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Araştırma Makalesi
Research Article

Enterotoxin Gene Profiles of *Staphylococcus aureus* and Other Staphylococcal Isolates from Various Foods and Food Ingredients*

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Summary: This study was aimed to identify the toxin gene profiles of 107 staphylococcal isolates from a variety of foods. Firstly, 102 CPS were analyzed by PCR and 70 (68.6%) of them were identified as *S. aureus*. Then, all isolates (70 *S. aureus*, 32 CPS and 5 CNS) were investigated for the presence of SE genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) by using multiplex PCR. Among 70 *S. aureus*, only 2 (2.9%) which were isolated form poultry meat shown to be positive for *sea* gene. None of the other isolates harboured any of the SE genes ascertained. Though these results of toxin gene positivity are too low, enterotoxin genes of enterotoxigenic staphylococci constitute a potential risk for consumers' health.

Key Words: Enterotoxin genes, food, *Staphylococcus aureus*

Çeşitli Gıda ve Gıda Katkılarından İzole Edilen *Staphylococcus aureus* ve Diğer Stafilokokların Enterotoksin Gen Profilleri

Özet: Bu çalışmada, çeşitli gıdalardan izole edilen 107 stafilokok suşunun toksin gen profillerinin belirlenmesi amaçlanmıştır. Bu kapsamda, 102 koagulaz pozitif stafilokok izolatının 70'i (%68.6) PCR ile *S. aureus* olarak identifiye edilmiştir. Daha sonra, tüm izolatlar (70 *S. aureus*, 32 koagulaz pozitif ve 5 koagulaz negative stafilokok) multipleks PCR ile SE genlerinin (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) varlığı yönünden incelenmiştir. *S. aureus* (70) izolatları arasından sadece 2 (%2.9) kanatlı eti izolatının *sea* genine sahip olduğu belirlenmiştir. Diğer izolatların hiçbirinden enterotoksin genleri tespit edilmemiştir. Stafilokok izolatlarında toksin geni prevalansı her ne kadar düşük bulunmuş olsa da, gıdalarda enterotoksin oluşturan stafilokoklar tüketici sağlığı yönünden potansiyel bir risk oluşturmaktadır.

Introduction

Staphylococcal intoxication, which is due to the consumption of food containing one or more preformed staphylococcal enterotoxins (SE), is one of the leading food-borne diseases worldwide (3). Contamination of food with enterotoxigenic staphylococci can occur, directly from infected food-producing animal or at any stage of food production, processing, transportation, storage or retail as a result of poor personal hygiene that leads colonized individuals (30-50 %) to provide the main source for dissemination of staphylococci (21). Staphylococcal food poisoning (SFP) is a self limiting gastroenteritis that develops with a main symptom of vomiting, which can be accompanied by nausea, abdominal pain and/or diarrhea. Even the mortality rates due to SFP are low, they can be elevated for populations such as; children, elderly or immunocompromised (2).

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Coagulase positive staphylococci (CPS) and especially *S. aureus* are known as the primary causes of SFPs (10, 12). Therefore, the coagulase negative staphylococci (CNS) are not detected by standard microbiological methods (18). However, it was shown in previous studies that some strains of both CPS and CNS could produce enterotoxins and might involve in staphylococcal food poisonings (5, 10, 24, 25).

Nine major antigenic types of SE's have been reported comprising the five classical (SEA, SEB, SEC, SED and SEE) and four newly described (SEG, SEH, SEI and SEJ) (23). Genes encoding SEs have different genetic supports, most of which are mobile genetic elements such as; phages (*sea*, *see*), transposons (*seb*), plasmids (*seb*, *sed* and *sej*), pathogenity islands (*seb*, *sec*) or chromosomal genes (*seb*, *sec*, *seg*, *seh* and *sei*) (2, 3, 10) which enables horizontal spread within staphylococci populations (4).

The SE production by staphylococcal strains has been studied in Turkey (7, 13, 14, 15, 20). However, there is a lack of information on foodborne staphylococcal SE genes and there are a few molecular studies on this subject in Turkey. The objectives of this study were to identify the *S.*

aureus by PCR analysis and to find out the toxin gene profiles of *S. aureus* and other staphylococcal isolates that were previously isolated from a variety of foods and food ingredients.

Material and Method

Bacterial strains

There was a total of 102 CPS and 5 CNS that were previously isolated from various kinds of foods. The number of the CPS and CNS isolates related to the food samples were as follows; 33 from beef (32 CPS, 1 CNS), 22 from poultry meat (20 CPS: 9 from chicken and 11 from turkey meat, 2 CNS from chicken), 24 from dairy products (23 CPS, 1 CNS), 19 from ready to eat foods (18 CPS, 1 CNS), and 9 CPS from some food ingredients. All of the isolates were kindly provided by Çağatay Çelik (Headquarters of 2nd Armoured Brigade of Turkish General Staff, Maltepe, Istanbul, Turkey) that was not published previously .

Reference strains of *S. aureus* D4508 (*sea*, *seh*), RIMD31092 (*seb*, *sec, seg, sei*), NCTC9393 (*sed*, *sej, seg, sei*), FRI326 (ATCC 27664) (*see*) and A900322 (*seg, sei*) were kindly and generously provided by G. Blaiotta, Dipartimento di Scienza degli Alimenti, Sezione di Microbiologia Agraria, Alimentare, Ambientale e di Igiene, Stazione di Microbiologia Industriale, Universita degli Studi di Napoli "Federico II", Via Universita, Portici, Italy.

Identification of *S. aureus* by PCR analysis

In the study, for the identification of *S. aureus*, 102 CPS were analyzed by PCR assay. For this purpose 16S rRNA specific primers (Integrated DNA Technologies- IDT, Leuven, Belgium) were used (17, 19) (Table 1).

Table 1. PCR Primers and expected product sizes for the identification of *S. aureus* and detection of enterotoxin genes.

Gene	Oligonucleotide sequence (5'-3')	Product size	Reference
sea	F- GCA GGG AAC AGC TTT AGG C	521 bp	(19)
	R- GTT CTG TAG AAG TAT GAA ACA CG		
seb	F- ACA TGT AAT TTT GAT ATT CGC ACT G	667 bp	(17)
	R- TGC AGG CAT CAT GTC ATA CCA		
sec	F- CTT GTA TGT ATG GAG GAA TAA CAA	284 bp	(19)
	R- TGC AGG CAT CAT ATC ATA CCA		
sed	F- GTG GTG AAA TAG ATA GGA CTG C	385 bp	(19)
	R- ATA TGA AGG TGC TCT GTG G		
see	F- TAC CAA TTA ACT TGT GGA TAG AC	171 bp	(19)
	R- CTC TTT GCA CCT TAC CGC		
seg	F- CGT CTC CAC CTG TTG AAG G	328 bp	(19)
	R- CCA AGT GAT TGT CTA TTG TCG		
seh	F- CAA CTG CTG ATT TAG CTC AG	359 bp	(19)
	R- GTC GAA TGA GTA ATC TCT AGG		
sei	F- CAA CTC GAA TTT TCA ACA GGT ACC	466 bp	(17, 19)
	R- CAG GCA GTC CAT CTC CTG		
sej	F- CAT CAG AAC TGT TGT TCC GCT AG	142 bp	(19)
	R- CTG AAT TTT ACC ATC AAA GGT AC		
16S	F- GTA GGT GGC AAG CGT TAT CC	228 bp	(19)
rRNA	R- CGC ACA TCA GCG TCA G		

One ml overnight culture at 37°C in 5ml brain-heart infusion (BHI, Oxoid CM0225B, Hampshire, UK) of each strain was transferred into an eppendorf tube and centrifuged (Beckman Coulter Microfuge 22R, Fullerton, USA) at 12000*g* for 3 min. After the removal of the supernatant, pellet was suspended with 1 ml of sterile ultra pure water and mixed thoroughly. The washing step repeated. Two 2 µl of proteinase K [70 mg/ml (AppliChem GmbH, Darmstadt, Germany)] and 200µl of Chelex 100 [5 % (Bio-Rad, Hercules, CA, USA)] were added on the remaining pellet and mixed. Mixtures were floated in a water bath (Memmert, Schwabagh, Germany) at 95°C for 10 min after the former incubation at 55°C for 40 min. Consequently, centrifugation with the same parameters was done and 10 µl of the supernatant used as the template DNA.

PCR was performed in a total volume of 50 µl containing the following: 5 µl of 10xPCR buffer (Bioron GmbH, Ludwigshafen, Germany), 3 µl of 25mM $MgCl_2$ (Bioron), 2 µl of 10 mM dNTP mixture (Bioron), 0,5 µl of each primer which contains 100 pmol/µl, 0,5 µl of 5 U Taq DNA polymerase (Bioron). The volume of the master mix was adjusted to 40 µl with sterile ultra pure water and 10 µl of template DNA was added. DNA amplification was carried out in a thermocycler (Eppendorf mastercycler gradient, Hamburg, Germany) as described previously (19). After an incubation at 95°C for 10 min, continued as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, extension at 72° C for 1 min, and finally a terminating incubation at 72°C for 10 min.

A 10 µl aliquot of each resultant PCR product was further analyzed by agarose gel (1.5 % Agarose-Basica LE, Prona, Spain) electrophoresis (CSL MSMixi-Duo, Corston, UK), stained with 0.1 µg ml^{-1} ethidium bromide (BioChemica GmbH, Darmstadt, Germany), at 100 V for 1 h and visualized by a gel documentation and analysis system (Sygene Ingenius, Cambridge, UK).

Multiplex PCR assay for the detection of staphylococcal enterotoxin genes

The nine oligonucleotide primer pairs (Integrated DNA Technologies- IDT, Leuven, Belgium) used to amplify the genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej* and the expected amplicon sizes are listed in Table 1 (17, 19). For DNA extraction, method described above was used.

Two sets of primer mixtures were used. Mixture A consisted of the primer pairs; *sea, seb*, *sec*, *seh* and *sej*, while the mixture B contained primer pairs; *sed*, *see*, *seg* and *sei*. Multiplex PCR was performed in a total volume of 50 µl containing the following: 5 µl of 10xPCR buffer (Bioron GmbH, Ludwigshafen, Germany), 8 µl of 25mM $MgCl_2$ (Bioron), 3 µl of 10 mM dNTP mixture (Bioron), 0,5 µl of each primer which contains 100 pmol/µl, 0,5 µl of 5 U Taq DNA polymerase (Bioron) and 10 µl of template DNA. The volume of the master mix was adjusted to 40 µl with sterile ultra pure water. Reference strains were included as positive controls on every reaction depending on the mixture's primer contents. DNA amplification was carried out in a thermocycler (Eppendorf mastercycler gradient, Hamburg, Germany) as described previously (19) with a slight decrease in the annealing temperature. After an incubation at 95°C for 10 min, continued as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, extension at 72°C for 1 min, and finally a terminating incubation at 72°C for 10 min. Electrophoresis of the resultant PCR products were performed as described above.

Results

In the present study, a total of 102 CPS were analyzed by PCR and 70 (68.6%) of them were identified as *S. aureus*. According to the analysis, 65.6% of the beef isolates (21/32), 55.0% of the poultry meat isolates (11/20; 5/9 for chicken meat, 6/11 for turkey meat), 73.9% of the dairy products (17/23), 77.7% of the ready to eat foods (14/18), and 77.7% of the food ingredients (7/9) were identified as *S. aureus.*

Then, detection of enterotoxin gene profiles of 70 *S. aureus*, 32 CPS and 5 CNS was performed with multiplex PCR assay. Although in every multiplex PCR reaction, reference positive control strains were shown to be positive for the targeted genes, only 2 (2.9 %) of the tested 70 *S. aureus* strains belonging to turkey meat were shown to be positive for *sea* gene (Fig. 1). None of the CPS or CNS isolates harboured any of the SE genes (*sea*-*see*, *seg*-*sej*).

Discussion and Conclusion

It is widely accepted that SE production is characteristic of CPS, and most studies have dealt with *S. aureus* (8). Geniogeorgis (9) concluded that there is no prevailing type of SEs, apart from the strains isolated from foods involved in staphylococcal gastroenteritis, where SEA is the main type of SE recovered. In an epidemiological analysis, it has been reported that a total of 100 to

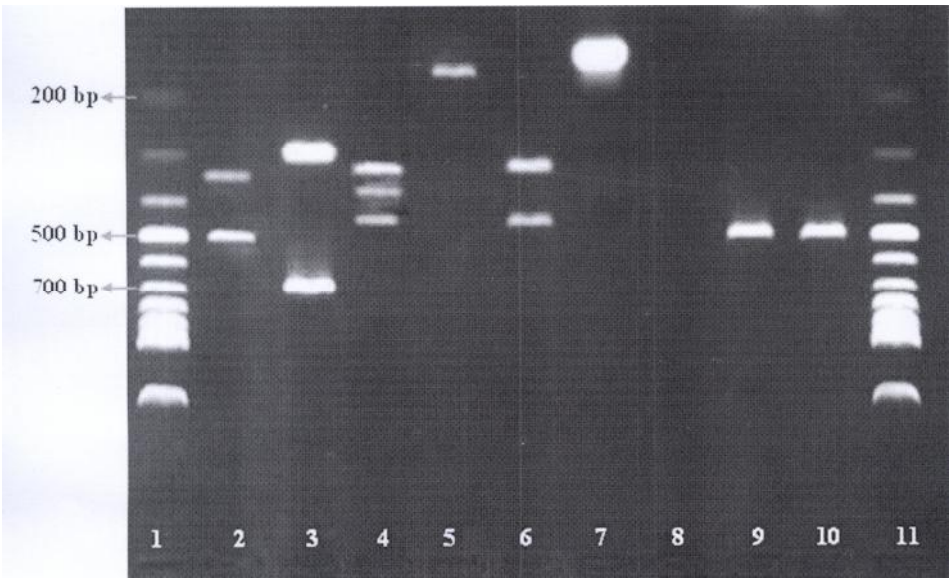

Figure 1. Detection of staphylococcal enterotoxin genes by multiplex PCR. [**1 and 11:** 100 bp DNA marker, **2 :** *S. aureus* D4508 (*sea*-521 bp and *seh*-359 bp), **3:** *S. aureus* RIMD31092 (*seb*-667 bp and *sec*-284 bp), **4:** *S. aureus* NCTC 9393 (*sed*-385 bp, *seg*-328 bp and *sei*-466 bp), **5:** *S.aureus* ATCC 27664 (*see*-171 bp), **6:** *S. aureus* A900322 (*seg*-328 bp and *sei*-466), **7:** *S. aureus* NCTC 9393 (*sej*-142 bp), **8:** Negative control, **9 and 10:** *sea* gene positive *S. aureus* isolates]

200 ng of SEA in food was sufficient to cause food poisoning (8). Previous data also indicate that SEA is mostly involved in outbreaks of staphylococcal food poisoning (16). In addition to *sea*, *sec* and *sed* genes, the occurrence of *seg*, *seh*, *sei* and *sej* genes in food-borne *S. aureus* strains was evaluated by some researchers (1, 23). Rosec and Gigaud (23) determined that the frequency of the strains harbouring *seg*, *seh*, *sei* and *sej* genes was very high (57%) and greater than that of the strains harbouring "classical" SE genes (*sea*, *seb*, *sec*, *sed*, and *see*). Akineden et al. (1) found that 75 of 103 *S. aureus* isolates had one or more toxin genes which were *sea*, *sec*, *sed*, *seg*, *sei* and *sej*. Omoe et al. (22) determined all of the 146 *S. aureus* isolates harboured SE genes and 36 of them (11.7%) had *sea* genes. Also, they reported that the most commonly detected SE genotypes were *seg* and *sei* (38.9%). However, Lim et al. (16) determined 37 of 166 *S. aureus* isolates harboured *sea*, seb and *sec* genes while *sea* (32 of 37 the SE positive isolates) was the most frequent SE gene. While the results of the present study confirms previous reports of high isolation rates of foodborne *S. aureus* (68.6%), the low incidence of SE gene positive isolates (2 of the 70 *S. aureus* isolates) of the present results is in disagreement with the results of some researchers (1, 22, 23).

For *S. aureus*, a large spectrum of different exotoxins which are responsible for toxin-mediated diseases has been described but only a little and conflicting data is available regarding the toxin production in all other staphylococcal species (5). The results of the previous studies indicate that the occurrence of SE genes in CNS is rare (6, 11). Our results about CNS are in agreement with some of the studies carried out on CNS (6, 11, 23). Valle et al. (26) isolated high percentages of enterotoxigenic CPS from milk. On the contrary, our results showed that none of the CPS other than *S. aureus* isolates harboured SE genes.

The results concluded that the incidence of *S. aureus* is high while the incidence of SE genes (*sea-see*, *seg-sej*) among foodborne *S. aureus* in Turkey is very low. Though the toxin gene positivity is too low, enterotoxin genes in enterotoxigenic staphylococci continue to constitute a potential risk for consumers' health. Therefore, not only enterotoxin production, but also detection of the enterotoxin genes must be taken into account for all food-borne staphylococcal isolates.

Referances

1. Akineden Ö, Annemüller C, Hassan AA, Lammler C, Wolter W, Zschöck M, 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. *Clin Diagn Lab Immunol*, 8: 959-964.
2. Alouf JE, Müller-Alouf H, 2003. Staphylococcal and streptococcal superantigens: Molecular, biological and clinical aspects. *Int J Med Microbiol*, 292: 429–440.
3. Balaban N, Rasooly A, 2000. Staphylococcal enterotoxins. *Int J Food Microbiol*, 61: 1–10.
4. Bania J, Dabrowska A, Korzekwa K, Zarczynska A, Bystron J, Chrzanowska J, Molenda J 2006. The profiles of enterotoxin genes in *Staphylococcus aureus* from nasal carriers. *Lett Appl Microbiol*, 42: 315-320.
5. Becker K, Haverkämper G, von Eiff C, Roth R, Peters G, 2001. Survey of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene in non-*Staphylococcus aureus* species. *Eur J Clin Microbiol Infect Dis*, 20: 407-409.
6. Blaiotta G, Ercolini D, Pennacchia C, Fusco V, Casaburi A, Pepe O, Villani F, 2004. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of *se*G and *se*I in *S. aureus* AB-8802. *J Appl Microbiol*, 97: 719-730.
7. Erol I, Usca A, 1996. Detecting the enterotoxigenity of coagulase-positive staphylococci strains isolated from frozen chicken carcasses by SET-RPLA. *Ankara Üniv Vet Fak Derg*, 43: 443-448.
8. Evenson ML, Hinds MW, Bernstein RS, Bergdoll MS, 1988. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. *Int J Food Microbiol*, 7: 311-316.
9. Geniogeorgis CA, 1989. Present state of knowledge on staphylococcal intoxication. *Int J Food Microbiol*, 9: 327-360.
10. Jablonski LM, Bohach GA, 2001. *Staphylococcus aureus*. *Food Microbiology: Fundamentals and Frontiers*. Washington DC, ASM Press, pp. 411-434.
11. Jaulhac B, Bes M, Bornstein N, Piémont Y, Brun Y, Fleurette J, 1992. Synthetic DNA probes for detection of genes for enterotoxins A, B, C, D, E and TSST-1 in staphylococcal strains. *J Appl Bacteriol*, 72: 386-392.
12. Jay MJ, Loessner MJ, Golden DA, 2001. *Staphylococcal Gastroenteritis*, *Modern Food Microbiology.* New York, Springer Science and Business Media, pp. 545-566.
13. Kılıç S, 2007. Determination of the Enterotoxigenicity of Coagulase Positive Staphylococci Isolated from Turkey Meats by EIA. *Ph.D. Thesis*. Institute of Medical Sciences of Ankara University.
14. Kısa Ö, Albay A, Erol I, Sırıken B, Esin N, Gün H, Yurtyeri A, 1996. Detecting the enterotoxigenicity of coagulase-positive staphylococci isolated from cream pastries by VIDAS technique. *Ankara Üniv Vet Fak Derg*, 43: 405-411.
15. Küplülü Ö, Sarımehmetoğlu B, Kaymaz Ş, 2002. Detection of staphylococcal enterotoxins in pasteurized milk with ELISA technique. *Turk J Vet Anim Sci*, 26: 631-637.
16. Lim S, Joo Y, Moon J, Lee A, Nam H, Wee S, Koh H, 2004. Molecular typing of enterotoxigenic *Staphylococcus aureus* isolated from bovine mastitis in Korea. *J Vet Med Sci*, 66: 581-584.
17. Løvseth A, Loncarevic S, Berdal KG, 2004. Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. *J Clin Microbiol*, 42: 3869-3872.
18. Minor TE, Marth EH, 1976. *Physiological Characteristics Suggested as Indices of Staphylococcal Enterotoxigenicity*. *Staphylococci and their Significance in Foods.* Amsterdam, Elsevier, pp. 53-78.
19. Monday SR, Bohach GA, 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. *J Clin Microbiol*, 37: 3411-3414.
20. Mutluer B, Erol I, Kaymaz Ş, Akgün S, 1993. Growth and enterotoxin production of enterotoxigenic *Staphylococcus aureus* strains during the manufacture of white cheese. *Ankara Üniv Vet Fak Derg*, 40: 413-426.

21. Normanno G, La Salandra G, Dambrosio A, Quaglia NC, Corrente M, Parisi A, Santagada G, Firinu A, Crisetti E, Celano GV, 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int J Food Microbiol*, 115: 290–296.

22. Omoe K, Ishikawa M, Shimoda Y, Hu D, Ueda S, Shinagawa K, 2002. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg*, *seh*, or *sei* genes. *J Clin Microbiol*, 40: 857-862.

23. Rosec JP, Gigaud O, 2002. Staphylococcal enterotoxin genes of classical and new type detected by PCR in France. *Int J Food Microbiol*, 77, 61-70.

24. Souza Da Cunha MLR, Peresi E, Calsolari RAO, Junior JPA, 2006. Detection of enterotoxin genes in coagulase negative staphylococci isolated from foods. *Braz J Microbiol*, 37: 70-74.

25. Udo EE, Al-Bustan MA, Jacob LE, Chugh TD, 1999. Enterotoxin production by coagulase-negative staphylococci in restaurant workers from Kuwait City may be a potential cause of food poisoning. *Soc General Microbiol*, 48: 819-823.

26. Valle J, Gomez-Lucia E, Piriz S, Goyache J, Orden JA, Vadillo S, 1990. Enterotoxin production by staphylococci isolated from healthy goats. *Appl Environ Microbiol*, 56: 1323-1326.

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