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RESEARCH ARTICLE

Investigation of *Staphylococcus aureus* Enterotoxins A to E Via Real-Time PCR from Various Food Samples in Turkey

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ABSTRACT

The aim of this study was to investigate the Staphylococcal enterotoxin (SE) and directly detect the five classical SEA, SEB, SEC, SED and SEE gene in *Staphylococcus aureus* strains from different food samples by real-time PCR. We studied totally 3650 different food samples such as milk and dairy products, meat and meat products, poultry and eggs, canned food, coffee, cocoa and derived products, honey, confectionery and bakery, ready-to-eat foods, beverages in food control laboratory. We found that a total of 36 (0.98%) *S. aureus* strains were isolated and only 14 (0.38%) out of 36 *S. aureus* strains were found to be enterotoxingenic. Milk samples were found to be most contaminant among the products. The most prevalent SE types were SEA 7 (19.4%). Commercial EIA kit results were used to compare with the real-time PCR results. SE results were found to be same with these two methods. This study showed that prevalence and type of the staphylococcal enterotoxin may vary from food to food. It is important to know this data to prevent outbreaks. Additionally, automated DNA isolation and real-time PCR methods can be performed to direct enterotoxin gene detection rapidly and reliably. This technique can be also used for food safety and clinical diagnosis applications.

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INTRODUCTION

Bacterial foodborne intoxications are important for human health. (Fischer *et al.*, 2007). All over the world, in humans and animals, *Staphylococcus aureus* is one of the most important pathogenic bacteria and cause of severe infections (Hata *et al.*, 2006; Bitrus *et al*., 2016). Bacterial foodborne intoxications via *S. aureus*, is the most frequent situations (Letertre *et al.*, 2003; 2005). Most common cause of this poisoning is the ingestion of one or more enterotoxin containing foods. Staphylococcal enterotoxin (SE) is highly stable and heat-resistant (Mossong *et al*., 2015). Small amount (<1 mg) of these toxins escalate the symptoms (Aitichou *et al.*, 2004). Abdominal cramps, vomiting, nausea, and diarrhea are the most common symptoms and full recovery takes approximately 1 to 3 days (Pinto *et al.*, 2005). Foods high in protein and starch are considered to support SEs production (Fletcher *et al.*, 2015). Currently, *S. aureus* strains are produce 23 different Staphylococcal enterotoxins (Podkowik *et al.*, 2013). Staphylococcal poisoning via enterotoxins is one of the leading economically important causes of foodborne outbreaks in the European Union countries (EFSA, 2013). In the European Union, SEs were responsible for 6.4% of all 346 reported foodborne outbreaks (Macori *et al.*, 2016). Recent studies were mentioned different prevalence rates with these outbreaks and were reported different products as a source of SEs (Mossong *et al*., 2015; Tarekgne *et al*., 2016). For this reason, it is important to know food source of SE epidemiologically to prevent outbreaks.

Different methods have been developed for reliably detecting the enterotoxins production of *S. aureus* (Klotz *et al.*, 2003; Fischer *et al.*, 2007). SEs can be usually detected by latex agglutination assay (LAA) or enzyme immune assay (EIA). The major disadvantages of these assays are false positivity due to cross reactivity, weak specificity and have limitations. PCR based methods such as real-time PCR have been used as an alternative to EIA and LAA methods (Letertre *et al.*, 2003). Conventional PCR technique is not appropriate to screen the large number of

samples rapidly (Klotz *et al.*, 2003). Real-time PCR and using combine with automated nucleic acid identification might be the right alternative.

The aim of this study was to investigate contamination sources and determine the prevalence rates and type of the SEs in different food products and examines of SEs detection assays directly from various food products by real-time PCR.

MATERIALS AND METHODS

Sample storage: In this study, 3650 food samples were collected. 2117 (58%) audit request samples, 1350 (37%) official request samples and 183 (5%) special request samples out of 3650 samples were analyzed at Ministry of Food, Agriculture and Livestock, Istanbul food control Biogenetic and Microbiology laboratories. Distributions of these 3650 samples are shown in Table 1.

Table 1: Distribution of food categories of all samples.

Food Category	Subcategory	N	%
Milk and Dairy Products	Milk	317	8.68
(n: 1178)	Cheese	576	15.78
	Ice cream	81	2.22
	Butter	204	5.59
Meat and Meat Products	Raw meat	590	16.16
(n: 1357)	products		
	Cooked meat products	767	21.01
Poultry and Eggs		263	7.21
Canned Food		269	7.37
Coffee, Cocoa and Derived Products		23	0.63
Honey, Confectionery and Bakery		309	8.47
Ready-to-eat foods		227	6.22
Beverages		24	0.66

Conventional *S. aureus* isolation and identification: Conventional microbiological analysis of the totally 3650 samples were performed according to TS EN ISO 7218, TS 10524, TS 6582-3 and EN ISO 6888-3 methods. In this study, we used the following strains as positive controls; *S. aureus* ATCC 13565 (SEA), *S. aureus ATCC* 14458 (SEB), *S. aureus* ATCC 19095 (SEC), *S. aureus* ATCC 23235 (SED), *S. aureus* ATCC 27664 (SEE) and *S. epidermidis* DSM 20044 (nontoxigenic). These strains were also used as a control for ELISA and real-time PCR assays.

Staphylococcal enterotoxin detection immunologically by RIDASCREEN Set A, B, C, D, E kit: To detect *S. aureus* enterotoxins. SEA, SEB, SEC, SED and SEE, commercial EIA kit of Ridascreen Set A, B, C, D, E (R-Biopharm, Germany) were used per manufacturer's instructions.

Automated DNA extraction: DNA extraction was performed directly to all 3650 samples. 50 mg of food products were taking for homogenization with Magna Lyser green bead kit and Magna Lyser instrument (Roche Diagnostics, Germany). 800 µl extraction lysis buffer was used that process. Extraction lysis buffer contains 10mM tris (pH 8.0), 100 mM NaCl, 2 mM EDTA, 1% w/v sodium dodecyl sulfate, after homogenization process, added 100 µl, 5M guanidine thiocyanate and incubated 60ºC for 10 min. After incubation, was added 1 ml chloroform and mixed well. Centrifugation was made at 12.000xg for 5 minutes and the upper phase transfer to new microcentrifuge tube and 200 µl of the upper phase sample was used to made DNA extraction (Sakai *et al.*, 2002). The MagNA Pure LC DNA Isolation Kit III (Roche Diagnostics, Germany) was used for DNA isolation on Magna Pure LC2.0 instrument (Roche Diagnostics, Germany). We used an external Lysis protocols per manufacturer instructions. DNA concentration and purity were measured using ND-1000 spectrophotometer (NanoDrop Technologies, USA) for each isolation. Each measurement was repeated three times. DNA isolates were stored at -20ºC until real-time PCR run.

Primers and Hydrolysis probes design: To detect the SEA, SEB, SEC1, SED and SEE gene, primer and hydrolysis probes were used (Table 2). These were provided from Integrated DNA Technologies (IDT, USA).

Real-time PCR assays to investigate *S. aureus* enterotoxins: 3650 food samples were investigated directly by qualitative real-time PCR analysis. Real-Time PCR amplification was carried out in 20 µl volumes. To perform a real-time PCR assay, Light Cycler 480 Probe Master kit (Roche Diagnostics, Germany), primers, probes, template DNA and PCR grade water were added to reaction mixture per manufacturer's instructions. 0.5 µM primers and 0.1 µM probes were added in reaction. Real-time PCR assays were performed on Light Cycler 480 II (Roche Diagnostics, Germany) instrument. Following PCR profile was used; denaturation step at 95ºC for 10 min, followed by 40 cycles, of 10s at 95ºC, 30s at 55ºC, 1s at 72ºC. The real-time PCR runs were performed in duplicate for each gene and software determined the crossing point (Cp) automatically for each reaction to qualitative analysis.

RESULTS

In this study, 3650 food samples were studied (Table 1). These products were categorized 1178 (32.27%) different milk and dairy products in 4 different categories, 1357 (37.17%) meat and meat products in 2 different categories, 263 (7.21%) poultry and eggs, 269 (7.37%) canned food, 23 (0.63%) coffee, cocoa and derived products, 309 (8.47%) honey, confectionery and bakery, 227 (6.22%) ready-to-eat foods and 24 (0.66%) beverages. *S. aureus* strains were isolated in 36 (0.98%) by culture techniques. All *S. aureus* strains were investigated commercial EIA Ridascreen Set A, B, C, D, E assay to detect enterotoxin presence and we also investigated all food samples directly to detect *S. aureus* enterotoxins by real-time PCR. Real-time PCR software automatically collected the fluorescent signal from appropriate well, analyzed the each cp of amplification curve and performed the qualitative analysis (Fig. 1). EIA and real-time PCR results were found to be same. According to results, among 36 *S. aureus* isolates, 14 (38%) of these *S. aureus* strains were contained one or more enterotoxigenic gene. The most prevalent enterotoxigenic type was SEA [7, (19.4%)] and 5 (13.8%) of these 7 SEA strains were just positive for SEA. The other 1 (2.7%) strain was positive for SEA, SEC and SED and the other 1 strain (2.7%) was positive for SEA and SED. 1 (2.7%) out of 14 enterotoxigenic strains among 36 *S. aureus* was positive for SEB. 3 (8.3%) strains were positive for SEC, 2 (5.5%) strains were positive for SED and 1 strain was positive for SEE (Table 3).

In milk and dairy products SE was investigated in 576 (15.78%) cheese, 81 (2.22%) ice cream and 201 (5.59%) butter. 3 (0.94%) and 4 (0.69%) different SE types were found in milk and cheese samples respectively. SE was not found in ice cream and butter products. In meat products, SE was found in 2 raw meat products and 2 in cooked meat products. SEA was found 3 of 4 positive products. 2 sample in poultry and eggs products and 1 sample in honey, confectionery and bakery sample were found to be SE positive. SE types found to be change food to food. Especially SEA type was found frequently (Table 4). At this study, most common SE was found in 3 (0.94%) out of 317 milk samples in dairy products.

DISCUSSION

S. aureus is considered one of the important foodborne pathogen. Different kind of foods can be a good growth medium for *S. aureus* and it is able to produce SEs. SEs are one of the most common reasons in foodborne outbreaks (Jung *et al.*, 2015).

In United Kingdom, Wieneke *et al.* (1993), reported that among the cases in 1969 to 1990; of the cases poultry products were responsible for 22%, milk products were responsible for 8%, fish and shellfish were responsible for 7% and eggs were responsible for 3.5% in total of 53% of the staphylococcal food poisonings (Wieneke *et al.*, 1993). In France, Haeghebaert *et al*. (2002) reported that among the cases in 1999 to 2000; 32% of the cases due to milk products and cheeses, 22% of the cases due to meats, 15% of the cases due to sausages and pies, 11% of the cases due to fish and seafood, 11% of the cases due to eggs and egg products and 9.5% of the cases due to poultry of the total staphylococcal food poisonings (Haeghebaert *et al.,* 2002). In Japan, Asao *et al.,* (2003) observed an outbreak of staphylococcal food-poisoning in 2002 that 13420 people were influenced after ingesting skim milk and yoghurt contaminated with 0.38 ng/ml and 3.7 ng/g of SEA, respectively. In our study, detection limit of our EIA kit was 0.25 ng/g. In Italy, Morandi *et al.* (2007) analyzed incidence of the *S. aureus* strains 67% of the samples milk and dairy products were positive for the presence of genes that encode enterotoxins., Tarekgne *et al.* (2016) detected 160 (29.1%) milk samples were positive for *S. aureus*, of which 22 strains were positive for the presence of SEA to SEE gene. In Japan, Katsuda *et al.* (2005) reported that 183 (67.8%) out of 270 *S. aureus* isolates were positive for the presence of genes that coding for one or more toxins. In South Korea, Jang *et al.,* (2013) reported that 1.3% *S. aureus* isolates out of 1120 sandwiches in cafes, sandwich bars and bakeries. In France, Mossong *et al.* (2015) reported that 300 (23%) out of 1,288 foodborne outbreaks due to SEs. Macori *et al. (*2016) reported that in the European union, SEs were responsible for 6.4% of all 346 reported foodborne outbreaks. Also Fletcher *et al.* (2015) reported that epidemiological findings at food samples, were linked the outbreaks.

Table 2: Oligonucleotide primers and hydrolysis probes used in the Real-time PCR assay

Gene	Primer & Probe	Sequence	Amplicon size	Genbank	References
SEA	SEA fw	5-AAAATACAGTACCTTTGGAAACGGTT-3	92	M18970	Klotz *et al.* (2003)
	SEA Rv	5-TTTCCTGTAAATAACGTCTTGCTTGA-3			
	SEA Probe	FAM-AACGAATAAGAAAAATGTAACTGTTCAGGAGTTGGATC-Tamra			
SEB	SEB fw	5-ACACCCAACGTTTTAGCAGAGAG-3	81	M11118	Klotz *et al.* (2003)
	SEB Rv	5-CCATCAAACCAGTGAATTTACTCG-3			
	SEB Probe	FAM-CAACCAGATCCTAAACCAGATGAGTTGCACA-Tamra			
SEC	SEC fw	5-AATAAAACGGTTGATTCTAAAAGTGTGAA-3	80	X05815	Klotz *et al.* (2003)
	SEC Rv	5-ATCAAAATCGGATTAACATTATCCATTC-3			
	SEC Probe	FAM-TAGAAGTCCACCTTACAACAA-Tamra			
SED	SED fw	5-TGATTCTTCTGATGGGTCTAAAGTCTC-3	115	M28521	Klotz *et al.* (2003)
	SED Rv	5-GAAGGTGCTCTGTGGATAATGTTTT-3			
	SED Probe	FAM-TATGATTTATTTGATGTTAAGGGTGATTTTCCCGAA-Tamra			
SEE	SEE fw	5-GCTTTGGCGGTAAGGTGC-3	68	M21319	Chiefari *et al.* (2005)
	SEE Rv	5-ATAACTTACCGTGGACCCTTCAGA-3			
	SEE Probe	FAM-AGGCTTGATTGTGTTTCATT-Tamra			

Table 3: Real-Time PCR results and distribution of enterotoxin types

S. aureus (n=36)	Types of Enterotoxin								
	Nontoxigenic	Enterotoxigenic	SEA	SEB	SEC	SED	SEA+D	SEA+C+D	SEE
N	22	14	5	1	3	2	1	1	1
%	60	38	13.8	2.7	8.3	5.4	2.7	2.7	2.7

Table 4: Real-Time PCR result distribution of enterotoxin types according to food categories.

Food Category	Subcategory	N (%)	SE (+) (n(%))	SE Type
Milk and Dairy Products	Milk	317 (8.68)	3 (0.94)	SEA, SEC, SEA+SEC+SED
(n: 1178)	Cheese	576 (15.78)	4 (0.69)	SEA, SEC, SEE, SEA+SED
	Ice cream	81 (2.22)	0 (0)	-
	Butter	204 (5.59)	0 (0)	-
Meat and Meat Products	Raw meat products	590 (16.16)	2 (0.33)	2 SEA
(n: 1357)	Cooked meat products	767 (21.01)	2 (0.26)	SEA, SED
Poultry and Eggs		263 (7.21)	2 (0.76)	SEB, SEC
Canned Food		269 (7.37)	0 (0)	-
Coffee, Cocoa and Derived Products		23 (0.63)	0 (0)	-
Honey, Confectionery and Bakery		309 (8.47)	1 (0.32)	SED
Ready-to-eat foods		227 (6.22)	0 (0)	-
Beverages		24 (0.66)	0 (0)	-

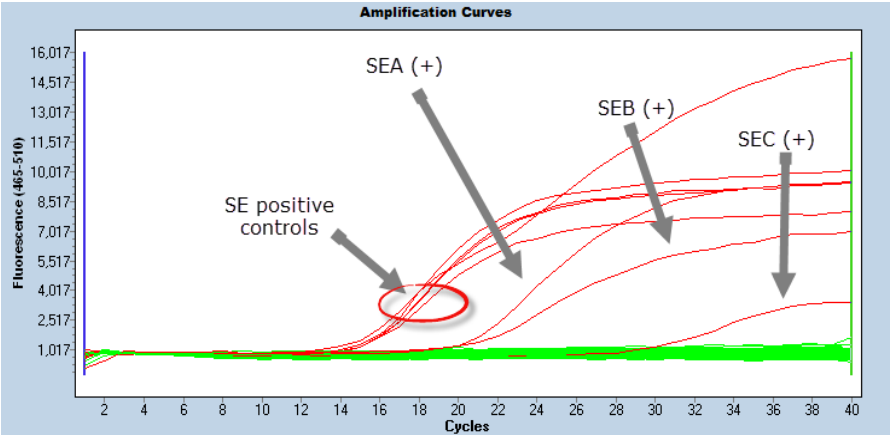

Fig. 1: Real-time PCR amplification curves graphs of SE genes (red: positive controls and representative positive samples, green: negative control and negative samples.).

Table 4 showed that our results in this study and these results thought us, incidence of Staphylococcal enterotoxin may vary from food to food and it is important to check epidemiologically to understand source of contamination. Foods high in protein and starch are considered to support SEs production (Fletcher *et al.*, 2015). Our SE positive results lower than the previous reports; it could be depending on the type of the samples working at this study but similar to the recent reports, milk samples were found the one of the most contaminant products for SEs in our study. Fletcher *et al.* (2015) reported that diagnosis of staphylococcal poisoning is primarily clinical, for this reason, this epidemiological data is important to support clinicians.

Prevalence and typing of SEA, SEB, SEC, SED and SEE genes by *S. aureus* strains has already been reported many authors at different country (Sokari, 1991; Lim *et al.*, 2004; Rall *et al.*, 2008; Pereira *et al.*, 2009). These authors reported that most prevalent strain was SEA producers in different food products. Our results were the same previous reports about the SEA gene. There are some studies to investigate enterotoxigenic *S. aureus* in Turkey with different methods (Bingöl *et al.*, 2012; Gücükoğlu *et al.*, 2013; Akkaya *et al.*, 2014). But our study is the first large study to investigate these enterotoxins from different food products directly by real-time PCR. In spite of different techniques of these previous reports, SEA seems to be the most common type in our country. Our results are also compatible with these results.

Conclusions: In summary, the prevalence of enterotoxin genes types of various food products investigated in this study and enhances our current knowledge. Rapid and reliable molecular methods could be used to detect these toxins. Prevalence and types of the staphylococcal enterotoxin might be different from food to food. Staphylococcal food poisoning diagnosis is mainly clinical but knowing this epidemiological data of the country to help clinicians to consider these toxins.

Authors contribution: Development of original idea: MD and FT. Study concept and Design: MD and FT. Analysis and Interpretation of data: MD, FT, SKA, AY. Data collection: FT and MD. Preparation of manuscript: MD, SKA, AY. Laboratory testing: for real-time PCR: FT and MD, for Elisa method: SKA. For DNA isolation: FT and AY.

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