Short Paper

Association between the enterotoxin production and presence of *Coa*, *Nuc* genes among *Staphylococcus aureus* isolated from various sources, in Shiraz

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Summary

The present study was aimed to identify the frequency of coagulase (*Coa*) and thermonuclease (*Nuc*) genes and Staphylococcal enterotoxin A (Sea) production among *Staphylococcus aureus* isolated from various sources in Shiraz. Moreover, the correlation between the *Sea* gene and coagulase and thermonuclease enzymes is also considered. A total of 100 *S. aureus* were isolated from various sources including 40 humans, 30 animals and 30 food samples by the routine biochemical tests. The frequency of *Coa*, *Nuc* and *Sea* genes was evaluated by PCR assay. Correlation among those genes was finally evaluated by statistical analysis. The PCR results showed that the prevalence of *Coa*, *Nuc* and *Sea* genes was 91%, 100% and 14%, respectively. The evaluation of the enterotoxin production indicated that 78.6% of the *Sea* gene was expressed. The presence of enterotoxin A was not necessarily correlated to the production of toxin. As a final conclusion to detect the enterotoxigenic strains, both genotypic and phenotypic methods are highly recommended.

Key words: Coagulase, Enterotoxin, *Staphylococcus aureus*, Thermonuclease

Introduction

The major characteristics of staphylococcal enterotoxins (SEs) are low molecular weight, resistance to heat, pepsin digestion and superantigenicity (Bergdoll *et al.*, 1979; Klotz *et al.*, 2003) Symptoms of SEs include increased saliva, vomiting, abdominal cramping and diarrhea which can be accompanied by blood in some cases (Gómez *et al.*, 2007). Approximately 5% of food poisoning illnesses are estimated to occur by staphylococcal enterotoxins (Vimercati *et al.*, 2006).

Various types of *Staphylococcus aureus* enterotoxins including SEA, SEB, SEC1, SEC2, SEC3, SED and SEE are currently recognized (Morandi *et al.*, 2007). Most strains produce one or more enterotoxins (Pourmand *et al.*, 2010). Of the numerous physiochemical characteristics of *S. aureus* used for its classification, production of coagulase and thermonuclease is the most reliable practical criterion used in identifying the bacteria (Brakstad *et al.*, 1992).

Enterotoxin A is the most common type among food-related strains of *Staphylococcus* (Barati *et al.*, 2006). Several studies have shown that 15% to 80% of *S. aureus* isolates from various sources were able to produce enterotoxin (Omoe *et al.*, 2005; Bania *et al.*, 2006). The present study was aimed to investigate the correlation between enterotoxin A gene, producing enterotoxin A protein and coagulase and thermonuclease enzymes in *S. aureus* isolated from various sources in Shiraz.

Materials and Methods

Samples were collected from different sources including human pus, mastitic milks, and foodstuffs in order to identify *S. aureus* using the bacteriological standard method. Briefly, each sample was cultured on Baird Parker agar (Merck, Germany) and incubated at 35°C for 36 h. The suspected black colonies were further analyzed using Gram stain and biochemical tests (Quinn *et al.*, 2002).

Coagulase and thermonuclease tests were performed, as was previously described. The presence of thermonuclease was confirmed based on the formation of a clear zone around the colonies (Baron and Finegold, 2013).

DNA extraction was performed using Cinnapure DNA kit (Cinnagen, Iran) based on manufacturer's instruction.

PCR was performed with a final volume of 25 µL containing final concentrations of 50 mM KCl, 10 mM Tris-HCl (pH = 8.3), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 25 pmol of each primer (Brakstad *et al.*, 1992;

Mehrotra *et al.*, 2005; Ahmadi *et al.*, 2010) and 1 U of Taq DNA polymerase (Vivantis, Malaysia). The amplified products were electrophoresed through a 1.5% agarose gel and visualized by staining using ethidium bromide.

Improving technique for gel electrophoresis of unknown protein using bacteriophage T_4 was firstly established by Laemmli (1970) in which, four major protein components of the head of the bacteriophage was firstly recognized. The method was then extensively used worldwide.

Correlation between the presence of enterotoxin A gene and the production of coagulase and thermonuclease enzymes, was investigated using a Spearman's rho correlation and Chi-square analysis. The statistical differences were considered significant when the p-values were equal and/or less than 0.05.

Results

One hundred *S. aureus* strains were isolated from human pus (n=40), mastitic milk (n=30) and foodstuffs (n=30) andconfirmed by biochemical test. All the strains were positive for the thermonuclease test. For the coagulase test, 54% of them presented a strong reaction (+3), while 46% were considered +1 or +2 (Table 1).

The frequency of *Coa* gene in the isolated strains of human, animals and foodstuffs was 90%, 93.3% and 90%, respectively (Fig. 1). The PCR assay confirmed the *Nuc* gene in all isolates (Fig. 2), whereas the *Coa* gene was detected in 91 strains which were phenotypically considered as 1+ (Table 1).

The prevalence of the enterotoxin A gene was 14% (human samples: 22.5%, food samples: 16.6%), but it was not found in any of the animal isolates. Moreover, the results showed that the occurrence of the enterotoxin A in animal samples was significantly lower than that in the human and food isolates (P<0.05) (Fig. 3, Table 1).

The expression of *Sea* using SDS-PAGE analysis gene was confirmed in 11 strains (Fig. 4). As such, 78.6% of enterotoxin A genes were expressed.

Statistical correlation of the genes

No correlation was shown among the presence of enterotoxin A gene and *Coa* gene (P=0.21) and production of the coagulase (P=0.17) (Table 2).

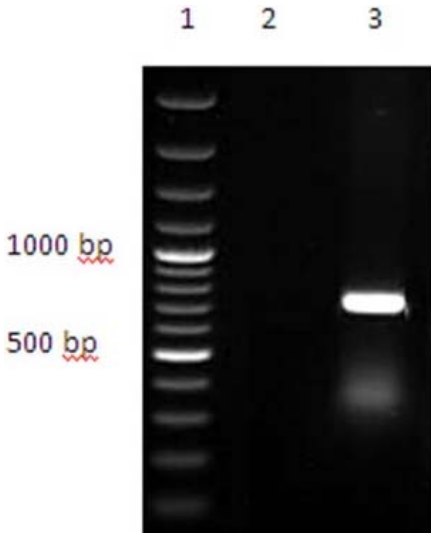

Fig. 1: Representation of PCR product of *Coa* gene. Lane 1: Marker 1000 bp, Lane 2: Negative control (no template), and Lane 3: *Coa* gene 720 bp

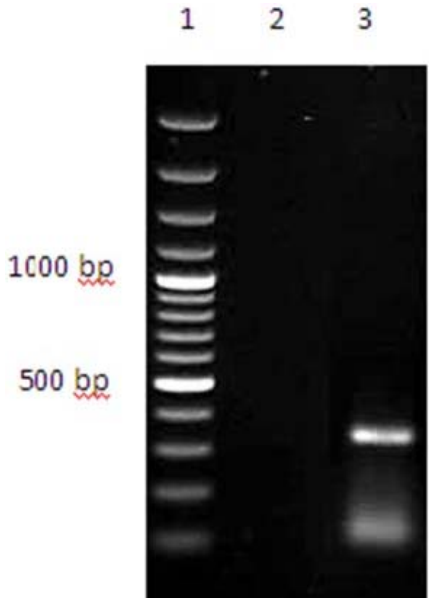

Fig. 2: Representation of PCR product of *Nuc* gene. Lane 1: Marker 1000 bp, Lane 2: Negative control (no template), and Lane 3: *Nuc* gene 397 bp

Table 1: Frequency of *Coa*, *Nuc* and *Sea* gene in *S. aureus*

Source	Number	Coagulase +1	Coagulase +2	Coagulase +3	*Coa* gene	Nuclease test	*Nuc* gene	*Sea* gene	Expression of *Sea* gene
Human	40	6	11	23	36 (90%)	40	40 (100%)	9 (22.5%)	7/26
Animal	30	4	11	15	28 (93.3%)	30	30 (100%)	0 (0%)	0/13
Food	30	5	9	16	27 (90%)	30	30 (100%)	5 (16.6%)	4/16
Total	100	15	31	54	91 (91%)	100	100 (100%)	14 (14%)	11/54

Table 2: Correlation between various tests and presence of *Coa* and *Sea* genes

Specific test	Source	*Coa* gene	*Sea* gene
Coagulase test	P=0.88, R=-0.015	P<0.001, R=0.453	P=0.09, R=0.169
Coa gene	P=0.95, R=-0.006	–	P=0.21, R=0.127
Sea gene	P=0.32, R=-0.101	–	–
Sea gene expression	P=0.65, R=-0.064	–	P<0.001, R=0.85

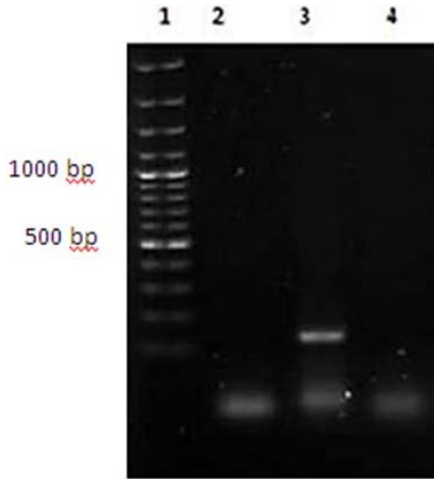

Fig. 3: Representation of PCR product of *Sea* gene. Lane 1: Marker 1000 bp, Lanes 2, 4: Negative control (no template), and Lane 3: *Sea* gene 102 bp

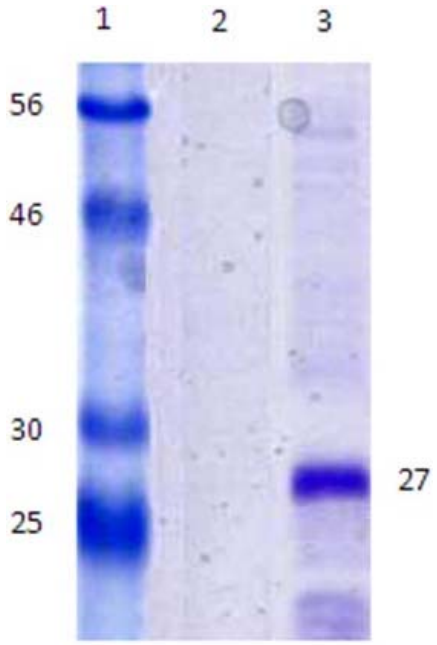

Fig. 4: SDS PAGE analysis of enterotoxin on a 10% polyacrylamide gel. Lane 1: Marker (KDa), Lane 2: Negative control, and Lane 3: SEA protein

Discussion

The present study revealed a prevalence of 22.5% enterotoxin A gene among the human isolates. Previous studies using different techniques showed the prevalence of 15%, 12% and 46.9% of *Sea* gene in Jordan, Germany and Iran, respectively (Klotz *et al*., 2003; Naffa *et al*., 2006; Pourmand *et al*., 2010).

Former studies have shown variability in the enterotoxin gene prevalence in *S. aureus* isolated from mastitic milk (Ahmadi *et al*., 2010). In Italy, Vimercati *et al*. (2006) reported more than 70% enterotoxin prevalence in *S. aureus* isolates recovered from cows with mastitis (Vimercati *et al*., 2006). 85 of the isolates (73%) harbored at least one enterotoxin gene (*Se*) with a predominance of *Sea*, *Sed* and *Sej* among isolates from bovine. In the current study, none of our isolates showed the occurrence of *Sea* gene. Our results were supported by the work performed by Gomez *et al*. (2007), in which none of *Sea*, *Seb* and *Sec* was identified from the *S. aureus* isolates.

In this study, prevalence of *Sea* among 30 food samples was 16.7%. Many studies have been carried out on the detection of enterotoxin gene in food. Asao *et al*. (2003) reported a food poisoning outbreak resulting from the consumption of milk and yogurt made from milk contaminated with enterotoxin A in Japan. The prevalence of 15.6% of *Sea* gene among *S. aureus* isolated from dairy products was reported in Tehran (Imanifooladi *et al*., 2010). According to the study of Ertas *et al*. (2010), the highest frequency of *Sea* gene among different foods was demonstrated. Attention to the hygiene condition during production and use of heat process is critical. As shown, PCR method was able to identify potential strains to produce enterotoxin (Najera-Sanchez *et al*., 2003). Gene expression assessment in isolated strains using SDS-PAGE showed 11 out of 14 strains contained enterotoxin A gene. The difference is possibly associated with the lack of expression of some genes. Furthermore, some strains produce lower level of enterotoxin below the sensitivity of phenotypic method. No significant correlation was observed between presence of enterotoxin A gene and production of coagulase and thermonuclease enzymes. In the study conducted on some clinical isolates, no significant correlation was shown among the enterotoxigenic strains and coagulase gene (Demir *et al*., 2011). Similar results were obtained by Rojasandda-Cunha Mde L. (Da *et al*., 2007; Suarez *et al*., 2008; Rojas *et al*., 2012). Although the coagulase and nuclease tests are valuable to identify *S. aureus*, the tests do not necessarily imply enterotoxigenic strains.

It could be concluded from the study, the sensitivity and reliability of the PCR assay detects the staphylococcal enterotoxin. Additionally, the presence of the genes was associated with the enterotoxigenic bacteria and therefore, to detect the enterotoxigenic types of *S. aureus*, genotypic methods are highly recommended. Also, no correlation was shown between the production of enterotoxin, and presence of coagulase and thermonuclease genes.

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References

Ahmadi, M; Razavi Rohani, SM and Ayremlou, N (2010). Detection of *Staphylococcus aureus* in milk by PCR. Comp. Clin. Pathol., 19: 91-94.

Asao, T; Kumeda, Y; Kawai, T; Shibata, T; Oda, H; Haruki, K; Nakazawa, H and Kozaki, S (2003). An extensive outbreak of staphylococcal food poisoning due to

low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiol. Infect., 130: 33-40.

Bania, J; Dabrowska, A; Korzekwa, K; Zarczynska, A; Bystron, J; Chrzanowska, J and Molenda, J (2006). The profiles of enterotoxin genes in *Staphylococcus aureus* from nasal carriers. Lett. Appl. Microbiol., 42: 315-320.

Barati, B; Saadati, M and Bahmani, MK (2006). Isolation and detection of enterotoxigenic *Staphylococcus aureus* type A by multiplex PCR. J. Mil. Med., 8: 119-128.

Baron, E and Finegold, S (2013). *Bailey and scotts diagnostic microbiology*. 8th Edn., ST Louis, The CV Mosby Company. PP: 111, 325.

Bergdoll, MS; Riemann, H and Bryan, FL (1979). *Staphylococcal intoxications in foodborn infections and intoxications*. 4th Edn., San Diego, Academic Press Inc., PP: 443-493.

Brakstad, OG; Aasbakk, K and Maeland, JA (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J. Clin. Microbiol., 3: 1654-1660.

Da Cunha Mde, L; Calsolari, RA and Júnior, JP (2007). Detection of enterotoxin and toxic shock syndrome toxin 1 genes in *Staphylococcus*, with emphasis on coagulase-negative staphylococci. Microbiol. Immunol., 51: 381-390.

Demir, C; Aslantas, O; Duran, N; Ocak, S and Ozer, B (2011). Investigation of toxin genes in *Staphylococcus aureus* strains isolated in Mustafa Kemal University Hospital. Turk. J. Med. Sci., 41: 343-352.

Ertas, N; Gonulalan, Z; Yildirim, Y and Kum, E (2010). Detection of *Staphylococcus aureus* enterotoxins in sheep cheese and dairy desserts by multiplex PCR technique. Int. J. Food. Microbiol., 142: 74-77.

Gómez, C; Pinal, L and Franco, J (2007). Identification of *Staphylococcus aureus* strains negative for enterotoxins A, B and C isolated from bovine mastitis in México. Vet. Immunol. Immunopathol., 117: 249-253.

Imanifooladi, AA; Tavakoli, HR and Naderi, A (2010). Detection of enterotoxigenic *Staphylococcus aureus* isolates in domestic dairy products. Iran. J. Microbiol., 2: 137-142.

Klotz, M; Opper, S; Heeg, K and Zimmermann, S (2003). Detection of *Staphylococcus aureus* enterotoxin A to D by real-time fluorescence PCR assay. J. Clin. Microbiol., 41: 4683-4687.

Laemmli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Mehrotra, M; Wang, G and Johnson, WM (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. J. Clin. Microbiol., 38: 1032-1035.

Morandi, S; Brasca, M; Lodi, R; Cremonesi, P and Castiglioni, B (2007). Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. Vet. Microbiol., 124: 66-72.

Naffa, RG; Bdour, SM; Migdadi, HM and Shehabi, AA (2006). Enterotoxicity and genetic variation among *Staphylococcus aureus* isolates in Jordan. J. Med. Microbiol., 55: 183-187.

Najera-Sanchez, G; Maldonado-Rodriguez, R; Olvera, RR and de la Garza, LM (2003). Development of two multiplex polymerase chain reactions for the detection of enterotoxigenic strains of *Staphylococcus aureus* isolated from foods. J. Food. Prot., 66: 1055-1062.

Omoe, K; Hu, DL; Takahashi-Omoe, H; Nakane, A and Shinagawa, K (2005). Comprehensive analysis of classical and newly described staphylococcal super-antigenictoxin genes in *Staphylococcus aureus* isolates. FEMS Microbiol. Lett., 246: 191-198.

Pourmand, MR; Memariani, M; Hoseini, M and Bagherzadeh Yazdchi, S (2010). High prevalence of SEA gene among clinical isolates of *Staphylococcus aureus* in Tehran. Acta Medica. Iranica., 5: 357-361.

Quinn, PJ; Markey, BK; Bryan, K; Leonard, FC; Carter, ME and Donnelly, WJ (2002). *Veterinary microbiology and microbial disease*. 1st Edn., Chichester, West Sussex, UK, Wiley-Blackwell. PP: 44-45.

Rojas, MB; Antonelli, CM; Pereira Franchi, EPL and da Cunha, MLRS (2012). Detection of enterotoxin A in coagulase-negative staohylococci isolated from nutrition students. Arch. Clin. Microbiol., 3:6. doi: org/10.3823/262.

Suarez, MJ; Arias, ML and Del Mar Gamboa, M (2008). *Staphylococcus aureus* enterotoxin A detection using the polymerase chain reaction (PCR) and its correlation with coagulase and thermonuclease tests. Arch. Latinoam. Nutr., 58: 59-63.

Vimercati, C; Cremonesi, P; Castiglioni, B; Pisoni, G; Boettcher, PJ; Stella, A; Vicenzoni, G and Moroni, P (2006). Molecular typing of *Staphylococcus aureus* isolated from cows, goats and sheep with intramammary infections on the basis of gene polymorphisms and toxins genes. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 53: 423-428.