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Abstract

Staphylococcal foodborne intoxication, occurring after consumption of staphylococcal enterotoxins (SEs) in food, is considered one of the most common forms of bacterial foodborne outbreaks worldwide. Milk and dairy products account for 5% of all the incriminated foods in staphylococcal outbreaks, referring to Europe. The distribution of genes encoding for enterotoxins in Staphylococcus aureus strains is highly variable, with some carried on stable regions of the chromosome and others carried on mobile genetic elements. The aim of this study was to analyse the distribution of genes encoding for SEs in Staph. aureus strains isolated from milk and dairy products. In the period from January 2010 to June 2011, a total of 1245 dairy samples (848 of raw milk and 397 of dairy products) were collected and analysed for detection of genes encoding for 11 SEs and SEls (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SER SElJ and SElP) according to the procedures of the Italian National Reference Laboratory for coagulase-positive Staphylococci including Staph. aureus. Staphylococcus aureus strains were isolated in 481 (39%) samples. Of the 481 isolates of Staph. aureus tested, 255 (53%) were positive for one or more SE genes, and thirty-five different enterotoxin gene profiles were distinguished among the isolates. ser gene, found in 134 (28%) of the isolates, was the most frequent, followed by sed (25%) and selj genes (25%). The identification of new SEs increased the isolation frequency of enterotoxigenic staphylococci, thus suggesting that the pathogenic potential of Staph. aureus may be of greater importance than previously thought. Further studies are needed to quantify the expression of these new enterotoxins, and to assess their contribution to foodborne disease burden.

Introduction

Staphylococcal foodborne intoxication (SFP) is considered one of the most common forms of bacterial foodborne outbreaks worldwide. According to the outbreak reports from 15 European countries, milk and dairy products represented 1–9% (mean 4·8%) of all the incriminated foods in staphylococcal outbreaks (Balaban and Rasooly 2000). SFP is characterized by gastrointestinal symptoms and occurs after consumption of staphylococcal entero- toxins (SE) in food. SEs are produced by enterotoxino- genic strains of coagulase-positive staphylococci (mainly Staph. aureus) while coagulase negative staphylococci have never been reported as cause foodborne outbreaks. SFPs are not always notified in all Member States, therefore increasing the possibility of under reporting of cases (SCVPH 2000).

Coagulase-positive staphylococci are facultative anaero- bic bacteria which can grow over a relatively wide range of pH (4–10, with the optimum being 6–7), temperature (7–48°C) and water activity (minimum Aw is 0·83, pro- vided that all other conditions are optimal), while ranges for SE production are somewhat narrower (Hennekinne et al. 2011). The adaptability of the organism permits its growth in many types of food, leading to enterotoxins production and subsequent possibility of causing food poisoning. However, not all coagulase-positive staphylo- cocci are SE producers and even so, SE production will not occur in every food. Environmental factors and a conspicuous combinations of parameters of the food can influence and contribute to the formation of SE, such as water activity, pH, redox potential and temperature; besides, bacterial antagonism is known to play an impor- tant role (Genigeorgis 1989; Hennekinne et al. 2011; Schelin et al. 2011).

Growth of Staph. aureus and SE production in dairy products can be prevented during food processing by heat treatment of milk, or inhibited using starter cultures, antagonistic effect of natural flora, concentration of salt, drop of pH, low temperature of processing and storage of cheese and/or minimizing the pressing time. SEs, how- ever, are much more resistant to environmental effects and food-processing procedures than the staphylococcal bacterial cells; thus, although the bacteria are eliminated, the toxins will remain and can cause SFP (SCVPH 2000; Hennekinne et al. 2011; Schelin et al. 2011).

Several SEs are designated as SE-like (SEl) toxins because they lack the emetic properties or still have not been tested (Lina et al. 2004; Omoe et al. 2004). Thus, to date, a total of 21 SEs and SEls toxins have been reported. In addition to the five well-characterized classical staphylococcal enterotoxins SEA, SEB, SEC, SED

and SEE, 16 new types of SEs (SEG, SEH, SEI, SER, SES, SET) and SEls (SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SElU and SElV) have been introduced (Argudin et al. 2010).

The distribution of genes encoding for enterotoxins in Staph. aureus strains is highly variable, with some carried on stable regions of the chromosome (e.g. enterotoxigenic gene cluster—EGC) associated with particular lineages and others carried on mobile genetic elements (MGEs). MGEs are segments of DNA that encode enzymes and other proteins that confer their ability to move horizon- tally between bacterial cells (Frost et al. 2005). In Staph. aureus, the major MGEs are bacteriophages, Staph. aureus pathogenicity islands (SaPIs), plasmids, transposons and staphylococcal cassette chromosomes (SCCs). All have been reported to carry SE genes, except SCCs which typically carry antibiotic resistance genes, including mecA (Lindsay 2011). Most MGEs can move at high frequency between Staph. aureus isolates, including during the course of infection (Goerke and Wolz 2004; Lindsay and Holden 2006; Lindsay 2011).

The aim of this study was to analyse the distribution of genes encoding for SEs in Staph. aureus strains isolated from milk for human consumption and from dairy prod- ucts. Strains were analysed for detection of genes encod- ing for 11 SEs and SEls (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SER SElJ and SElP) according to the proce- dures of the Italian National Reference Laboratory for coagulase-positive Staphylococci including Staph. aureus.

Results and discussion

Staphylococcus aureus strains were isolated in 481 (39%) of 1245 milk and cheese samples, with Staph. aureus posi- tive samples accounting for 40% (343/848) and 35% (138/397), respectively. Table 1 shows the results of molecular tests for the detection of genes encoding the toxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SElJ, SElP and SER. Of the 481 strains of Staph. aureus tested, 255 (53%) were positive for one or more SE genes and 35 different enterotoxin gene profiles were distinguished among the isolates (Table 1). None of the isolates was positive for seb and see genes, while ser gene, found in 134 (28%) of the isolates, was the most frequent, followed by sed (25%) and selj (25%) genes. The genes encoding for SED, SER and SElJ are carried on the same plasmid and among all the profiles, sed-ser-selj (15%) was the most common, followed by seg-sei (12·3%). These latter genes are likely to be carried together on the EGC. SE genes carried on plasmid and EGC were the most fre- quently present within the isolates bearing SE genes: genes known to be carried by those elements were present in 142 (30%) and 89 (19%) isolates, respectively (Table 1, Figure 1).

In spite of the great discrepancy in data concerning the prevalence of enterotoxigenic Staph. aureus isolates found in the literature, which is attributable to different types of foods and biovars involved, SEA is the most frequently observed enterotoxin in enterotoxigenic strains of Staph. aureus (Normanno et al. 2005). Asao et al. (2003) reported an outbreak of foodborne disease in Kansai, Japan, where 13 420 people were affected after ingesting skimmed milk and yogurt (prepared with powdered milk) contaminated with 0·38 ng ml^{-1} and 3·7 ng g^{-1} of SEA, respectively.

Several studies have investigated the amount of ingested SE required to initiate symptoms: indeed, doses are supposed to change in relationship with SE types and the health status and age of patients.

One of the first trials demonstrated that ingestion of 20–25 lg of SEB (c. 0·4 lg kg^{-1} of body weight) is able to cause emesis (Raj and Bergdoll 1969). However, the average dose of SEA ingested by students in an outbreak caused by milk chocolate in the United States resulted to be 144 ± 50 ng (Evenson et al. 1988). Furthermore, an outbreak in Japan caused by low-fat milk contaminated with SEA showed that the total intake of SEA per individ- ual was estimated to be c. 20–100 ng (Asao et al. 2003). More recently, in a French outbreak caused by contami- nated cheese, doses of SEE ingested by symptomatic per- sons were estimated to be about 90 ng, based on the mean weight of the cheese portion (about 200 g) and the total amount of SEE of food sample (0·45 ng g^{-1}) (Ostyn et al. 2010). In this study, the SEA gene was found in 59 isolates (12%), which is in contrast with previous find- ings, where SEA gene was predominant (Lawrynowicz-Paciorek et al. 2007; Morandi et al. 2009; Ostyn et al. 2012). The most frequent SE gene found in the isolates has been SER, together with SED and SElJ, carried on the same plasmid (Rall et al. 2008). As shown in Fig. 1, in our research, these genes were found together in 106 (22%) isolates and in 35 (7%) separately. SED was previ- ously reported as the most frequently isolated toxin type, after SEA, in staphylococcal food-poisoning outbreaks involving dairy products (Lawrynowicz-Paciorek et al. 2007; Morandi et al. 2009). The SEG gene was observed in 85 (18%) isolates of Staph. aureus; just in one of the cases, it was not associated with SEI (Fig. 1). Similar val- ues were reported by Rosec and Gigaud (2002), who observed that SEG and SEI were associated in 80·6% of 155 strains. These genes are frequently found together because they are within the same genetic cluster (EGC), in a 3·2-kb DNA fragment (Jarraud et al. 2001). The small percentage of strains

carrying only one of these two enterotoxin genes could be explained by point mutations in seg or by variations in the cluster where these two genes are located (Jarraud et al. 2001). With PCR and enzyme-linked immunosorbent assays, Omoe et al. (2005) showed that most of the seg-positive Staph. aureus iso- lates, and about 60% of the SEI-positive isolates, did not produce detectable levels of SEG or SEI, while reverse transcription-PCR showed the presence of mRNA gener- ated from seg and sei genes. The gene products of seg, sei, selm, seln and selo may therefore be produced in small quantities only, resulting in a minor or negligible role in staphylococcal food poisoning (Bystron et al. 2010), although other authors suggested a relevant role for strains producing SEG and SEI in foodborne severe neonatal enteropathy (Naik et al. 2008) and toxic shock syndrome (Jarraud et al. 1999; Holtfreter et al. 2004).

The recent identification of new SEs has considerably increased the isolation frequency of enterotoxigenic staph- ylococci, thus suggesting that the pathogenic potential of Staph. aureus may be greater than previously thought. Little is known, however, about the role of these new enterotoxins by Staph. aureus in the occurrence of SFPs, and this is particularly true for SEs encoded by genes located on the EGC (seg and sei). Further studies are needed to quantify the expression of these, and to assess their contribution to foodborne disease burden. More- over, due to the involvement of recently described SEs, commercially available kits able to detect these toxins should also be developed, as, to date, available kits are only able to detect SEA to SEE toxins (Hennekinne et al. 2010). In particular, this could be the case with SEH, which has been responsible for milk-based food-poisoning outbreaks (Jørgensen et al. 2005; Ostyn et al. 2012).

Materials and methods

In the period from January 2010 to June 2011, a total of 1245 dairy samples were collected in Turin area (north- western Italy) and carried to local public laboratory (Isti- tuto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, Italy) at a controlled temperature +1/+8°C according to ISO 7218:2007 (Anonymous 2007). In total, 848 samples of raw cow milk, derived from unpasteurized milk dispensers, and 397 samples of unpasteurized cheese products were analysed for CPS isolation and for Staph. aureus identification.

For coagulase-positive staphylococci isolation, serial dilutions of each sample homogenate were plated on Baird–Parker agar + rabbit plasma fibrinogen (BP-RPF) (Liofilchem srl, Roseto degli Abruzzi - TE, Italy) and incubated at 37 ± 1°C for 48 h. At least one characteris- tic colony per sample was tested with commercial biochemical identification kits API® ID32 STAPH (bioMerieux, Marcy l'Etoile, France) to identify Staph. aureus strains.

Genomic DNA was obtained from Staph. aureus strains using InstaGeneTM Matrix (Bio-Rad, Milano, Italy). Each colony was mixed in 100 ll of matrix buffer, incubated for 60 min at 56°C and then for 45 min at 95°C. Finally, the suspension was centrifuged for 5 min at 20 000 g, and supernatant was used for PCR amplification.

To detect SEs genes, two multiplex PCR protocols were used according to European Union Reference Laboratory for Coagulase-Positive Staphylococci (EU-RL CPS) meth- ods (Kerouanton et al. 2007). The eleven primer pairs designed for PCR targeting genes (Tables 2 and 3) were purchased from Invitrogen (Carlsbad, CA, USA). Each PCR contained 1 U of FastStart Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany), 19 FastStart Buffer without $MgCl_2$ (Roche Diagnostics), 2·5 mmol l^{-1} $MgCl_2$ (Roche Diagnostics), 0·2 mmol l^{-1} dNTPs (Fer- mentas, Vilnius, Lithuania), primers (concentration reported in Tables 2 and 3) and 2 ll of DNA samples. The final volume was adjusted to 25 ll by adding sterile ultrapure water.

Reference strains of Staph. aureus, FRI S6 (SEA, SEB), FRI 137 (SEG, SEH, SEI), FRI 326 (SEE), FRI 361 (SEC, SED, SER), HMPL 280 (SEG, SEI, SEIJ, SEIP), were used as positive controls (provided by EU-RL of CPS).

PCR were performed on a GeneAmp System 9700 ther- mal cycler (Applied Biosystems, Foster City, CA, USA). Two thermal profiles were set according to EU-RL of CPS protocols (Tables 2 and 3).

Reaction products were separated by standard gel elec- trophoresis using 10 ll of the PCR product mixture on 2·5% agarose gels in TAE buffer (0·1 mol l^{-1} Tris, 0·1 mol l^{-1} acetic acid and 0·002 mol l^{-1} Na_2EDTA) add- ing Gel Green (Biotium, Hayward, CA, USA). Molecular marker (Bio-Rad) was used as molecular weight standard.

The gels were visually inspected under a UV transillumina- tor (GelDoc, Bio-Rad).

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Table 1 Enterotoxin gene profiles with putative MGEs location. The number at the end of each line represents the number of isolates bearing a specific enterotoxin gene profile. The number at the bottom of each column represents the number of isolates bearing a specific enterotoxin gene

Phage		SaPI		Plasmid		EGC		Transp.	
sea	selp	sec	sed	selj	ser	seg	sei	seh	No of isolates
			X	X	X				72
						X	X		59
X			X	X	X				27
		X							23
X				X	X				10
								X	10
		X				X	X		7
			X	X	X	X	X		6
			X						4
X					X				4
			X		X				3
X							X		3
X									3
			X		X	X	X		2
X					X	X	X		2
				X					1
				X	X				1
					X				1
							X		1
	X		X		X				1
X			X		X				1
X				X					1
			X			X	X		1
X						X			1
X						X	X		1
	X					X	X		1
X			X			X	X		1
X			X		X	X	X		1
X			X	X	X	X	X		1
	X								1
X								X	1
		X			X				1
						X	X	X	1
X		X				X	X		1
X		X			X				1
59	3	33	120	119	134	85	88	12	

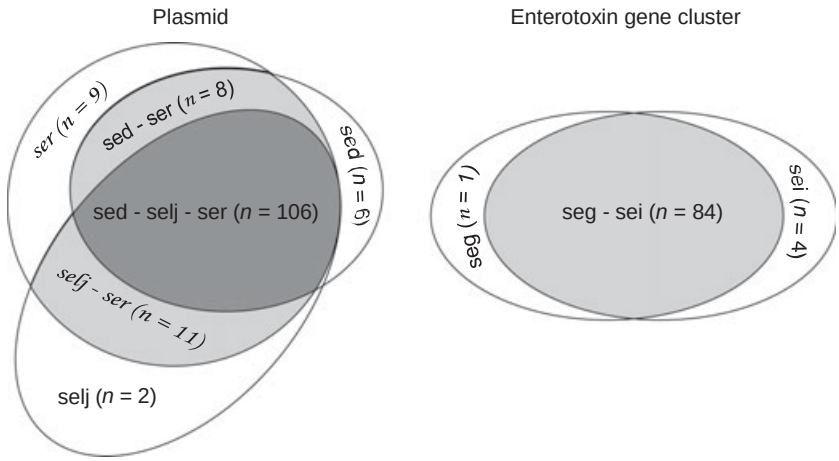

Figure 1 Number of isolates bearing SE genes and SE genes profiles located on Plasmid and EGC.

Table 2 Assay conditions for sea, seb, sec, sed, see, ser genes amplification tests

Gene	Primer	Sequence (5′–3′)	Concentration (lmol l^{-1})	Size (bp)	Reference
sea	GSEAR-1	GGT TAT CAA TGT GCG GGT GG	0·2	102	Mehrotra et al. (2000)
	GSEAR-2	CGG CAC TTT TTT CTC TTC GG	0·2		
seb	GSEBR-1	GTA TGG TGG TGT AAC TGA GC	0·2	164	Mehrotra et al. (2000)
	GSEBR-2	CCA AAT AGT GAC GAG TTA GG	0·2		
sec	GSECR-1	AGA TGA AGT AGT TGA TGT GTA TGG	0·2	451	Mehrotra et al. (2000)
	GSECR-2	CAC ACT TTT AGA ATC AAC CG	0·2		
sed	GSEDR-1	CCA ATA ATA GGA GAA AAT AAA AG	0·8	278	Mehrotra et al. (2000)
	GSEDR-2	ATT GGT ATT TTT TTT CGT TC	0·8		
see	SA-U	TGT ATG TAT GGA GGT GTA AC	0·6	213	Sharma et al. (2000)
	SA-E rev	GCC AAA GCT GTC TGA G	0·6		
ser	SER 1	AGA TGT GTT TGG AAT ACC CTA T	0·2	123	Chiang et al. (2008)
	SER 2	CTA TCA GCT GTG GAG TGC AT	0·2		

Table 3 Assay conditions for seg, seh, sei, selj, selp genes amplification tests

Gene	Primer	Sequence (5′–3′)	Concentration (lM)	Size (bp)	Reference
seg	SEG-F	GTT AGA GGA GGT TTT ATG	0·6	198	Bania et al. (2006)
	SEG-R	TTC CTT CAA CAG GTG GAG A	0·6		
seh	SEH-F	CAA CTG CTG ATT TAG CTC AG	0·4	173	Bania et al. (2006)
	SEH-R	CCC AAA CAT TAG CAC CA	0·4		
sei	SEI-F	GGC CAC TTT ATC AGG ACA	0·8	328	Bania et al. (2006)
	SEI-R	AAC TTA CAG GCA GTC CA	0·8		
selj	SEJ-F	GTT CTG GTG GTA AAC CA	1·0	131	Bania et al. (2006)
	SEJ-R	GCG GAA CAA CAG TTC TGA	1·0		
selp	SEP-F	TCA AAA GAC ACC GCC AA	0·8	396	Bania et al. (2006)
	SEP-R	ATT GTC CTT GAG CAC CA	0·8		