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Prevalence of staphylococcal enterotoxins, toxin genes and genetic-relatedness of foodborne *Staphylococcus aureus* strains isolated in the Marmara Region of Turkey

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ABSTRACT

Staphylococcus aureus is a major foodborne pathogen and it has the ability to produce a number of extracellular toxins. We analyzed 1070 food samples obtained from retail markets and dairy farms in the Marmara Region of Turkey for the presence of *S. aureus*. Out of 147 isolates, 92 (62.6%) were enterotoxigenic. PCR was used to investigate the presence of staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq* and *seu*), exfoliative toxin genes (*eta* and *etb*) and the toxic−shock syndrome toxin gene (*tst*). The PCR results showed that 53.3% of the isolates contained staphylococcal enterotoxin-like (SEl) toxin genes (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq* and *seu*) which were more frequent than classical enterotoxin genes (*sea* to *see*). Furthermore, *seo*, *sei*, *sem*, *seg*, *seu* and *sec* were found in 37.0, 32.7, 30.4, 29.3, 29.3 and 27.2% of the isolates, respectively. The *tst* gene was detected and confirmed by DNA sequencing in 9 isolates. The presence of *eta* and *etb* were not found in the isolates. Enterotoxigenic capabilities of isolates with SEA–SEE were investigated by ELISA. Enterotoxigenic *S. aureus* isolates produced one to three enterotoxins, with the most frequently produced types being enterotoxin A and C. There was a correlation of 72.1% between production of a specific toxin and the presence of the respective genes. PFGE analysis was used to identify genetic-relatedness of enterotoxigenic *S. aureus* isolates and the results revealed that 13 groups of isolates from different or the same origin that contained the same genes showed 100% homology with indistinguishable band patterns. The other enterotoxigenic isolates showed related band patterns with 72–86% homology in *sea*-, 61–90% homology in *sec*-, 80–96% homology in *seh*-, and 69–96% homology in *sep*-positive isolates. To our knowledge, this is the first study to examine enterotoxins and related gene contents of *S. aureus* food isolates in the Marmara Region of Turkey.

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1. Introduction

Staphylococcus aureus is a pathogen associated with serious community and hospital-acquired diseases. Although the number of outbreaks reported annually has decreased in the last few decades, staphylococcal food poisoning is still reported as the third most prevalent cause of foodborne illness worldwide (Zhang et al., 1998). Illness is caused by enterotoxin-producing *S. aureus* (Cha et al., 2006; Sudagidan and Aydin, 2009; Wieneke et al., 1993). Raw meat (Pereira et al., 2009), meat products, including fermented products such as sucuk (Guven et al., 2010), raw milk (Rall et al., 2008), dairy products (Normanno et al., 2007) and ready-to-eat foods, including bakery products (Oh et al., 2007) are among the foods reported to be associated with *S. aureus* enterotoxin-induced food poisoning.

S. aureus produces more than 30 different extracellular by-products (Rogolsky, 1979) and staphylococcal toxins can be categorized into groups: pyrogenic toxin superantigens (PTSAgs), exfoliative toxins, leukocidins and other toxins. The family of PTSAgs includes staphylococcal enterotoxins (SEs), SE-like (SEl) toxins and toxic-shock syndrome toxin-1 (TSST-1) (Lina et al., 2004). Generally, five classical antigenic SE types (SEA to SEE) are recognized. Recently, the existence of new SEs including SEl toxins (i.e., SElG to SElQ, SElR and SElU) has been reported (Bania et al., 2006; Jarraud et al., 2002; Nashev et al., 2007). However, their potential role in staphylococcal food poisoning has not yet been clarified (Lina et al., 2004). TSST-1 is associated with staphylococcal toxic-shock syndrome and is considered to be the cause of nearly all cases of menstrual toxic-shock syndrome and at least 50% of nonmenstrual cases (Bergdoll and Schlievert, 1984). The presence of toxic-shock syndrome toxin gene (*tst*) in *S. aureus* isolated from foods has been reported (Cha et al., 2006). Moreover, clinical strains of *S. aureus* are known to produce immunologically distinct exfoliative toxin A and/or B (Bailey et al., 1980). The production of exfoliative toxin A by *S. aureus* isolated from foods and animals has been reported (Adesiyun et al., 1991; Hayakawa et al., 1998).

Various typing methods have been used to characterize *S. aureus* isolates. PCR has been used as a simple technique for detecting

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enterotoxigenic strains (Asperger and Zangerl, 2003). Although the PCR-based approach is specific, highly sensitive and rapid, it can only detect the presence of enterotoxigenic genes, not the production of the SE proteins (Boerema et al., 2006). Immunological methods are preferred for the detection of enterotoxins (Chiang et al., 2008). ELISA is the method of choice, because reagents are commercially available in polyvalent and monovalent formats for both toxin screening and serotype specific identification assays (Bennett, 2001). Numerous techniques [amplified fragment length polymorphism, multi locus sequence typing and pulsed-field gel electrophoresis (PFGE)] have been described for *S. aureus* genotyping (Melles et al., 2007), of which PFGE is the "gold standard" technique for determining genetic-relatedness, especially in outbreaks, due to its high discriminatory power (Weller, 2000).

The aim of this study was to determine the frequency of PTSAgs, including SE genes (*sea*, *seb*, *sec*, *sed* and *see*), SEl genes (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq* and *seu*), *tst* and the coexistence of exfoliative genes (*eta* and *etb*) in *S. aureus* food isolates, to determine the ability of the isolates to produce staphylococcal enterotoxins A–E and to investigate the genetic-relatedness of enterotoxigenic *S. aureus* isolates by PFGE.

2. Materials and methods

2.1. Sample collection and bacterial isolates

During the period of July 2007 and December 2008, 1070 food samples were collected from supermarkets, conventional markets, bazaars and dairy farms in and near large cities, including Balikesir, Bursa, Canakkale, Edirne, Istanbul, Kirklareli and Tekirdag, in the Marmara Region of Turkey. These samples included 115 meats (beef, mutton, chicken and turkey meat), 15 meat products [Turkish type fermented sausage (sucuk)], salami and sausage), 303 raw milk, 452 dairy products (cheese, butter, yoghurt and cream), 141 bakery products [pasta, thin sheets of dough (yufka) and cake] and 44 ready-to-eat foods. Raw milk samples were obtained from dairy farms located near these cities. Isolation of *S. aureus* from food samples was performed using EN ISO 6881-1 standard procedures described by the International Organization for Standardization (Anonymous, 1999). Procedures for identification of the isolates were previously described (Aydin et al., 2011).

2.2. Detection of toxin genes

Bacterial genomic DNA isolation was carried out as described previously (Sudagidan et al., 2008). The presence of staphylococcal enterotoxin genes *sea*, *seb*, *sec* and *see* (Johnson et al., 1991), *sed* (Jarraud et al., 2002; Johnson et al., 1991), *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sep* and *seq* (Bania et al., 2006), *sem*, *sen* and *seo* (Jarraud et al., 2002) and *seu* (Nashev et al., 2007), and genes for exfoliative toxins (*eta* and *etb*) (Johnson et al., 1991) and toxic-shock syndrome toxin (*tst*) (Booth et al., 2001) was determined by either monoplex or multiplex PCR. The PCR products were resolved in 1.5% (w/v) agarose gel electrophoresis in 1× TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA). PCR experiments were done twice for each isolate. Positive controls were *S. aureus* subsp. *aureus* NCTC (National Collection of Type Cultures) 10652 for *sea*, *S. aureus* subsp. *aureus* NCTC 10654 for *seb*, *S. aureus* subsp. *aureus* NCTC 10655 for *sec* and *S. aureus* subsp. *aureus* NCTC 10656 for *sed*. *S. aureus* N315, positive for *tst*, *sem*, *sen*, *seo* and *seu*, was kindly supplied by Dr. Teruyo Ito and Prof. Keiichi Hiramatsu from Juntendo University, Japan. *S. aureus* strains positive for *seg*, *sei*, *sej*, *seh* and *seo* genes were obtained from Department for Veterinary Public Health, Institute for Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Wien, Austria.

2.3. DNA sequencing of tst gene

The DNA sequences of *tst* positive PCR products were determined using PCR primers described by Booth et al. (2001). The following conditions were used for cycle sequencing of *tst* genes: 1 min at 96 °C, 30 cycles of 10 s at 96 °C, 5 s at 56 °C (*tst*-forward and reverse) and

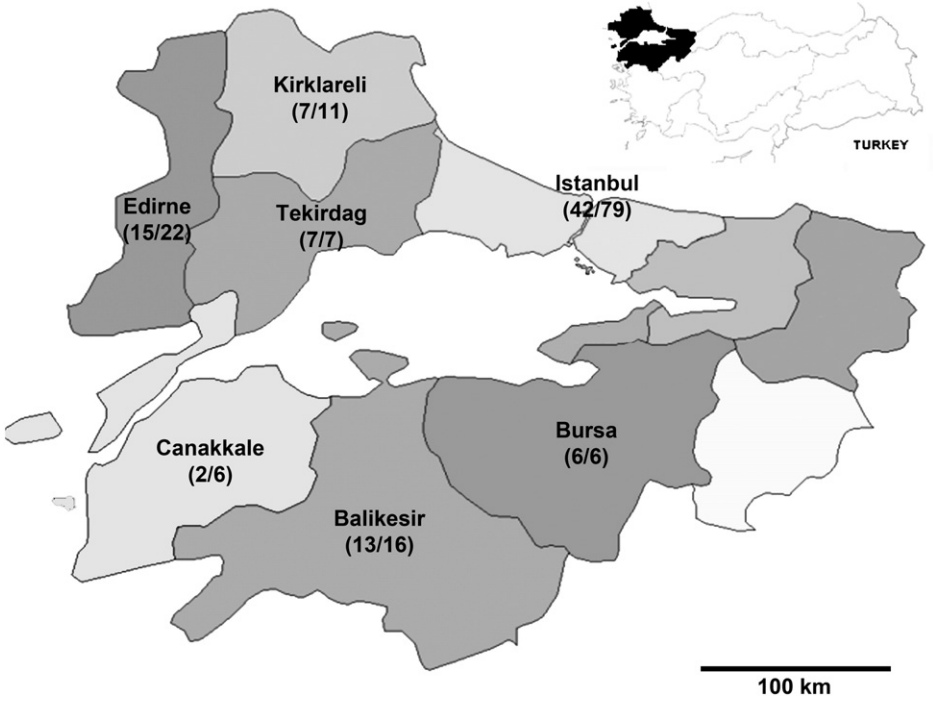

Fig. 1. Distribution of enterotoxigenic *S. aureus* isolates/total number of *S. aureus* isolates which were isolated from foods collected in the Marmara Region of Turkey.

Table 1
The presence of enterotoxin genes in *S. aureus* isolates.

Enterotoxin genes	Meat (n = 13) (%)	Meat products (%) (n = 6)	Raw milk (n = 31) (%)	Dairy products (n = 36) (%)	Bakery products (n = 5) (%)	Ready-to-eat food (n = 1) (%)	Total (n = 92) (%)
sea	1 (7.7)		3 (9.7)	4 (11.1)			8 (8.6)
sec			7 (22.6)	3 (8.3)			10 (10.9)
seh	4 (30.7)		1 (3.2)	3 (8.3)			8 (8.6)
sei			1 (3.2)				1 (1.1)
sel	1 (7.7)						1 (1.1)
sen				1 (2.8)			1 (1.1)
seo			1 (3.2)	2 (5.5)			3 (3.2)
sep	1 (7.7)		4 (13.0)	6 (16.6)	1 (20.0)		12 (13.0)
seu				1 (2.8)			1 (1.1)
sea, sec			1 (3.2)	1 (2.8)		1 (100)	3 (3.2)
sea, seh			1 (3.2)				1 (1.1)
sea, seq				1 (2.8)			1 (1.1)
seb, sec				1 (2.8)			1 (1.1)
seb, sep	1 (7.7)			1 (2.8)			2 (2.2)
sec, seo					1 (20.0)		1 (1.1)
seg, sei				1 (2.8)			1 (1.1)
sel, sep			2 (6.5)				2 (2.2)
sem, seo			1 (3.2)				1 (1.1)
sec, sel, sep				1 (2.8)			1 (1.1)
seh, sek, seq					1 (20.0)		1 (1.1)
sem, seo, sep			1 (3.2)				1 (1.1)
sea, seh, sek, seq			1 (3.2)		1 (20.0)		2 (2.2)
sec, sei, sel, sep				1 (2.8)			1 (1.1)
sea, seg, sei, seo, seu		1 (16.7)					1 (1.1)
seb, sek, sen, seo, seq	1 (7.7)						1 (1.1)
seg, sei, sem, sen, seo				1 (2.8)			1 (1.1)
seg, sei, sem, seo, seu		1 (16.7)		4 (11.1)			5 (5.4)
seb, seg, sei, sem, seo, seu				1 (2.8)			1 (1.1)
seg, sei, sem, sen, seo, seu	1 (7.7)		3 (9.7)	2 (5.5)	1 (20.0)		7 (7.6)
seg, sei, sem, seo, seq, seu			1 (3.2)	1 (2.8)			2 (2.2)
sea, seg, sei, sem, sen, seo, seu		1 (16.7)					1 (1.1)
sec, seg, sei, sel, sem, seo, seu	1 (7.7)						1 (1.1)
sec, seg, sei, sem, sen, seo, seu			1 (3.2)				1 (1.1)
sec, seh, sei, sem, sen, seo, seu		1 (16.7)					1 (1.1)
seg, sei, sem, sen, seo, seq, seu	1 (7.7)						1 (1.1)
sec, seg, seh, sei, sem, sen, seo, seu		2 (33.2)					2 (2.2)
sec, seg, sei, sel, sem, sen, seo, seu	1 (7.7)		2 (6.5)				3 (3.2)

4 min at 60 °C. The DNA sequencing was carried out using Applied Biosystems 3130*xl* Genetic Analyzer (Foster City, California, USA).

2.4. Determination of enterotoxin production

Enterotoxin production was determined by ELISA and enterotoxins were detected using a Ridascreen SET A, B, C, D, E assay kit (R-Biopharm AG, Germany). Briefly, the supernatant of 24 h cultures of *S. aureus* (9 log CFU/ml) grown at 37 °C in Brain Hearth Infusion Broth (BHI, Oxoid) was separated from cells by centrifugation at 4500 × *g* for 5 min at 4 °C. The supernatant was passed through 0.20 μm filter (Millipore) and 100 μl of the filtrate were transferred to an ELISA plate. The solutions were mixed gently by rocking of the plate for 1 h at 23 °C in the dark. To each well, 250 μl of washing buffer (0.55 g $NaH_2PO_4 \cdot H_2O$, 2.85 g $Na_2HPO_4 \cdot 2H_2O$, 8.7 g NaCl; pH 7.2) was added, followed by aspiration. This procedure was repeated three times. Subsequently, 100 μl of peroxidase conjugated anti-SET antibodies were added to each well and incubated for 60 min at 23 °C in the dark. The liquid was then aspirated from the wells and the wells were rinsed three times with 250 μl of wash buffer. Urea peroxide (50 μl) and tetramethyl-benzidine (50 μg) were added to each well, mixed thoroughly and incubated for 30 min at 23 °C in the dark. Stop reagent (100 μl of 1 N H_2SO_4) was added to each well and the absorbance was measured at 450 nm in an ELISA reader (ELX 800, Bio-tek Inst., Winooski, Vermont, USA) (Anonymous, 2003; Bennett, 2001).

2.5. PFGE analysis

Genetic-relatedness of 92 exterotoxigenic *S. aureus* food isolates was determined by PFGE analysis. Agarose plugs were prepared as described previously (Aydin et al., 2011; Sudagidan and Aydin, 2010). Bacterial DNA in plugs was digested with 30U *Sma*I (Fermentas) for 18 h, followed by applying to 1% (w/v) pulsed-field certified agarose (Bio-Rad) with a 5–40 s pulse time, 6 V/cm, 120° angle, at 14 °C for 22 h using the CHEF-Mapper PFGE system (Bio-Rad). After electrophoresis, the gel was stained with ethidium bromide (5 μg/ml) and visualized with VersaDoc 4000MP image analyzer system (Bio-Rad). The obtained band patterns were analyzed using BIO-PROFIL Bio-1D++ software (Vilber Lourmat, France) at 13% homology coefficient. The similarity between the isolates was determined automatically by specifying the formula of Nei and Li (1979). The clustering was performed by the unweighted pair group method with arithmetic mean (UPGMA) (Vilber Lourmat).

3. Results and discussion

3.1. Prevalence of enterotoxin producing S. aureus in food samples

Enterotoxin-producing *S. aureus* is one of the causative agents of foodborne intoxication and for this reason determination of its prevalence in foods is important with respect to assessing public health risks. In this study, 147 (13.8%) *S. aureus* strains, which were isolated from 1070 food samples, were analyzed for toxigenic capabilities. Ninety-two strains (62.6%) isolated from meat (13/13), meat products (6/6), raw milk (31/63), dairy products (36/54), bakery products (5/9) and ready-to-eat foods (1/2) were enterotoxigenic. These strains were isolated mainly from samples collected in Istanbul (n = 42), Edirne (n = 15), Balikesir (n = 13), Tekirdag (n = 7), Kirklareli (n = 7), Bursa (n = 6) and Canakkale (n = 2) (Fig. 1). Similar results were reported by Guven et al. (2010). They isolated 138 *S. aureus* strains from 413 food samples collected in the

central Anatolia region of Turkey and 83 (60.1%) of the strains synthesized one or two enterotoxins. In Portugal, Pereira et al. (2009) observed that 101 (68.2%) out of 148 *S. aureus* isolates from various foods were positive for the presence of genes coding for one or more enterotoxins. In another study, Normanno et al. (2007) found a similar prevalence in Italy, in which 59.8% of the *S. aureus* strains isolated from milk, dairy and meat products produced enterotoxins.

3.2. Detection of genes encoding staphylococcal enterotoxins

Studies on *S. aureus* isolated from foods have shown that the percentage of enterotoxigenic strains is considerably higher if the newly described SEl genes are considered together with genes encoding for the so-called classical enterotoxins (Bania et al., 2006; Le Loir et al., 2003). Enterotoxigenic *S. aureus* strains have different SE gene contents and they can harbor several of the genes. SE genes are located on plasmids (*sed* and *sej*), phages (*sea*, *see* and *sep*) and chromosomes (*seb*, *sec*, *seg*, *seh*, *sei*, *sek*, *sel*, *sem*, *sen*, *seo* and *seq*) (Kuroda et al., 2001). In this study, 17 SE and SEl genes were investigated. It was found that 53.3% of the isolates negative for *sea* to *see* carried SEl genes. This is in agreement with other studies showing higher percentages of SEl genes relative to classical SE genes (Bania et al., 2006; Nashev et al., 2007; Rosec and Gigaud, 2002).

In the present study, 8.6% of the enterotoxigenic isolates were found to encode only *sea*. In other studies, the *sea* was reported to be present in 15.4% (Cha et al., 2006) and 18.8% (Oh et al., 2007) of *S. aureus* strains isolated from foods. In our study, the highest percentage of single enterotoxigenic gene was *sep* (13%) (Table 1). The *sep* gene is another potential superantigen and was detected in 19 enterotoxigenic *S. aureus* isolates (20.7%), most commonly in dairy products (n = 8) and raw milk (n = 7). The *seb* gene was detected in 5 *S. aureus* isolates (5.4%) isolated from meat and dairy products (Table 1). However, in one isolate (HE4D), *seb* was accompanied by the *sek* gene. Another study reported all *seb*-positive strains (n = 3) to be *sek*-negative (Bania et al., 2006). SEK has many of the biological activities associated with the SEs, including superantigenicity, pyrogenicity and the ability to enhance the lethality of endotoxin (Orwin et al., 2001). Enterotoxigenic *S. aureus* strains containing *seq*, known to occur jointly with *sek* (Bania et al., 2006). In this study, it was found that all *sek*-positive isolates (HE4D, S133A, YF62A and YF62B) were also positive for *seq*, whereas, 4 *seq*-positive isolates (PY38BY/1, PY92BY/2, S137AY and TE15A) did not contain *sek*. Furthermore, our results showed that none of the enterotoxigenic isolates contained *sed*, *see* and *sej* genes. Similar observations have been reported for the absence of *see* (Bania et al., 2006; Nashev et al., 2007; Pereira et al., 2009).

Kuroda et al. (2001) suggested a correlation among *sec*, *sel* and *sep* genes. We found that only two isolates, both from dairy products (PY178A and PY178B), contained *sec*, *sel*, and *sep* and only two isolates (HE25A and HE25B) contained *sec* and *sel* genes. Bania et al. (2006) reported that *sec*/*sel*-positive strains were *sep*-negative; thus, any relationship between these genes is not obvious. In this study, *sec* was detected in 27.2% of enterotoxigenic *S. aureus* isolates and among these isolates, 8% were found to carry *sel*. These results were in agreement with those reported by Bania et al. (2006) which demonstrated that 7.1% of *S. aureus* isolates contained *sec* and *sel*.

The coexistence of *seg* and *sei* genes on a common genetic element was presented by Jarraud et al. (2001) and, in our study, all *seg*-positive isolates (n = 28) were also positive for *sei*. Interestingly, 3 isolates (EU6A, PY178A and S226) containing *sei* did not contain *seg*. Similarly, MacLauchlin et al. (2000) detected 20 *S. aureus* isolates which were positive for *seg* and *sei* (n = 19) or for only *sei* (n = 1). Moreover, the SEU enterotoxin, encoded by *seu*, was homologous, but not identical to any known enterotoxins (Letertre et al., 2003) and the majority of our strains (29.4%) contained *seu* gene and combinations with other enterotoxin genes (Table 1).

SElH has been shown to have emetic activity and is considered as a potential causative agent for food poisoning (Su and Wong, 1995). In the present study, *seh* was detected the only enterotoxin genes in 8 (8.6%) of the isolates and totally in 15 (16.3%) of the isolates *seh* and *seh* combinations with other enterotoxin genes were detected (Table 1). This prevalence was relatively lower than that (52%) reported by Bania et al. (2006), but comparable to that found by Nashev et al. (2004), who reported a prevalence of 13.6% in clinical *S. aureus* isolates.

3.3. Determination of enterotoxin production

Enterotoxins that cause human illness are referred to as classical staphylococcal enterotoxins (SEA to SEE) (Cha et al., 2006). In approximately 5% of cases, classical enterotoxins are not detected and these cases have been attributed to the production of new enterotoxins (Kokan and Bergdoll, 1987; Su and Wong, 1995). The specificity of ELISA is very high and the detection of enterotoxins A, B, C, D and E is highly sensitive (Bennett, 2001). In the present study, 46.7% *S. aureus* isolates possessed the targeted classical SEs genes (*sea*, *seb* and *sec*). In addition, 72.1% of the enterotoxigenic *S. aureus* isolates produced enterotoxins SEA–SED, as detected by ELISA (Table 2). The most frequently encountered enterotoxins in foods are SEA (Oh et al., 2007; Tsen et al., 1998) and SEC (Soriano et al., 2002; Tamarapu et al., 2001). Our observations were in agreement with other studies. We found that 2, 8 and 10 of the isolates produced only SEB, SEA and SEC, respectively, however, in 11 of the

Table 2
Presence of the genes and the produced enterotoxins in *S. aureus* isolates.

Isolates (food[a], origin[b])	SEs detected by ELISA				The genes detected by PCR			
	SEA	SEB	SEC	SED	*sea*	*seb*	*sec*	Other SEl genes
SE21A, M, Bu[c]	+			+	+			−
HE4D, M, E		+				+		*sek*, *sen*, *seo*, *seq*
HE25A, M,E			+				+	*seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*
HE25B, M, E			+				+	*seg*, *sei*, *sel*, *sem*, *seo*, *seu*
TE2, M, I		+	+			+		*sep*
EU6B, MP, I			+				+	*seg*, *seh*, *sei*, *sem*, *sen*, *seo*, *seu*
EU6C, MP, I			+				+	*seg*, *seh*, *sei*, *sem*, *sen*, *seo*, *seu*
EU7A, MP, I	+		+	+	+			*seg*, *sei*, *seo*, *seu*
EU7B, MP, I	+				+			*seg*, *sei*, *sem*, *sen*, *seo*, *seu*
S35A, RM, K			+				+	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*
S133A, RM, T	+				+			*seh*, *sek*, *seq*
S158B, RM, T		+	+				+	*seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*
S235, RM, I	+		+	+	+		+	−
S255, RM, I			+				+	−
S269, RM, I	+			+	+			*seh*
S272, RM, I	+		+	+	+			−
S273, RM, I	+			+	+			−
PY6, DP, I		+				+		*sep*
PY92BY2, DP, I	+				+			*seq*
PY100C, DP, I	+				+		+	−
PY104A, DP, I	+				+			−
PY104B, DP, I	+				+			−
PY178A, DP, K			+				+	*sei*, *sel*, *sep*
PY178B, DP, K			+				+	*sel*, *sep*
PY186A, DP, E		+	+			+		*seg*, *sei*, *sem*, *seo*, *seu*
PY276, DP, B	+			+	+			−
PY311B, DP, B	+				+			−
PY351A, DP, B			+				+	−
PY368A, DP, C			+			+	+	−
YF62A, BP, B	+				+			−
YE15A, RTE, I	+			+	+		+	−

[a] M: Meat; MP: Meat Products; RM: Raw Milk; DP: Dairy Products; BP: Bakery Products and RTE: Ready-To-Eat Food.
[b] B: Balikesir; Bu: Bursa; C: Canakkale; E: Edirne; I: Istanbul; K: Kirklareli; and T: Tekirdag.
[c] In 13 isolates, there was not a correlation between enterotoxin production and the related genes are shown in boldface type.

isolates produced more than one enterotoxin (Table 2). There was 72.1% correlation between enterotoxin types and presence of the respective genes. Similarly, Pereira et al. (2009) demonstrated 80% correlation in *S. aureus* isolates using the VIDAS assay and PCR.

In 12 *S. aureus* isolates (EU6A, S35B, S182, S215, S217, S242, S258, S266, S267, P33A, PY376A and PY406B) positive for SE genes, the presence of enterotoxins were not detected by ELISA. The difference between PCR and ELISA results can also be explained by the low-level of staphylococcal enterotoxin production, *i.e.* below the threshold of immunoassay detection (Bystron et al., 2006). The concentration of toxin should be above 1 ng/ml, which is the lowest detection limit of the test used in this study. Another explanation might be the incomplete expression of enterotoxigenic genes. Furthermore, SE production can be affected by environmental conditions such as temperature, pH and water activity (Nájera-Sánchez et al., 2003). The environment of enterotoxigenic *S. aureus* in food is important for both growth and production of enterotoxins.

According to the ELISA results, 8 of the enterotoxigenic isolates produced SED and 4 isolates (TE2, EU7A, S272 and PY186A) produced SEC; however, *sed* and *sec* were not detected in these isolates by PCR (Table 2). Five isolates were positive for SED, whereas 3 were positive for SEC and 3 were positive for SEC and SED. It is possible that these pairs of PCR primers (SEC1–SEC2 and SED1–SED2) had low specificity owing to small sequence differences between strains (Nájera-Sánchez et al., 2003). Moreover, one isolate (S158B) that produced SEB and SEC tested negative for the presence of *seb*, but positive for *sec* and SEl genes. According to the other reports, SEB and SEC share 62 to 64% amino acid identity (Bohach and Schlievert, 1987; Munson et al., 1998) and SEG is similar to SEB and SEC (Munson et al., 1998). In addition, 2 isolates (EU7A and PY186A) were positive SEC and SED and these isolates contained also SEl genes (*seg*, *sei*, *sem*, *seo* and *seu*). These differences might have resulted from the use of prepared polyclonal antibodies against each SE, which sometimes exhibit antigenic similarities among SEs and SEl toxins, thereby causing cross-reactions in the tests (Edwin et al., 1986). Because enterotoxins SEA–SEE and SEH also share nucleotide sequence identity (≥32%), it is possible that a new enterotoxin gene could also share nucleotide sequence identity with the characterized enterotoxins (Munson et al., 1998).

3.4. Prevalence of tst gene

TSST-1 has been detected in *S. aureus* isolates most commonly isolated from clinical samples with important clinical symptoms (Lappin and Ferguson, 2009), moreover, *tst*-positive isolates have been also isolated from various foods (Cha et al., 2006), poultry (Evans et al., 1983) and milk (Valle et al., 1991). In our study, 9.8% of the enterotoxigenic *S. aureus* isolates were positive for *tst*. Moreover, PCR products of *tst* were also confirmed by DNA sequencing. This is in agreement with Cha et al. (2006), who reported a 12% prevalence of *tst* in foodborne *S. aureus* isolates in Korea. Conversly, Tsen et al. (1998) found *tst* in 4.8% of clinical *S. aureus* isolates, but not in food

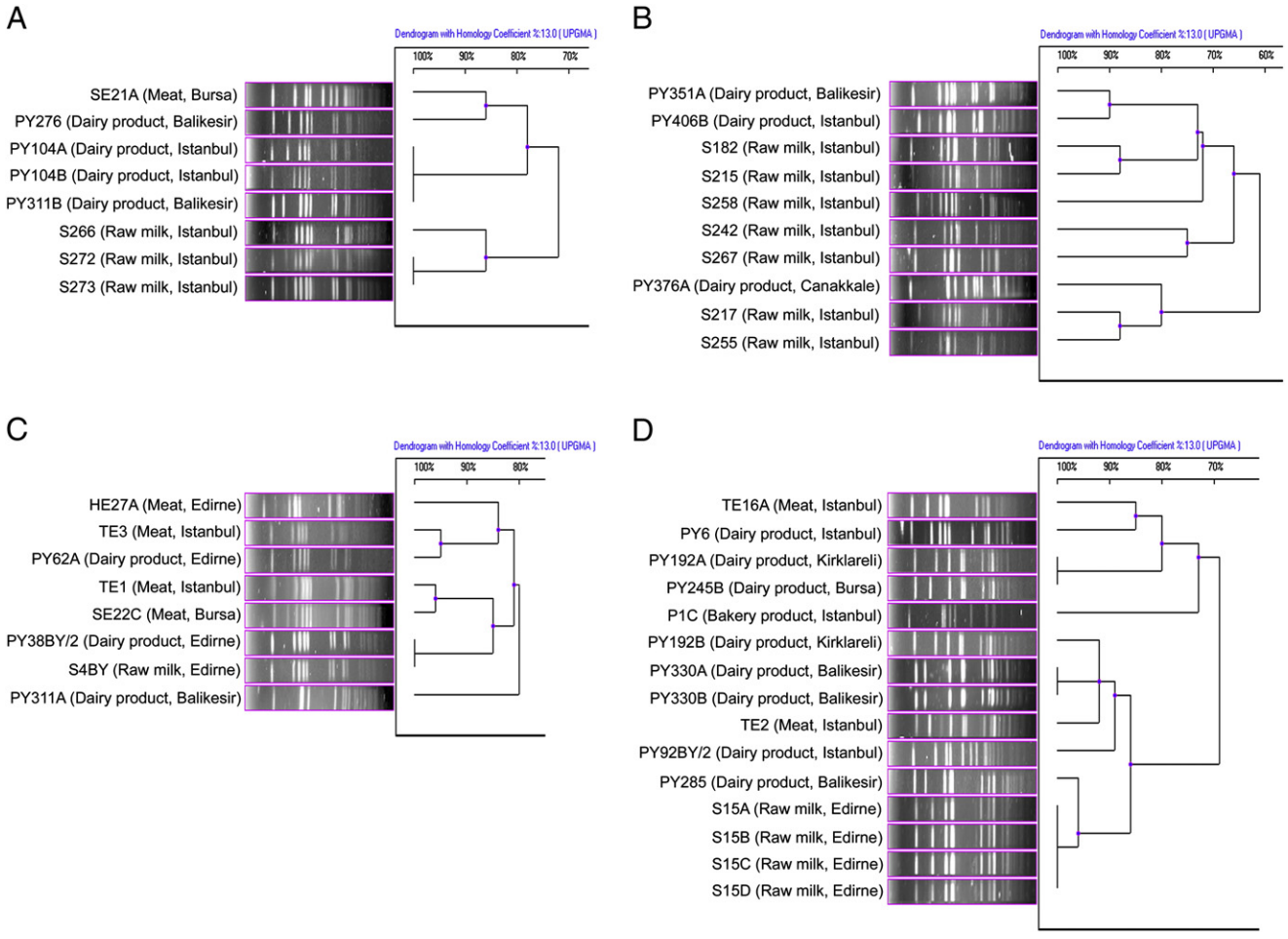

Fig. 2. Genetic-relatedness of *sea* (A), *sec* (B), *seh* (C) and *sep*-positive (D) *S. aureus* isolates.

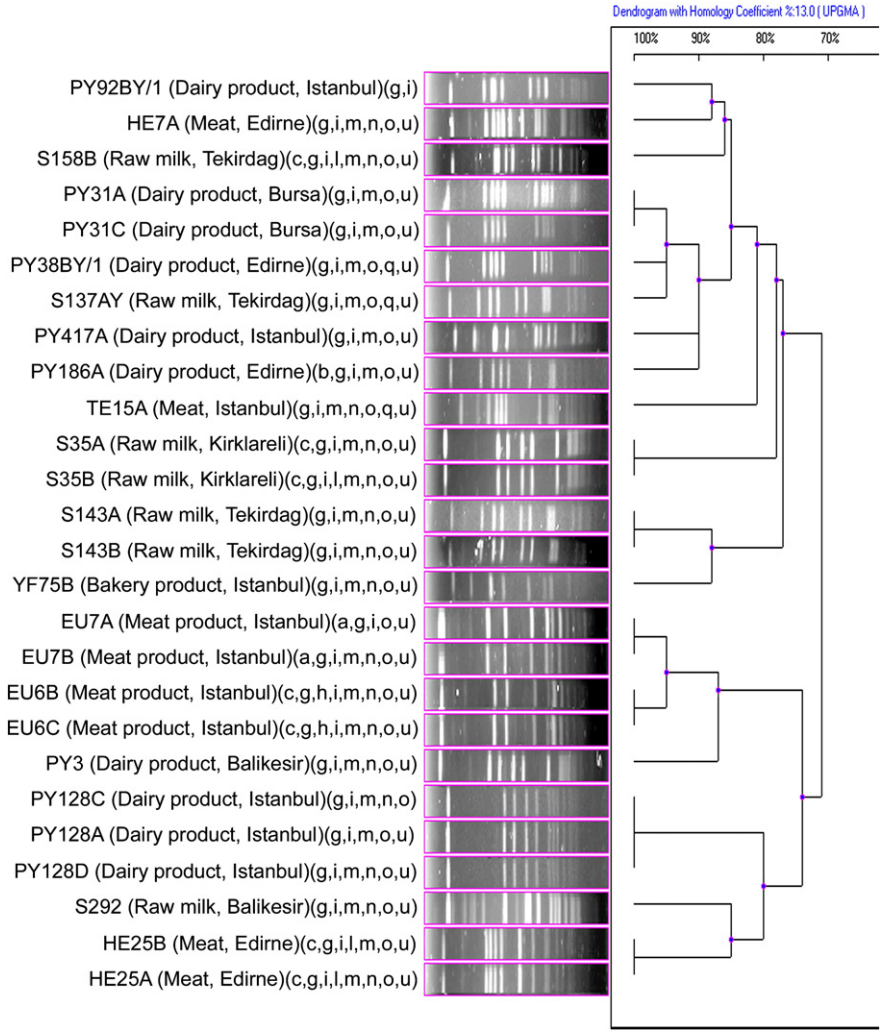

Fig. 3. Genetic-relatedness of 26 *S. aureus* isolates containing multi enterotoxin genes.

isolates. The probability of finding *tst*-positive foodborne *S. aureus* isolates would appear to be low (Tsen et al., 1998).

3.5. Detection of eta and etb

Genes encoding *eta* and *etb*-linked virulence factors are associated with bullous impetigo. In our study, none of the *S. aureus* isolates contained *eta* and *etb* genes. Similar to our results, Oh et al. (2007) and Karahan et al. (2009) could not find *eta* and *etb* in *S. aureus* isolated from ready-to-eat food and mastitic milk samples. On the other hand, Hayakawa et al. (1998) reported ETA in 1.2% (2/162) and 0.6% (1/166) of *S. aureus* isolates from mastitic cows' milk and bulk milk, respectively. Interestingly, these *eta*-positive *S. aureus* isolates did not cause general exfoliation of the epidermis accompanied by the so-called Nikolsky sign when inoculated into neonatal mice.

3.6. PFGE analysis of the enterotoxigenic strains

In the PFGE analysis, digestion of enterotoxigenic *S. aureus* strains' DNA with *Sma*I resulted in 9–15 fragments and the band patterns were grouped and compared to each other. Fig. 2 shows the genetic-relatedness of 8 *sea*-, 10 *sec*-, 8 *seh*- and 15 *sep*-positive isolates. Three *S. aureus* isolates (PY104A, PY104B and PY311B) isolated from dairy products obtained in Istanbul and Balikesir and two isolates (S272 and S273) from raw milk samples obtained in Istanbul showed 100% homology with indistinguishable band patterns. The other *sea*-positive isolates displayed 72–86% homology (Fig. 2A). However, the band patterns of ten *sec*-positive isolates showed variations (61–90% homology) (Fig. 2B). There was no unique clonal type among *sec*-positive isolates. *S. aureus* containing the *sec* were isolated mainly from raw milk samples collected in Istanbul. When the band patterns of *seh*-positive isolates were examined, only 2 isolates (PY38BY/2 and S4BY) from dairy products collected in Istanbul and raw milk in Edirne showed 100% homology. Other *seh*-containing isolates from meat and

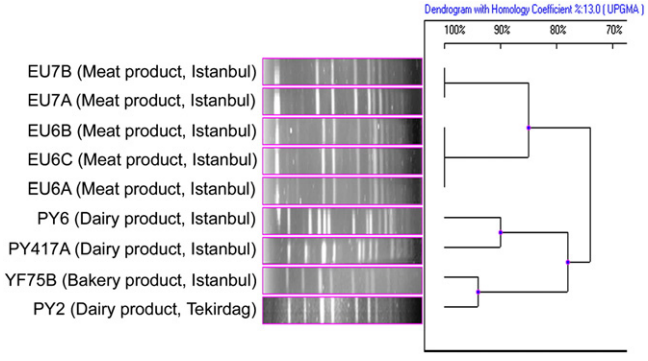

Fig. 4. Genetic-relatedness of *tst*-positive *S. aureus* isolates.

dairy product samples were closely related (80–96% homology) (Fig. 2C). Fifteen *sep*-positive isolates displayed 69–100% homology. Four isolates (S15A, S15B, S15C and S15D) from raw milk samples collected in Edirne and 2 isolates (PY330A and PY330B) from dairy products in Balikesir had the same band patterns. Interestingly, 2 isolates (PY192A and PY245B) from dairy products collected in different cities (Kirklareli and Bursa) showed 100% homology (Fig. 2D). *S. aureus* isolates obtained from dairy products and raw milk samples frequently contained the *sep*-gene.

The genetic-relatedness of *S. aureus* isolates containing multi enterotoxin genes is shown in Fig. 3. Aside from the identical strains, 11 *S. aureus* isolates displayed 71–95% homology. Seven groups of the isolates showed 100% homology. In these groups, 3 isolates (PY128A, PY128C and PY128D) and 6 groups of isolates (EU6B and EU6C, EU7A and EU7B, HE25A and HE25B, PY31A and PY31C, S35A and S35B and S143A and S143B) displayed 100% homology. These isolates were obtained from meat products, dairy products and raw milk samples. Isolates containing the same genes were closely related with more than 80% homology (Fig. 3).

The presence of *tst* in our *S. aureus* isolates was low. The genetic relatedness of 9 *tst*-positive isolates is shown in Fig. 4. The dendrogram of *tst*-positive isolates is composed of two main groups with 74% homology. One group is composed of 3 isolates (EU6A, EU6B and EU6C) and 2 isolates (EU7A and EU7B) all of which isolated from meat products and showed 100% homology. Another group contains 4 isolates (Fig. 4). *S. aureus* YF75B (from a bakery product) and PY2 (from a dairy product) showed 94% homology and they were isolated from samples collected in different cities. Moreover, 2 *S. aureus* isolates, which were isolated from dairy products in Istanbul, showed 90% homology. All *tst*-positive *S. aureus* isolates were closely related, with more than 74% homology.

4. Conclusions

The recent discovery and characterization of new SEs resulted in an increased frequency of potentially enterotoxigenic *S. aureus* isolates from foods, suggesting that the prevalence pathogenic *S. aureus* may be higher than the previously recognized. The high prevalence of newly discovered enterotoxin genes, including those encoding emetic toxins, is evident in foodborne isolates. In light of these observations, additional work is needed to better understanding the role of *S. aureus* in food poisoning and also to monitor the presence of these genes in foodborne isolates. The results demonstrated the high prevalence of newly discovered genes in foodborne *S. aureus*, especially in the isolates positive for classical enterotoxins, thus contributing to the prevalence of isolates potentially capable of causing food poisoning. The distribution of other genes suggests the possibility of the presence of yet unknown genetic elements encoding SEls. PFGE analysis showed a high genetic diversity among strains, indicating that contamination of various food products with *S. aureus* could originate from numerous sources, preprocessing environments, processing areas and the market place.

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