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Research Article

Occurrence of enterotoxins, exfoliative toxins and toxic shock syndrome toxin-1 genes in *Staphylococcus aureus* and CoNS isolated from clinical and food samples in Algeria

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Abstract. The aim of this study was to determine the occurrence of toxin-genes carried by strains of *Staphylococcus aureus* and Coagulase Negative Staphylococci (CoNS) in Algeria. The present study performed two set multiplex PCR assay using specific primers for screening of 8 genes encoding for classical enterotoxins (SEs) (*sea* to *see*), Exfoliative Toxins (*eta, etb*) and Toxic Shock Syndrome Toxin-1 (*tst*). We analyzed 51 strains isolated from food samples and 45 strains originated from clinical cases. We observed that more than half of food strains (52.94%) possessed at least one of SEs genes; where *S. aureus* appears to be potentially enterotoxigenic than CoNS (68.18% vs 41.37%). From all the SEs genes amplified (27), *sed* gene (19;70.37%) was the most frequently detected. In clinical isolates, only 6 (13%) *S. aureus* harboured at least one SEs genes. However, 55.55% of clinical isolates (*S. aureus* or CoNS) possessed *tst* gene for Toxic Shock Syndrome Toxin-1. There were no foods or clinical isolates detected to possess exfoliative toxins genes (*eta, etb*). In conclusion, this study showed high frequency of SEs genes in food isolates, and *tst* gene in clinical isolates; our findings provide updated data on the Staphylococci toxins carriage in Algeria.

Key Words: *Staphylococcus aureus*; Enterotoxins; PCR; CoNS; TSST-1.

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Introduction

Staphylococci are responsible for nosocomial/community acquired infections and foodborne illnesses worldwide (Le Loir et al 2003). This bacterium remains a persistent opportunistic of the respiratory tract and skin, either for human or animals (Monecke et al 2011) and constitutes a contamination source in food or health-care facilities (Kluytmans et al 1997; Von Eiff et al 2001).

Staphylococci produce a variety of toxins responsible for foodborne illness or toxic affections in patients with serious health problems (Hennekinne et al 2010). Consumption of contaminated meals with enterotoxins causes vomiting, diarrhoea and enteritis within six hours (Le Loir et al 2003; Seo and Bohach 2007). In addition, Staphylococci cause severe systemic affections (pneumonia, endocarditis), associated with variety of exotoxins such as toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins A and B, and Panton-Valentine leucocidin (PVL) (Balaban and Rasooly 2000; Dinges et al 2000).

Moreover, Staphylococci have a wide ability to acquire toxin-encoding genes. Most of these genes are located and transmitted via mobile genetic elements. For example, plasmids for *seb* (SEB), *sed* (SED) and *etb* (exfoliative toxin B) genes (Bayles and Iandolo 1989; Argudin et al 2010); prophages for *sea* (SEA) and *see* (SEE) genes (Balaban and Rasooly2000; Le Loir et al 2003);Staphylococcal pathogenicity islands for *sec* (SEC) and *tst* (TSST-1) gene (Dinges et al 2000; Argudin et al 2010). These aspects of mobile carriage accentuate horizontal transmission among isolates as well as modify their virulent ability and their evolution state (Becker et al 2003; Argudin et al 2010; Grumann et al 2014).

Coagulase positive Staphylococci (CoPS) especially *Staphylococcus aureus* were considered as the most pathogenic species (Hennekinne et al 2010), while, Coagulase Negative Staphylococci (CoNS) had gained little attention and thought to be non-pathogenic (Kloos and Schleifer 1975). However, recently, CoNS ability to carry multiple pathogenic factors such as enterotoxins and TSST-1 toxin have been demonstrated (da Cunha et al 2007; Zell et al 2008; Vasconcelos et al 2011;Batista et al 2013).Toxin-associated genes carried by CoNS can be transmitted by cohabitation with other pathogenic Staphylococci (Ławrynowicz-Paciorek et al 2007; Vasconcelos and da Cunha 2010). Nevertheless, CoNS can contaminate foodstuffs and their clinical importance and toxigenic capacity cannot be ignored (Kloos and Bannerman 1995; Otto 2009).

Algerian studies were focused on Staphylococci antibiotic resistance (Ramdani-Bouguessa et al 2006; Rebiahi et al 2011;

Table 1.Characteristics of primers used for PCR analysis

Gene	Primer	Oligonucleotide sequence (5'-3')	Location within gene	Size of amplified product (bp)
sea	GSEAR-1 GSEAR-2	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	349–368 431–450	102
seb	GSEBR-1 GSEBR-2	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	666–685 810–829	164
sec	GSECR-1 GSECR-2	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	432–455 863–882	451
sed	GSEDR-1 GSEDR-2	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	492–514 750–769	278
see	GSEER-1 GSEER-2	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	237–257 425–445	209
femA	GFEMAR-1 GFEMAR-2	AAAAAAGCACATAACAAGCG GATAAAGAAGAAACCAGCAG	1444–1463 1556–1575	132
eta	GETAR-1 GETAR-2	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTTGCTG	775–794 848–867	93
etb	GETBR-1 GETBR-2	ACAAGCAAAAGAATACAGCG GTTTTTGGCTGCTTCTCTTG	509–528 715–734	226
tst	GTSSTR-1 GTSSTR-2	ACCCCTGTTCCCTTATCATC TTTTCAGTATTTGTAACGCC	88–107 394–113	326

Ouchenane et al 2011) and rarely on virulence factors such as:TSST-1 and PVL toxins (Ramdani-Bouguessa et al 2006).The aim of the present study was to determine by PCR the frequency of genes encoding staphylococcal enterotoxins, exfoliative toxins, and toxic shock syndrome toxin-1 genes, in Staphylococci strains isolated from food and clinical samples in Algeria.

Materials and methods

Bacterial Strains

A total of 96 *Staphylococcus* spp. isolates were used. Fifty one (53.13%) isolates were obtained from food matrix, samples including raw milk, minced beef meat, sausages and creamy cake were collected from retail market (supermarkets, conventional markets, or bazaars) in large cities at two regions of Médéa and Ain defla provinces, Algeria.

Forty five (46.88%) strains were isolated from clinical samples, clinical samples included: pus, sperm, urine, vaginal discharge, wound, catheter tips and secretions in general were aseptically collected in different services of Mohamed Boudiaf hospital, Médéa, Algeria. Clinical samples were taken to the microbiology laboratory of the same hospital for analysis.

Detection and enumeration of coagulase-positive Staphylococci in food matrix was done according to ISO 6888-1:1999/A1:2003. Baird Parker agar plates were incubated for 24 to 48 h at 37 °C. Microbiological analysis of clinical samples was done according to Quinn et al (1994). For coagulase-positive Staphylococci (CoPS) confirmation, catalase and coagulase tests were used (Rabbit plasma, Oxoid, UK). *Staphylococcus aureus* identification for both food and clinical isolates was made by Pastorex staph plus (Bio-Rad, France 2016).

DNA extraction

All isolates were purified by inoculation in Trypticase Soya Broth (TSB), and then transferred to Trypticase Soya Agar (TSA) plates for 18h incubation at 37°C. For bacterial cell lyses, lysozyme (100mg/ml) and proteinase-K (10mg/ml)were used. Nucleic acid extraction was performed with Phenol/chloroform extraction method according to Sambrook and Russel (2001). DNA pellet was re-suspended in Diethyl pyrocarbonate (DEPC) treated water and stored at –20 °C until PCR analyses.

PCR Procedure

Specific primers were used for the amplification of the *sea, seb, sec, sed, see* (SEs) genes; *eta, etb* (Exfoliative Toxins A, B associated genes), and *tst* (Toxic Shock Syndrome Toxin-1 gene) (Table 1). The *femA* primers were used for confirmation of *S. aureus* among studied strains. PCR procedures were applied according to Mehrotra et al (2000). For the PCR analysis, positive control DNAs were obtained from Mustafa Kemal University, Faculty of Veterinary Medicine, Microbiology Department.

Two sets of multiplex PCR procedures were performed according to Mehrotra et al (2000) with minor modifications. Briefly, for SEs, amplification mix containing 200µM deoxynucleoside triphosphates; 5 µl of 10X reaction buffer (100 mMTris-HCl [pH 8.3], 500 mMKCl); 1.5 mM MgCl2; 20 pmol of each *sea, seb, sec, see*, and *femA* primers; 40 pmol of *sed* primer; 2.5 U of Taq DNA polymerase (Thermo scientific), was added to 3 µl of template DNA. For others toxins genes, PCR reaction had the same constituents as in SEs except for the MgCl2 concentration (2.0 mM) and the primers, which were used at 50 pmol for *eta* and 20 pmol for *etb* and *tst*. The final volume was adjusted to 25 µl by adding sterile ultrapure water. Amplification (CFX 96 thermal cycler, Bio-Rad) programme was as follows:

Table 2. Distribution of the enterotoxins, Exfoliative toxins and Toxic Shock Syndrome Toxin-1 genes for food isolates.

Target genes Amplicon size (bp)	Food isolates (n = 51)		P value	Samples origin
	S. aureus (n = 22; 43.14%)	CoNS (n = 29; 56.86%)		
***femA*(132)**	22	-	ND	(10) Raw milk (2) Creamy cake (2) Sausages (8)Minced beef meat
***sea*(102)**	2 (9.09%)	-	0.181	(2) Creamy cake
***seb*(164)**	-	-	ND	-
***sec*(451)**	1(4.55%)	2(6.90%)	1	(1)Creamy cake (2) Minced beef meat
***sed*(278)**	11(50%)	8(27.59%)	0.145	(10)Raw milk (1)Creamy cake (2) Sausages (6)Minced beef meat
see (208)	1(4.55%)	2(6.90%)	1	(3)Raw milk
***eta*(93)**	-	-	ND	-
***etb*(266)**	-	-	ND	-
***tst*(326)**	-	-	ND	-

initial denaturation at 94°C for 3 min followed by 29 cycles of amplification (denaturation at 94°C for 1.5 min, annealing at 54°C for 1.5 min, and extension at 72°C for 1 min). A final extension step (72°C for 7 min) was performed after the completion of cycles.

PCR products, were loaded into 2% agarose gel (BioMax) stained with 5 µl of RedSafeTM nucleic acid (iNtROn Biotechnology, Korea) and visualized using a UV transluminator (EC3, UVP Bioimaging systems, Inc).

Statistical analysis

Fisher's exact test was used to compare the frequency of each enterotoxin, exfoliative toxin and TSST-1 toxin genes. P value of <0.05 was considered as statistically significant.

Results

In the present study, the amplification of SEs, other toxin-associated genes and *femA* was successfully obtained. Table 2 and Table 3 show the PCR results for the detection of classic enterotoxins encoding genes; exfoliative toxins and toxic shock syndrome toxin-1 encoding genes of all Staphylococci isolates. According to phenotypic methods and femA gene detection, 61 isolates (63.54%) (22 food, 39 clinical) were confirmed as *Staphylococcus aureus*.

Among food isolates (n=51), 27 (52.94%) had at least one gene encoding for SEs production. The detection of *sed* gene was the most predominant (70.37%) being found in 19 isolates (11 in S. *aureus* and 8 in CoNS strains). However, three isolates carried *sec* and *see* genes. Also, *sea* gene was confirmed in only two *S. aureus* isolates, while *seb* gene was not detected.

Regarding to food-matrix origins, raw milk and minced beef meat were the most common matrix which contained Staphylococci harbouring *sed* gene (10 and 6 isolates, respectively) (Table 2). In addition, three *see* genes were detected in raw milk isolates only. However, the two *S. aureus* isolates possessing the *sea* gene were exclusively isolated from creamy cake samples. In all food isolates, *S. aureus* was more enterotoxigenic than CoNS (68.18% vs41.37% respectively) but not significantly different (P=0.089), this difference was remarkably observed for *sed* and *sea* genes(P=0.145, P=0.181). However, the frequency of *sec* and *see* genes in CoNS (6.9%) was higher but not significant compared to *S. aureus* isolates (4.54%) (P=1.000). The exfoliative encoding genes (*eta, etb*) and *tst* gene were not detected in any food isolates.

Regarding clinical isolates, the rate of staphylococcal-harbouring enterotoxin genes isolated from clinical samples was significantly lower (6/13.33%) compared to that of food isolates (27/55.94%) (P<0.001). All clinical CoNS isolates were negative for SEs genes. Among the six SEs genes positive isolates, three hospital acquired isolates possessed the *sed* gene and one isolate harboured *seb* gene. Exceptionally, one community acquired *S.aureus* isolate had the *seb* gene.

The exfoliative encoding genes (*eta, etb*) were not found in any clinical isolates. Nevertheless, a high percentage of the *tst* gene (55.55%) was detected, with predominance of pus samples (9 isolates) (Table 3). For all clinical CoNS isolates, the *tst* gene was found only in one community acquired strain.

Table 4 lists the genotype profiles for all isolates harbouring one or multiple toxin genes. Among all profiles observed, the most commonly identified gene profiles were those containing a single

Table 3. Distribution of the enterotoxins, Exfoliative toxins and Toxic Shock Syndrome Toxin-1 genes for clinical isolates

Target genes Amplicon size (bp)	Clinical isolates (n = 45)			P value	Samples origin
	Hospital acquired Isolates (n = 18)	Community acquired Isolates (n = 27)			
	S. aureus (n = 18; 40%)	*S. aureus* (n = 21; 46.67%)	CoNS (n = 06; 13.33%)		
***femA*(132)**	18	21	-	ND	-
***sea*(102)**	-	-	-	ND	-
***seb*(164)**	1(5.56%)	1(4.76%)	-	1	(1)Sperm (1) Vaginal discharge
***sec*(451)**	1(5.56%)	-	-	1	(1) Throat samples
***sed*(278)**	3(16.67%)	-	-	1	(1) Blood culture (1) Urinary probe (1)Vaginal discharge
***see* (208)**	-	-	-	ND	-
***eta*(93)**	-	-	-	ND	-
***etb*(266)**	-	-	-	ND	-
***tst*(326)**	9(50%)	15(71.43%)	1(16.67%)	0.558	(9) Pus (5) Urine (5) Sperm (3) Vaginal discharge (1) Joint fluid (1)Surgical wound (1) Urinary probe

Table 4. Genotypes of Staphylococcal enterotoxins, exfoliative and toxins of toxic shock syndrome toxin-1 genes

Genotypic profiles	Food isolates (n = 51)		Clinicalisolates (n = 45)	
	S. aureus (n = 22) (43.14%)	CoNS (n = 29) (56.86%)	*S.aureus* (n = 39) (86.67%)	CoNS (n = 06) (13.33%)
sea	1	-	-	-
seb	-	-	1	-
sec	-	2	1	-
sed	9	8	-	1
see	1	2	-	-
sed-sea	1	-	-	-
sed-sec	1	-	-	-
sed-seb- tst	-	-	1	-
sed -tst	-	-	1	-
tst	-	-	22	1

toxin gene (*sea, seb, sec, sed, see* and *tst*).Twenty six (27.08%) isolates had one type of enterotoxins gene, and 23 (23.95%) possessed only the *tst* gene. Besides, the *sed* and *tst* genotypes were the most observed with 18 (%) and 23 (%) strains, respectively. Combinations genes had been found in four genotypes (*sed-sea, sed-sec, sed-tst, sed-seb-tst*); each multi-toxin genotype had only one strain.

The present results showed that s*ea, seb, sed-sea, sed-sec, sed-seb-tst* and *sed-tst* genotypes had occurred in *S. aureus* isolates. The remaining genotypes included both *S. aureus* and CoNS isolates.

Discussion

In Algeria, few studies have focused on the toxigenic potential of staphylococcal strains and their occurrence in food poisoning

or hospital/community acquired infections. Algerian reports had studied PVL and TSST-1 genes carriage in MRSA (Ramdani-Bouguessa et al 2006; Bittar et al 2009; Ouchenane et al 2010). Antri et al (2010) studied the *Staphylococcus aureus* enterotoxin genes carriage isolated in hospital and recorded a low frequency for classical enterotoxin genes (8.6%). For the knowledge of the authors, no data has been published about the frequency of toxins genes carriage in Staphylococci isolated form food matrix. Our results showed that the staphylococcal isolates harbouring genes for classical enterotoxins were responsible for more than half of all cases of food contamination. These results are in accordance with many previous works (Chapaval et al 2006; Pereira et al 2009; Carfora et al 2015). In the present study, *sed* was the most frequent toxin-encoding gene (19 of 51 isolates, 37.25%) isolated from food. This gene was mainly detected in Staphylococci isolated from raw milk and minced beef meat. These results are in agreement with other studies, such as those of Pu et al (2011) and Bianchi et al (2013) who reported that 15% (23/152) and 25% (120/481) of retail meat isolates and dairy product isolates had amplified the *sed* gene. In contrast, Zouharova and Rysanek (2008) reported that a low percentage of *S.aureus* isolates encoding the *sed* gene (2/70; 2.9%) in raw milk, whereas Balaban and Rasooly (2000) and Normanno et al (2005) found no evidence that the SED enterotoxin was involved in Staphylococcal food poisoning (SFP).

In our study, no isolates taken from raw milk encoded the *sea* gene. The only detected *sea* gene came from isolates which originated from cream cake samples. In contrast, Chapaval et al (2006) and Rall et al (2008) reported that *sea* was the most common toxin gene detected in raw milk isolates, finding 67.78% (61 out 90 isolates) and 28.9% (11 out 38 isolates), respectively. Omoe et al (2005), Chiang et al (2008) and Tang et al (2011) detected the *sea* gene mainly in Staphylococci isolated from food matrix. They reported that it was responsible for 5.8%, 29.2% and 50% of cases of SFP, respectively. However, Pereira et al (2009) did not detect the *sea* gene in 20 *S. aureus* isolated from raw milk, which is in accordance with our results.

In terms of the frequency of *seb* gene detection, our results are in accordance with those of Cremonesi et al (2005) and Bianchi et al (2013), who reported that all of Staphylococcal strains isolated from milk and dairy products were negative for the *seb* gene. In our study, *see* gene was harboured only by three Staphylococci strains isolated from raw milk; Rall et al(2008) detected the *see* gene in 5.26% (2 out of 38 isolates) *S. aureus* isolated from raw milk, while Zouharova and Rysanek (2008) did not. For *sec* gene carriage, we reported two strains isolated from minced beef meat, while Pu et al (2011) did not detect any *sec* gene in 152 *S. aureus* isolates.

Regarding clinical isolates, the rate of staphylococcal-harbouring enterotoxin genes observed in our study was lower than that reported in earlier clinical investigations (Becker et al 2003; Nashev et al 2007; Chiang et al 2008). Our clinical samples consisted mainly of samples obtained from patients with hospital- and community-acquired infections. Thus, the discordance may be explained by differences in the origin of the specimens, which included samples from food poisoning cases, or potential contamination by carriage sources of Staphylococci (nasal cavities and hands). However, some authors (Naffa et al 2006; da Cunha et al 2007 and Demir et al 2011) reported a high percentage of staphylococcal-positive SE-encoding genes obtained from samples population similar to ours: 23/100 (23%), 56/120 (47.5%) and 66/120 (55%), respectively. Moreover, considering that our results are in concordance with a local report of Antri et al (2010), we can consider that geographical location might explain the large difference between our findings and those reported earlier.

Irrespective of the clinical sampling origin, several studies reported a high frequency of the sea gene in Staphylococci isolates (Naffa et al 2006; Nashev et al 2007; Demir et al 2011). However, it should be pointed out that in the present study, sea gene was not detected in any of the clinical isolates. The frequency of *see* gene found in our study was in accordance with that of all the above studies, except the study by Becker et al (2003). The occurrence enterotoxin-encoding genes detected in CoNS isolated from food samples was 41.37% (12 out of 29 CoNS isolates), which was higher than reported in other studies (Blaiotta et al 2004; da Cunha et al 2006) but similar to another (Fijałkowski et al 2016). In our study, the most frequently detected enterotoxin gene was *sed*, and neither *sea* nor *seb* were detected in the CoNS isolates. Da Cuhna et al (2006) reported that 15% (3 out 20 isolates) of CoNS isolated from foods harboured the sea gene. Other studies also detected enterotoxin genes in CoNS strains from both dairy and meat products (Vernozy-Rozand et al1996; Rodriguez et al 1996).

In the present study we did not detect *eta* or *etb* genes in any of the tested samples. Some studies reported that *S. aureus* produced one or both exfoliative toxins (Hayakawa et al 2000; Becker et al 2003; Demir et al 2011). Jarraud et al (2001) did not detect *eta* and *etb* genes in 58 *S. aureus* isolates responsible for suppurative diseases.

In our study, *tst* gene was not detected in the food origin isolates, this result corroborate the Fijałkowski et al (2016) works. In contrast, we observed a high frequency of the *tst* gene (25/45; 55.55%) in clinical isolates, this result being similar to that found by Chiang et al (2008), who reported a frequency of 59.1% (87 out of 147 isolates). This was higher than that detected in earlier studies of Becker et al 2003; da Cunha et al 2007 and Demir et al 2011, who reported frequencies of (87/429; 20.3%), (11/104; 10.58%) and (17/120; 14.17%), respectively.

The TSST-1 toxin is a causative agent of systemic infections, such as the staphylococcal toxic shock syndrome (Dinges et al 2000) but is rarely implicated in SFP. A number of studies reported that TSST-1, was frequently detected in *S. aureus* clinical isolates but rarely in food isolates (Lappin and Ferguson 2009; Tsen et al 1998; El-Ghodban et al 2006).

In the present study, most genotypes contained a single toxin gene, regardless of the origin of the sample, with *tst* and *sed* as the most common genes. This result is in accordance with those of several other studies (Nashev et al 2007; Zouharova and Rysanek2008; Rall et al 2010), although the *sea* genotype was predominant in these studies. However, in other studies (Bianchi et al 2013; Pu et al 2011) the *sed* genotype was the most common genotype detected. In the present study, in clinical samples, the frequency of the *tst* genotype was in agreement with that found in an earlier study (Chiang et al 2008).

In the current study, frequency of toxin gene combinations was low, being observed in only four isolates. The *tst* gene was detected in combination with enterotoxin genes in two genotypes

(*sed-seb-tst* and *sed-tst*). A number of previous studies reported that Staphylococci strains harboured the *tst* gene, either alone or in combination with SE-encoding genes (Becker et al 2003; Chapaval et al 2006; da Cunha et al 2007; Chiang et al 2008; Demir et al 2011). Others studies recorded a *tst* combination with *sec*, but not with *sed* or *seb* (Hwang et al 2007).

The toxins production by Staphylococci strains is complex and involves gene carriage and gene promoters, such as multiple global regulators of virulence (e.g. *agr, sarA, rot* and *sigB*). Previous studies suggested that toxin-encoding genes (SEs, TSST-1 and exfoliative toxins) could be located on a mobile genetic carrier, which would provide potential support for horizontal transfer or genotype combination (Omoe et al 2005; Chiang et al 2008, Grumann et al 2014). Jarraud et al (2001) suggested that SEs and TSST-1 share common structural and biological properties and those that are derived from a common ancestor.

To conclude, this study reports the occurrence of the toxin genes in staphylococcal isolates from food and clinical samples in Algeria. Using a multiplex PCR method, a high frequency of SEs genes in food isolates and *tst* gene in clinical isolates were recorded. The pathogenic potential of CoNS points to the need for a greater surveillance in the area of hygiene and public health. There is an urgent need to establish legal standardized methods to be able to verify and quantify the degree of Staphylococci enterotoxins contamination in foods in Algeria. Further research is needed in order to investigate the contamination routes of food consumed in Algeria and the distribution of newly described Staphylococcal toxin genes.

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