein [17,23, 24].

This study was conducted to determine the prevalence of enterotoxin genes (*sea-seo)* in CPS isolates recovered from milk and milk products in the north of Morocco as this has, to our knowledge, not been investigated previously. The study allows a comparison of results from detection of SE (A-D) by SET-RPLA with the presence of the corresponding *se* genes shown by PCR.

Materials and Methods
Isolation of Staphylococcus *strains from samples*
A total of 46 coagulase positive strains, *S. aureus* (40), *S. hyicus hyicus* (4) and *S. intermedius* (2) were isolated from 81 samples of raw milk and the traditional milk products Lben and Jben. All the samples were obtained from different localities (weekly rural markets) in northern Morocco [Tangier, Tetouan and Larache] between May 2005 and May 2006. Staphylococcal strains were isolated as follows: twenty-five grams of each cheese sample and 25 ml of raw milk and Lben were stirred separately into 225 ml of sterile buffered peptone water. Baird-Parker plates (agar Rapid'staph, BIO-RAD) were then spread with 0.1ml of each diluted sample. Additional plates were prepared with successive 1/10 dilutions. The plates were incubated for 48 hours at 37°C. The identification of the *staphylococcus* genus was done by microscopic observation, Gram-staining, and catalase determination. All staphylococcal strains were checked for purity and tested for their ability to coagulate citrated rabbit plasma. Other identification/biochemical assays, including the API Staph system (BioMerieux, Marcy-l'Etoile, France) and the MicroScan® (WalkAway® 40/96 from Siemans) were used to determine the species. These bacteria were stored in brain heart infusion (BHI) broth (BIO-RAD) with 50% glycerol at -80°C and conserved in agar until use.

Preparation of staphylococcal DNA
One µl loop of a small quantity of growth, equivalent to about two small colonies, was scraped from the top of the culture and placed into 100 µl of sterile distilled water in a microcentrifuge tube. The mixture was vortexed for about 10 seconds, and then heated at 90°C for 17 minutes, after which time it was held at -20°C until needed. When required, the sample was allowed to thaw but while still cold was centrifuged in a minicentrifuge for two minutes at 8,000 g. For the PCR, 1µl of the supernatant was used.

Detection of enterotoxigenicity
For classical enterotoxins produced by *S. aureus* (SE: A, B, C, D), an immunological technique was used. The working cultures of the isolates were prepared in BHI broth at 37°C for 18 hours. One ml of the culture was centrifuged at 9,000 x g for 20 minutes at +4°C, and the resulting supernatants were tested for the presence of enterotoxins A to D by the

reversed-passive latex agglutination (RPLA) method, using SET-RPLA staphylococcal enterotoxins A, B, C, D (TD0900A, Oxoid) according to the manufacturer's instructions. Standard *S. aureus* enterotoxins A, B, C, and D were used as positive controls.

The isolates were additionally investigated by PCR technique for amplification of species-specific parts of the classical members of the pyrogenic toxin superantigen (PTSAg) gene family containing staphylococcal enterotoxin genes *sea-see* as well as recently described *se* genes, namely *seg, seh, sei, sej, sek, sel, sem, sen* and *seo*. Oligonucleotide primers used to detect *se* genes are listed in Table 1.

PCR protocols were performed for individual genes, as a series of separate reactions, using only one primer pair for each reaction mix. PCR amplification was conducted in a final reaction volume of 25 µl. The PCR mixture consisted of 0.25 µl of primer 1 (100 pmol /µl), 0.25µl of primer 2 (100 pmol/µl), 2 µl of deoxynucleoside triphosp hate (2.5 mmol/liter), 2.5 µl of 10 x thermophilic buffer, 1.5 µl of $MgCl_2$ (25 mmol/liter), 0.15 µl of Taq DNA polymerase (5 U/µl) (all the reagents from Applied Biosystems), and 17.35 µl of distilled water. Finally 1µl of DNA preparation was added to each 0.2 ml reaction tube. The tubes were subjected to thermal cycling (Techne-Progene; Thermodux, Wertheim,

Table 1. Oligonucletide primers and PCR programs for amplification of the genes encoding staphylococcal proteins including various toxins

Target gene	Primer name	Sequence (5'-3')	PCR program	PCR Fragment (bp)	Ref
sea	SEA_1 SEA_2	AAA GTG CCG ATC AAT TTA TGC CTA GTA ATT AAC CGA AGG TTC TGT AGA	a	**219**	c
seb	SEB_1 SEB_2	TCG CAT CAA ACT GAC AAA CGA CAC TTT TTC TTT GTC GTA AGA TAA	a	**410**	c
sec	SEC_1 SEC_2	AAC ATT AGT GAT AAA AAA GTG AAA TTG TAA GTT CCC ATT ATC AAA GTG	a	**234**	c
sed	SED_1 SED_2	GCA GAT AAA AAT CCA ATA ATA GGA TAC TAA AGA AAC TTC TTT TTG TAC	a	**331**	c
see	SEE_1 SEE_2	TTA CAA AGA AAT GCT TTA AGC TAA ACC AAA TTT TCC GTG	a	**456**	c
seg	SEG_1 SEG_2	GCT ATC GAC ACA CTA CAA CC CCA AGT GAT TGT CTA TTG TCG	a	**583**	c
seh	SEH_1 SEH_2	CAC ATC ATA TGC GAA AGC CGA ATG AGT AAT CTC TAG G	a	**548**	c
sei	SEI_1 SEI_2	GAT ACT GGA ACA GGA CAA GC CTT ACA GGC AGT CCA TCT CC	a	**789**	c
sej	SEJ_1 SEJ_2	CTC CCT GAC GTT AAC ACT ACT AAT TTG TCT GGA TAT TGA CCT ATA ACA	a	**666**	c
sek	SEK_1 SEK_2	CAC AGC TAC TAA CGA ATA TC TGG AAT TTC TCA GAC TCT AC	a	**378**	c
sel	SEL_1 SEL_2	CAT ACA GTC TTA TCT AAC GG TTT TCT GCT TTA GTA ACA CC	a	**275**	c
sem	SEM_1 SEM_2	CTT GTC CTG TTC CAG TAT C ATA CGG TGG AGT TAC ATT AG	a	**329**	c
seo	SEO_1 SEO_2	AGT CAA GTG TAG ACC CTA TT TAT GCT CCG AAT GAG AAT GA	a	**533**	d
sen	SEN_1 SEN_2	ATT GTT CTA CAT AGC TGC AA TTG AAA AAA CTC TGC TCC CA	a	**681**	d

[a] [DNA denaturation at 95°C , 1 min]; [30 times (95°C, 15 s; 50°C, 30 s; 72°C, 30s)]
  Extension at 72°C, 8 min.
[c] [25]
[d] [26]

Germany) with the program shown in Table 1.

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. The results were visualized and photographed with Image Master®VDS (Pharmacia Biotech). The sizes of the amplification products were estimated by comparison with a 123 bp DNA step ladder (Invitrogen, life technologies).

Results

Respectively, 40, four, and two CPS, *S. aureus*, *S. hyicus hyicus* and *S. intermedius,* isolates from milk and milk products from northern Morocco were tested by reversed passive latex agglutination (SET-RPLA). Eighteen strains (39%) were found to be positive for production of one or more classical staphylococcal enterotoxins: SEA, SEB, SEC and SED. The four toxins screened were found at different frequencies. SEA (with 11 isolates, 24 %) and SED (with 3 isolates, 6.5 %) were the most frequently found enterotoxins. SEA was most often found either alone (eight isolates) or together with SEC (two isolates); SEA was found less frequently with SED (one isolate) and with SEB and SEC (each with 2 isolates, 4.5%) (Table 2). A production of three enterotoxins by one isolate has not been observed, and neither has a combination of (SEA – SEB), (SEB-SEC), (SEB-SED) and (SEC-SED).

genes. Four (8,7%) isolates carried *seh* gene (Figure 1B). All of the isolates were found to be negative for the other enterotoxin genes (*see, seg, sei, sej, sek, sel, sem, sen* and *seo*) Figure 1A. shows the expected size of the amplified PCR fragments of enterotoxin genes A to D.

The comparison between the PCR results and the results of phenotypical detection of *S. aureus* toxins is summarized in Table 2.

The production of SEs as shown by SET-RPLA and the existence of *se* genes as demonstrated by PCR corresponded in 18 of the 46 CPS isolates analysed by both methods. The strains giving discordant results were sea (2), *seb* (1) and *sed* (5) positive by PCR and SEA, SEB and SED, respectively, which were negative by the phenotypic method used in this study.

Finally, in our study, none of the six non-*S. aureus* coagulase-positive (four *S. hyicus hyicus*) and two *S. intermedius*) produced enterotoxin SEA, SEB, SEC and SED using SET-RPLA. Furthermore, none of these strains harbored any of the genes *sea, seb, sec* and *sed* nor *see, seg, sei, sej, sek, sel, sem, sen* and *seo* using PCR technique.

Discussion

Examination of SE production and the presence of *se* in SCP (including *S. aureus* as well as *S. intermedius* and *S. hyicus hyicus* [27]) isolated from 81 samples of raw milk and raw milk products

Table 2. Comparison of genotypic (PCR) and phenotypic RPLA results for *S. aureus* toxin detection

	PCR					SET-RPLA (OXOID)	
Gene detection	Isolates n	SEA	SEB	SEC	SED	SE (A+C)	SE (A+D)
sea	13	11	-----	-----	-----	01	01
seb	03	-----	02	-----	-----	-----	-----
sec	02	-----	-----	02	-----	01	-----
sed	08	-----	-----	-----	03	-----	01
Total	26	11	02	02	03	02	01

The PCR technique was applied to all strains of CPS. Thirty of 46 CPS (65, 2%) isolates from raw milk and raw milk products (lben and Jben) were found to be positive for at least one PTSAg gene (*sea, seb, sec, sed* and *seh*). Twenty-six (56.5%) isolates tested harbored genes of the classical enterotoxins (*sea, seb, sec, sed)*. Thirteen isolates (28.3%) carried *sea*, three (6.5%) were *seb*-positive, two (4.3%) were *sec*-positive, and eight (17.4%) were *sed*-positive

revealed considerable diversity, especially in the *S. aureus* population. Some *S. aureus* strains (eighteen) produce one or more enterotoxigenic toxins including SEA-SED. These toxins represent the main cause of staphylococcal food poisoning. It has been estimated that about 95% of these outbreaks have been due to these classical SEs [28,29].Considerable variation in SE production and the presence of *se* was observed

Figure 1. Gel electrophoresis of the amplified PCR fragments.

A
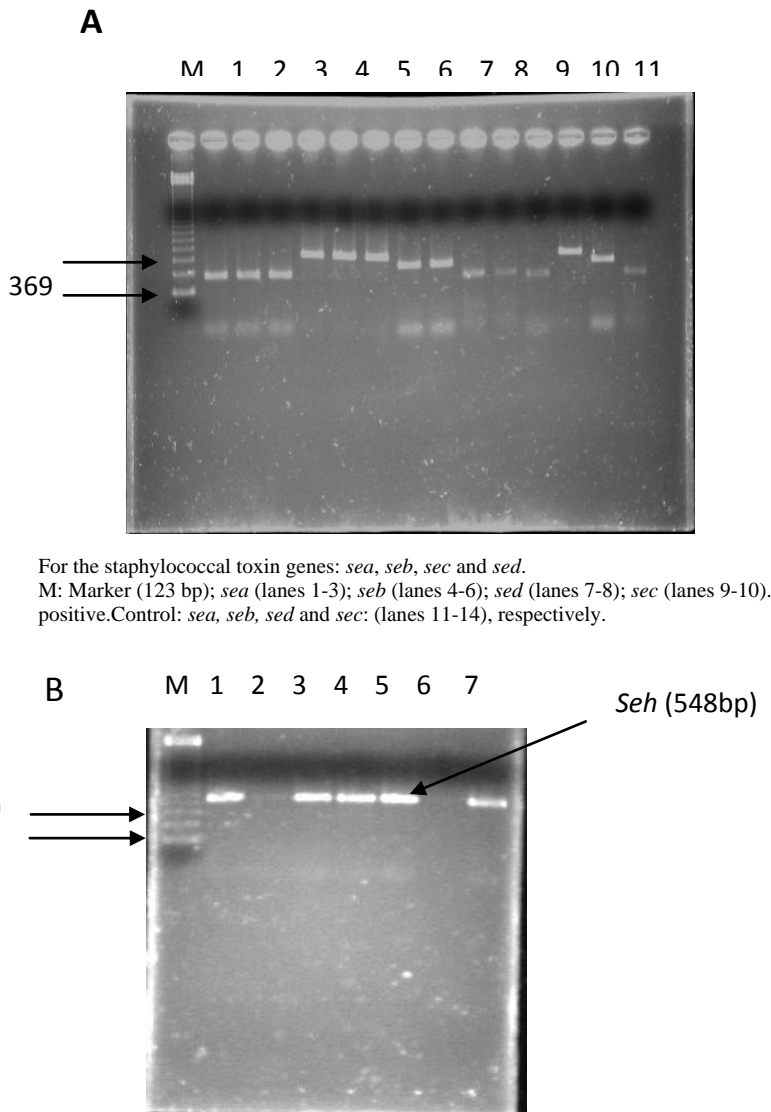
For the staphylococcal toxin genes: *sea*, *seb*, *sec* and *sed*.
M: Marker (123 bp); *sea* (lanes 1-3); *seb* (lanes 4-6); *sed* (lanes 7-8); *sec* (lanes 9-10).
positive.Control: *sea, seb, sed* and *sec*: (lanes 11-14), respectively.

B

(**B**) For the staphylococcal toxin gene *seh*:
M: Marker (123 bp); *seh* (lane 1 and lanes 3-5), *S. aureus* (neg. control, lane 2),
S. aureus (pos. control, lane 7) and distillate water (lane 6)

among the isolates from raw milk and raw milk products. In a first step, the SCP strains collected were screened for the production of the most frequent and classical types of enterotoxins using the SET-RPLA kit as well as for their genes by a PCR method. In a second step**,** all the strains were screened only by PCR for genes harboring more recently described enterotoxins (*see, seg, seh, sei, sej, sek, sel, sem, sen* and *seo).*

For the determination of SEs, we selected the most commonly used method, SET-RPLA, which can detect SEA-SED if it is expressed *in vitro*. It gave satisfactory results in the present study, since none of

the strains yielded nonspecific agglutination, *i.e.*, passive agglutination with both sensitized and control latex particles. The percentage of detected SE producing strains of *S. aureus* varied considerably among studies. In our study we found 39% out of 46 investigated SCP (with 40 *s. aureus*) strains from milk and milk products positive for the production of enterotoxins. RPLA was used in some studies and similar results were observed. Takeshige *et al*. examined 87 strains for enterotoxin production and they reported a prevalence of 33.3% [30]. Fueyo *et al*. [31] and Loncarevic *et al*. [32] found 34% and 28% strains positive for the production of enterotoxins among 386 isolates of raw milk and raw milk products and 224 isolates from human carriers and manually handled foods, respectively**.** Using the same method, a higher prevalence was reported by both Adesiyun [33] (53.6%) and Mork *et al.* [34] (64%) among *S. aureus* strains investigated for SEA-SED**.** On the other hand, we found that enterotoxin A was the toxin most commonly produced, followed by enterotoxin D, while enterotoxins B and C were the least common. Moreover, enterotoxin A is most often implicated in cases of staphylococcal food poisoning [35]. 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 [36], human nasal carriers, and manually handled foods [37]. These observations might be indicative of contamination by workers' hands during processing [35].

For the detection of enterotoxigenic *S. aureus* strains, a genotypic technique such as PCR was also used [23,37]. Table 2 shows the discrepancy in enterotoxigenic type detection when results from SET-RPLA and the PCR method were compared. In contrast, toxin production indicated in eight isolates by *sea* (2)*, seb* (1) and *sed* (5) was not verified with SET-RPLA. This result was consistent with previous reports, which showed that four raw bovine milk isolates were positive for *seb* and *sec* by PCR but negative for SEB and SEC by SET-RPLA [32]. Naresh *et al*. [38] identified the presence of a *sec* gene in two strains which did not produce detectable levels of SEC toxin when we used the SET-RPLA assay. Such a discrepancy could be due to the fact that the SET-RPLA kit detects the enterotoxin itself while the PCR method detects its gene. Under certain circumstances, the staphylococcal enterotoxins might not be produced or be produced only to a certain level below the detection limit of SET-RPLA [39]. Since the production of enterotoxins by

staphylococcal strains can be affected by the growth conditions used (inoculum level, temperature, pH, and water activity [4]), it is possible that for these particular isolates the standard culture conditions specified for the SET-RPLA assay are suboptimal for gene expression [38]. Alternatively, the *se* genes may not be expressed due to mutations either in the coding region or in a regulatory region (either the promoter or an accessory regulator, *e.g.*, *agr*) [29, 38]. Except for four *seh* positive strains (8.7%), the other 42 CPS strains investigated did not harbor any of the other new enterotoxin genes (*see, seg, sei, sej, sek, sel, sem, seo and sen*). However, in our study, a number of *seh* positive strains were higher than those noted by Larsen *et al.* [40]. Akineden *et al.* [41] and Omoe *et al.* [28] did not find *seh* genes in *S. aureus* from milk from cows with mastitis. The results obtained indicate that the presence of recently described *se* genes among *S. aureus* strains isolated from raw milk, Lben and Jben is important. Since, to date, no commercial tests for SEG-SEO exist, CPS isolates containing the corresponding genes should be considered potential SE producers [42].

We conclude that the combination of both phenotypic and genotypic characterization (RPLA and PCR) might provide a better understanding of diagnostic test results. In fact, PCR can serve as an early warning system for rapid detection of genes responsible for intoxication, and the products can then be tested further with immunological methods [29,43].

Conclusion

The presence of staphylococcal enterotoxin in raw milk and raw milk products in northern Morocco is a potential health hazard.

It demonstrates the need for proper examination by public health inspectors of the production, storage, and commercialization of milk, Jben, Lben, and other products made with unpasteurized milk. Further studies are needed to examine the presence of enterotoxigenic *S. aureus* isolates or their toxins in other types of food.

Lastly, the presence of enterotoxigenic strains (*se* positive by genotypic tests) in food does not always necessarily mean that the toxin will be produced. For that reason, the combination of both methods (RPLA and PCR) is a guarantee for success in diagnostic analysis tests. In fact, PCR can serve as an early warning system for rapid detection of genes responsible for intoxication because it gives information on the presence or absence of genes for staphylococcal enterotoxin); additionally, the products can then be tested further with immunological methods to obtain information on the expression of these genes.

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