

Detection of *Sea, Seb, Sec, Seq* genes in *staphylococcus aureus* isolated from nasal carriers in Tehran province, Iran; by multiplex PCR

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ABSTRACT

Staphylococcus(S.) aureus produces different extra-cellular protein toxins and virulence factors. One of the most important extra-cellular proteins is an enterotoxin which causes staphylococcal food poisoning (SFP) due to their enterotoxins. Different methods have been used to detect this toxin, each of which has advantages and disadvantages. DNA amplification methods, however, can show the presence of enterotoxigenic strains of *S. aureus* before the expression of enterotoxins on the basis of specific gene sequences. In this study, 150 *S. aureus* strains isolated from nasal carriers were confirmed by biochemical testing. PCR was used to amplify the staphylococcal enterotoxin A, B, C and Q genes, as well as the staphylococcal nuclease gene. Among the 150 healthy human isolates from the nasal carrier, 95 were confirmed as *S. aureus*. Only 58.9% of the isolates were diagnosed as *sea, b, c, q* positive. There were 24 (25.3%) isolates associated with the *sea* gene, 15.8% isolates associated with the *seb* gene, 9.5% of the isolates were associated with the *sec* gene, and 8.4% of the isolates associated with the *seq* gene. Of these isolates, 41% might be possessing additional *se* genes but they were not *see* (178 bp) and *sed* (319 bp) genes. The *nuc* gene, which encodes thermo nuclease, was used as a target DNA to identify *S. aureus*. Additionally, one of these enterotoxigenic isolates carried more than one toxin gene.

Keywords: *Staphylococcus aureus*; Enterotoxin; Multiplex PCR; Healthy Carrier; Iran

INTRODUCTION

Staphylococcus aureus, the most important genus of the micrococceae family, could, under certain conditions, cause food poisoning and severe infections in both animals and human beings by the enterotoxins it produces [1-3]. There are several types of staphylococcal enterotoxins: SEA, SEB, SEC1, SEC2, SEC3, SED and SEE. Recently, additional enterotoxins such as: SEO, SEP, SEQ, SEG, SHE, SEI, SEJ, SEK, SEL, SEM and SEN have been identified by new methods [4-6]. It was found that some strains of *S. aureus* produce more than one type of enterotoxin [7]. Therefore, the presence of *S. aureus* in food can be a potential risk for health, particularly, if the food is not stored at a prescribed temperature.

Most important specifications of staphylococcal enterotoxins are the resistance to heat, pepsin digestion and superantigenicity [8]. Symptoms of staphylococcal enterotoxin infection are: increased saliva, vomiting abdominal cramping, and diarrhea which can be accompanied by blood in some cases [9]. Approximately 5% of food poisoning illnesses are estimated to occur by this bacterial enterotoxin [10], with more illnesses reported [11-13]. It was reported by Bergdoll that staphylococcal enterotoxin types A, B, C, D and E were the cause of 95% of *S. aureus* infections and the remaining 5% were a result of infections from other types of bacteria [13]. Several reports on the isolation and detection of enterotoxigenic in food, healthy individuals carriers, infections and fecal have been written. In all, commonly used methods

for identifying bacterial toxins, such as latex agglutination, ELISA, immunodiffusion and RIA; a specific situation for enterotoxin gene expression is necessary [14-16], while it is possible that despite the presence of a potential toxin producing gene, in specific circumstances the bacteria are unable to produce toxin which would lead to negative results. Hence, efforts have been made by researchers to replace serological and biochemical methods by diagnostic molecular methods, by which the gene encoding for toxin would be, strains producing low level of enterotoxin to immunologic methods, low level of enterotoxin producing strain, could be identified by this method [7, 17, 18]. The aim of this study was to develop PCR to determine the presence of genes for staphylococcal enterotoxins from 150 samples. In this study, undertaken for the first time in Iran, *S. aureus* was isolated and the percentages of different strains that contained genes encoding types A, B, C, and Q enterotoxin were identified.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used included *S. aureus* strains characterized as positive for staphylococcal enterotoxin. Other strains used were: non-enterotoxigenic *S. aureus* (ATCC: 25923), *S. pyogenes*, *S. epidermidis*, *B. cereus* (ATCC: 11778), *B. polymyxa* (ATCC: 8094), *S. paratyphi* A (NCTC: 5702), *E. coli*, *P.*

vulgaris OX19 (ATCC: 6380), *Y. pseudo tuberculosis* (PCTC: 1070), and *M. luteus* (ATCC: 9341). These strains were used as either reference strains or controls in evaluating the specificity of the PCR primers. All strains were obtained from the Iranian Research Organization for Science and Technology (IROST). In this experimental study, nasal mucosal samples obtained by sterile swabs were prepared from 150 healthy carriers and cultured in mannitol salt agar media before being identified by biochemical testing. (Approval of study by university Institutional Review Board), informed consents were signed by patients who agreed to have nasal mucosal samples taken.

PCR primer design

Seven PCR primers sets were used to detect the deoxyribonuclease gene (*Nuc*) Specific for *S. aureus*, staphylococcal enterotoxin A gene (*Sea*), staphylococcal enterotoxin B gene (*Seb*), staphylococcal enterotoxin C gene (*Sec*), staphylococcal enterotoxin D gene (*Sed*), staphylococcal enterotoxin E gene (*See*) and staphylococcal enterotoxin Q gene (*Seq*) as described by Barati et al. [17] and Omoe et al. [7].

Primers were synthesized by MWG Company (Germany) (Table.1).

Table 1. PCR primers sets were used to detect genes *Nuc*, *Sea*, *Seb*, *Sec*, *Sed*, *See* and *Aeq*

DNA preparation and purification	Gene	Primer	Oligonucleotide sequence(5'-3')	Size of amplified product (bp)
Total genomic DNA was prepared	<i>nuc</i>	NUC1	5' CTG GCA TAT GTA TGG CAA TTG 3'	397 bp
		NUC2	5' AAT GCA CTT GCT TCA GGA CC 3'	
	<i>sea</i>	SEA 1	5' TTG CGA AAA AAG TCT GAA TTG C 3'	552 bp
		SEA2	5' ATT AAC CGA AGG TTC TGT AGA AGT A 3'	
	<i>seb</i>	SEB1	5' TCG CAT CAA ACT GAC AAA CG 3'	477 bp
		SEB2	5'AGG TAC TCT ATA AGT GCC TGC CT 3'	
	<i>sec</i>	SEC1	5' CTC AAG AAC TAG ACA TAA AAG CTA GG 3'	271 bp
		SEC2	5' TTA TAT CAA AAT CGG ATT AAC ATT ATC 3'	
	<i>sed</i>	SED1	5' CTA GTT TGG TAA TAT CTC CTT TAA ACG 3'	319 bp
		SED2	5'TTA ATG CTA TAT CTT ATA GGG TAA ACA TC3'	
	<i>see</i>	SEE1	5'CAG TAC CTA TAG ATA AAG TTA AAA CAA GC	178 bp
		SEE2	5' TAA CTT ACC GTG GAC CCT TCA G 3'	
	<i>seq</i>	SEQ 1	5' AAT CTC TGG GTC AAT GGT AAG C 3'	122bp
		SEQ 2	5'TTG TAT TCG TTT TGT AGG TAT TTT CG 3'	

according to the phenol-alcohol form extraction method. Before use, total genomic DNA were suspended in TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) buffer and stored at 4°C. To evaluate the quality of extracted DNA, it was located on a 1% agarose gel electrophoresed in 1X TBE (Tris-Borate-EDTE) buffer at 50 volts and then stained with ethidium bromide before being photographed by using ultraviolet illumination.

PCR amplification

The amplification reaction was performed in a final volume of 25 µl, containing 1 µl of genomic DNA, 0.5 µl of Taq polymerase (5 unit/µl), 0.5 µl of each of the primers (20 pmol/µl), 2 µl each of 2.5 mM dATP, dTTP, dCTP and dGTP, 2.5 µl of 10X PCR buffer (50 mM KCl), 10 mM Tris-HCl (pH 8.3 at 25 °C), and 1.5 µl of MgCl₂ (50 mM).

A total of 32 cycles was performed with the first denaturation at 94 °C for 3 min and the final extension at 72°C for 3 min. The amplified products were analyzed by electrophoresis with a 1% agarose gel followed by ethidium bromide staining and UV-transilluminator visualization.

Determination of specificity and sensitivity of the PCR reaction

To determine specificity, PCR was performed with purified extracted genomes from 11 bacteria, including strains of: *M. luteus*, *S. pyogenes*, *B. cereus*, *B. polymixia*, *S. paratyphi* A, *Y. pseudotuberculosis*, *E. coli*, *P. vulgaris*, *S. aureus* type A, and *S. epidermidis*. Sensitivity of the reaction was based on the numbers of bacteria. In this regard, after

Inoculating bacteria in nutrient broth media, serial dilutions up to 10⁻⁹ (15 cells µl⁻¹) were prepared and PCR reaction was accomplished for all dilutions, followed by colony counting for each.

RESULTS

In this investigation, 150 samples were obtained from 150 healthy carriers, of which 95 samples of *S. aureus* were detected biochemically. DNA extraction and PCR reaction with pre-prepared primers were then performed.

To detect *S. aureus* types *a*, *b*, *c* and *q* enterotoxins:

A total of 95 *S. aureus* strains originating from nasal carriers were tested for enterotoxin production by PCR assay. The specificity of PCR was tested for the positive and negative control strains. The six SE-encoding genes (*sea*, *seb*, *sec*, *sed*, *see* and *seq*) were detected in the positive control strains and not in the negative control strains (Fig 1). In addition, DNA sequences of all PCR products showed complete agreement with the sequences of the corresponding region of each SE-encoding gene (data not shown). Therefore, the six pairs of primers designed in this study were determined to be completely specific for each SE-encoding gene. PCR reaction with specific primers for *nuc*, *sea*, *seb*, *sec*, *sed*, *see* and *seq* genes were performed by which the existence of a 397 bp segment of *nuc* gene amplification led to the detection of *S. aureus* (Fig.1, lane 5). Furthermore, a 271 bp segment was related to the amplification of a specific fragment of gene *sec* that is responsible for enterotoxin type C (Fig.1, lane 4). DNA amplification fragments of 397 bp for the staphylococcal nuclease gene (*nuc*) (Fig.1, lane 3), 552 bp for staphylococcal enterotoxin A gene (*sea*) (Fig.1, lane 5), 477 bp for staphylococcal enterotoxin B gene (*seb*) (Fig.1, lane 2), 271 bp for staphylococcal enterotoxin C gene (*sec*) (Fig.1, lane 4) and 122 bp for staphylococcal enterotoxin Q gene (*seq*) (Fig.1, lanes 5, 6 and 8) were confirmed by digestion enzymes. *S. epidermidis* was used as a negative control and did not yield a PCR product (Fig.1, lane 7).

Specificity and sensitivity of the reaction

When different strains of bacteria, such as *M. luteus* (ATCC=9341), *S. pyogenes* (reference lab), *B. cereus*, *B. polymixie*, *S. parathify* A, *Y. pseudo tuberculosis*, *E. coli*, *P. vulgaris*, *S. aureus* strain type A and *S. epidermidis* were assayed with primers *nuc* (Fig. 2, lanes 2 through 11), only the *S. aureus* (lanes 2 and 10) generated positive PCR results. In order to test the sensitivity of the method, decided dilution series of enterotoxigenic *S. aureus* bacterial cultures (4×10³ to 60 cells µl⁻¹) were proposed. As depicted in Fig.3, PCR reaction was performed for dilutions of 125 cells and more of which the results of PCR products were observed on

1% agarose gel as 552 bp segments (data not shown). There was no band for dilutions of less than 125 cells (data not shown).

Therefore, the results of this experiment showed that under experimental conditions, DNA from 1.25×10^2 CFU/ml of the target cells could be detected (data not shown). In an attempt to determine the incidence of newly identified enterotoxins in SFP outbreaks in Iran, we obtained 150 *S. aureus* strains isolated from nasal carriers by using cotton swabs. Of these, 56 strains were positive for classical enterotoxins, *sea*, *seb*, *sec*, and *seq*.

Results showed that twenty four (25.3%) isolates were associated with the *sea* gene, fifteen (15.8%) isolates were associated with the *seb* gene, nine (9.5%) isolates were associated with the *sec* gene, eight (8.4%) isolates were associated with the *seq* gene and thirty-nine (41%) of these isolates might have possessed other *se* genes but which were not *see* and *sed* (319 genes). Only one of these 95 isolates harbored *sec* and *sea*. The *nuc* gene, which encodes thermonuclease was used as a target DNA to identify *S. aureus*.

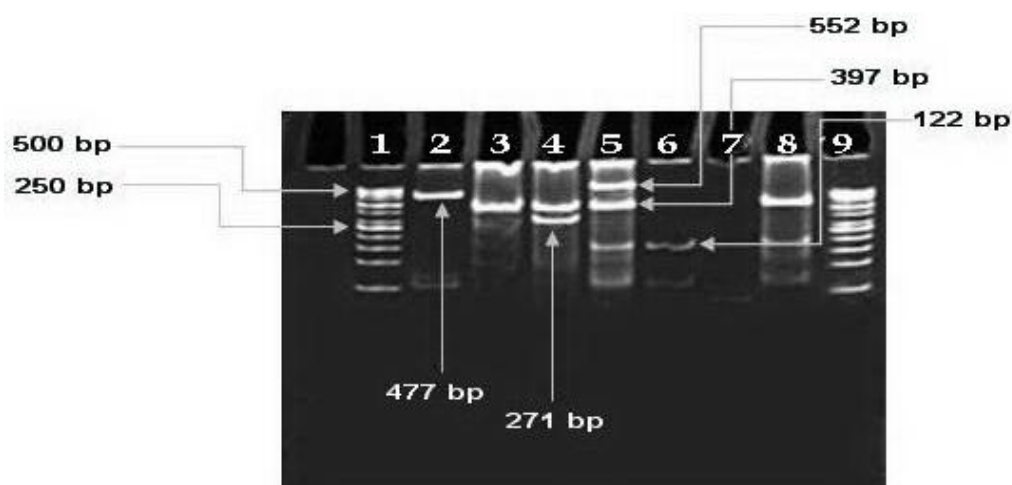


Fig1. Gel analysis of PCR-amplified toxin gene sequences. The individual toxin gene products were characterized by comparing them with a standard molecular size marker. Lanes 1 and 9, DNA ladder; lane 2, SEB (477 bp); lane 3, *nuc* (397 bp); lane 4, SEC (271 bp); lane 5, SEA and SEQ (552 bp and 122 bp); Lane 6, SEQ (122 bp); Lane 7, *S. epidermidis*; Lane 8, SEQ (122 bp).

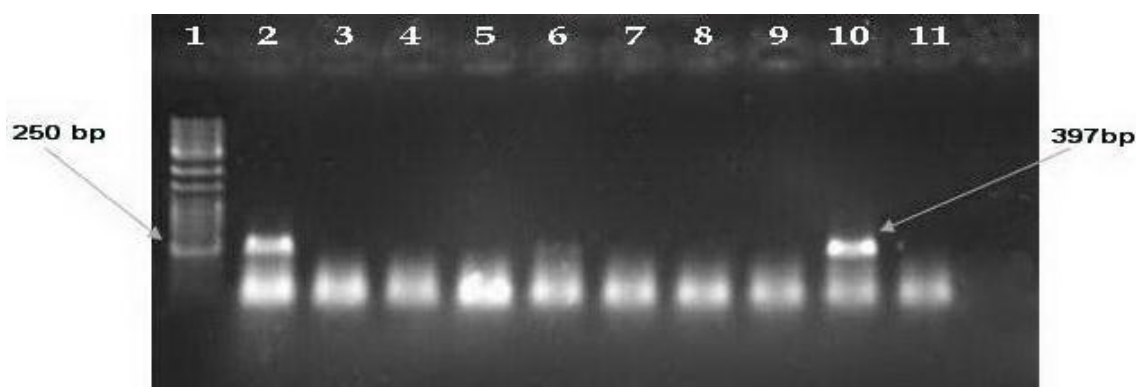


Fig 2. Gel electrophoresis of PCR with *nuc1* and *nuc2* genes in nine species of bacteria.

Lane 1: DNA Wight Marker
 Lane 2: *S. aureus* ATCC=25923
 Lane3: *Bacillus polymixa* ATCC = 10401 NCIB = 8094
 Lane 4: *B. cereus* ATCC = 11778 NCTC=10320
 Lane 5: *M. luteus* ATCC =9341
 Lane 6: *Strep. pyogenes*
 Lane 7: *P. vulgaris*, strain ox19 ATCC=6380
 Lane 8: *S. Typhimurium* NCTC=5702
 Lane 9: *E. coli* O111 Lane 10: *S. aureus*

DISCUSSION

We have described a multiplex PCR-based diagnostic protocol to detect the genes for enterotoxins *a*, *b*, *c*, *d*, *e* and *q* from human strains of *S. aureus*. These tests have variable sensitivities and depend on adequate gene expression for reliability and reproducibility.

Several methods have been used for detection and typing of these genes. It is possible to detect gene products immunologically by using a variety of enzyme-linked immunosorbent assays and radioimmunoassays [7,16,19], but these methods have some disadvantages. For example, misidentification by immunologic methods could easily occur because toxigenic strains of *S. aureus* have low levels of excreted toxin(s) or cross-reactive antigens. Also ELISA kits are commercially available but time-consuming and expensive, in addition to limitations in antigen detection and cross-reactions with other types of enterotoxins [16].

Other methods for gene identification, such as DNA hybridization, have been used to analyze strains for the presence of staphylococcal toxin genes. PCR has an advantage over DNA hybridization in that the sensitivity is sufficient to allow for detection of microbial DNA directly in pathological specimens [20-22]. Also, PCR can be performed by using whole bacterial cells without DNA extraction [7]; coupled with pre enrichment growth before the PCR, it dilutes out DNA not being biologically duplicated and permits the identification of organisms in samples containing numbers of pathogenic bacteria undetectable by other routine methods [23]. During the last decade, many studies extremely high capacity of have shown the and PCR for specifically detecting bacteria authors have already genes of interest. Several shown the feasibility of the PCR methodology for the identification of *S. aureus* strains [24]. On the other hand, different studies have also shown the applicability of PCR to the detection of staphylococcal enterotoxin genes.

Reported results by Holechava et al. indicate that there is no difference between RIA, PCR and dot-blot in detection of *S. aureus* strains [25], although dot-blot hybridization and RIA are more expensive than PCR. PCR identification of *S. aureus* has been based on

the detection of different specific target sequences such as *nuc*. The *nuc* gene was used as a target DNA to identify *S. aureus* [19,21, 26].

In this study, multiplex PCR was performed on distinct healthy carriers of *S. aureus* and typing of bacterium. Studies have focused on mostly *S. aureus* type A rather than the other types, including type C. In Iran, there is mostly an incidence of type A gastroenteritis [12]. A 2002 study in Slovakia by Beate Holeckara isolated 43 *S. aureus* strains from various food samples (sausage and noodle soup), of which fifteen strains (34.88%) were proven to be enterotoxigenic by multiple PCR. Among these seven strains (16.28%) possessed the enterotoxin type A gene [25]. The *see* genotype was classified into 12 genotypes. Letertre et al. (2003) has shown that eighteen strains of *S. aureus* possessed only one kind of *se* gene (*sea*, *sec* or *seh*), the remaining 39 isolates harboured more than one *se* gene. The *sea* gene was found in 58% (33 out of 57) isolates; eleven strains had *sea* alone and twenty-two had *sea* together with other *se* genes [24]. Coexistence of *sea*, *seb*, and *sec*, *sed* with *seg*, *seh* and *sei* has been reported by other laboratories [7] in this study, among the 150 healthy human isolates from nasal carriers, 95 isolates were confirmed as *S. aureus*. Only 58.9% of the isolates were diagnosed as *sea*, *b*, *c*, and *q* positive. The variation in reported rates results, at least partly, from differences in study populations, sampling and culture techniques, and criteria for the definition of persistent or intermittent carriers. [27]. Nasal Carrier of *S. aureus* have been identified as risk factors for community-acquired and nosocomial infections. Cole et al. in 2001 screened 230 donors of diverse ethnic and socioeconomic backgrounds and identified 62 (27%) whose nasal secretions were colonized by *S. aureus* [28]. The anterior nares have proven to be the primary reservoir of *S. aureus* in humans [29]. *S. aureus* nasal Carrier has been extensively studied in patients and healthy individuals [28]. Different studies shown that carrier patterns differ between individuals, and that 10 to 35% of individuals carry *S. aureus* persistently, 20 to 75% carry *S. aureus* intermittently, and 5 to 70% are persistently free of *S. aureus* (noncarriers)

[27,29]. In conclusion, this study offered novel PCR primers specific for the detection of *sea*, *seb*, *sec*, and *seq* genes of *S. aureus*. These primers could be used for an epidemiological study of the hazardous *S. aureus* in food-poisoning outbreaks. The identification of staphylococcal toxin genes in strains of *S. aureus* by PCR offers a very specific, sensitive, relatively rapid, and inexpensive alternative to traditional immunological assays which depend on adequate gene expression for reliability and sensitivity.

CONCLUSION

PCR Detection for encoded toxic genes in *Staphylococcus aureus* is simple, low cost, rapid and Very specific; in addition can identify several genes that encode toxin in the same time.

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