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Diversity of *Staphylococcus aureus* enterotoxin types within single samples of raw milk and raw milk products

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ABSTRACT

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Aim: To find out if testing of up to 10 *Staphylococcus aureus* isolates from each sample from raw milk and raw milk products for staphylococcal enterotoxin (SE) might increase the chances of identifying potential sources of food intoxication.

Methods and Results: Altogether 386 *S. aureus* isolates were tested for the presence of SE by reversed passive latex agglutination (SET-RPLA), and SE genes (*se*) by a multiplex polymerase chain reaction (PCR). In 18 of 34 (53%) *S. aureus* positive samples a mixture of SE and/or *se* positive and negative isolates were identified. Multiplex PCR increased the number of potential SE producing strains, i.e. isolates that harboured *se*, with 51% among the product and 48% among the raw bovine milk isolates. Examination by pulsed-field gel electrophoresis mostly confirmed clonal similarity among isolates sharing SE/*se* profile, but did not further differentiate between them.

Conclusions: Isolates of *S. aureus* collected from one sample may show great diversity in SE production and different plating media seem to suppress or favour different strains of *S. aureus*.

Significance and Impact of the Study: Several isolates of *S. aureus* from each sample should be tested for enterotoxin production in cases with typical SE intoxication symptoms with methods that are able to reveal new SE/*se*.

Keywords: multiplex PCR, pulsed-field gel electrophoresis, *Staphylococcus aureus* eneterotoxin, staphylococcal enterotoxin genes, reversed passive latex agglutination.

INTRODUCTION

Staphylococcus aureus food poisoning is an intoxication caused by ingestion of food containing staphylococcal enterotoxin (SE). It is characterized by an acute onset of nausea, vomiting, abdominal cramps and diarrhoea, and is one of the most common food-borne diseases in the world.

So far, 20 serologically distinct SEs have been identified. SEA, SEB, SEC, SED, SEE (Jones and Khan 1986; Betley and Mekalanos 1988; Couch *et al.* 1988; Bayles and Iandolo 1989) represent classical types, while SEG, SEH, SEI and SEJ are newly described enterotoxins (Ren *et al.* 1994; Su and Wong 1995; Munson *et al.* 1998; Zhang *et al.* 1998). SEC has been described with minor antigenic variations and designated SEC_1, SEC_2 and SEC_3 (Bergdoll *et al.* 1965; Avena and Bergdoll 1967; Reiser *et al.* 1984). Recent studies (Jarraud *et al.* 2001; Orwin *et al.* 2001; Letertre *et al.* 2003; Omoe *et al.* 2003) have described other SE genes (*se*); *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser* and *seu*) which point to the possible existence of new SE. Not all staphylococci are SE producers, or the amount of produced SE may be insufficient for food intoxication.

The detection of *S. aureus* and SE in food is often difficult. Food processing may kill the bacteria without destroying the thermostable SE. Methods currently used for

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direct detection of SE in food are enzyme-linked immunosorbent assays. These methods have limitations such as time consumption, cost and a detection limit of SE higher than the level required for staphylococcal intoxication. For presence of SE in bacterial culture, reversed passive latex agglutination (SET-RPLA) has been the method of choice. The detection limit of this method is <1 ng SE ml^{-1} and it is possible to detect and differentiate the five classical SE (SEA, SEB, SEC and SED) from bacterial isolates.

Sequencing of the genes encoding all the identified SE has given the opportunity for detection and differentiation of all *se* by the polymerase chain reaction (PCR) technique. The continuous identification of new SE, and the requirement for faster methods in the investigation of food poisoning, have led to the development of methods for simultaneous detection of all *se*, such as the multiplex PCR technique (Monday and Bohach 1999).

Recently there has been an increase in production and consumption of raw milk products in Norway and consequently increased attention to such products as a potential source of *S. aureus* food poisoning. An outbreak of *S. aureus* intoxication caused by raw milk cheese has been described (Kvellestad *et al.* 1988), and such products have also caused sporadic cases in Norway (S. Loncarevic, unpublished data). In many suspected staphylococcal food poisoning cases, however, the source for the illness has not been identified, and the number of cases is probably underestimated.

In cases of staphylococcal food poisoning or epidemiological investigations of food, it is common to only test one isolate of *S. aureus* for toxin production. For other bacterial species however, it has been shown that molecular typing and characterization of several isolates from the same food sample, obtained from different plating media, is necessary in order to associate a certain food item with food poisoning (Loncarevic *et al.* 1996, 1997).

The aim of this study was (i) to find out if testing of up to 10 colonies from each food sample might increase the identification of raw milk and raw milk products as potential sources for staphylococcal intoxication; (ii) to compare results from detection of SE (A-D) by SET-RPLA with the presence of the corresponding *se* shown by multiplex PCR; (iii) to explore clonal similarity between isolates with various SE/*se* profiles, by comparison of all isolates from one sample by use of restriction enzyme analysis (REA) with pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Isolates of *S. aureus* from raw milk and raw milk products

Staphylococcus aureus were collected from 26 samples of raw milk products and 18 samples of bovine and caprine bulk milk. The raw milk products were provided by 19 different producers and included fresh, soft, semi-hard and hard cheese from bovine (10), caprine (14) and reindeer (2) milk. Soft cheese included six imported cheese. The milk samples were taken from normal bulk milk at delivery to local dairies. To our knowledge, none of the samples had been involved in food poisoning.

The milk and milk products were analysed for the presence of *S. aureus*. Ten grams of each milk product were added to 90 g of sterile peptone water and stomached for 30–90 s. Three 10-fold dilutions were made and 0·1 ml of each step was inoculated on bovine blood agar (Oxoid, Basingstoke, Hampshire, UK) with washed erythrocytes (BA) and Baird Parker with Rabbit Plasma Fibrinogen supplement (BP + RPF) (bioMèrieux, Marcy-l'Etoile, France). The plates were incubated for 48 h at 37°C. Typical colonies were counted after 24 and 48 h. Typical colonies from both plates were investigated further. Up to five typical and three atypical presumptive *S. aureus* colonies from both blood agar plates and BP-RPF were further investigated. Gram-positive cocci that were catalase and coagulase positive and that exhibited growth on P-agar with 7 mg l^{-1} acriflavin (Roberson *et al.* 1992) were considered *S. aureus*.

In total 386 *S. aureus* isolates from the raw milk products (209 isolates) and raw bovine (100) and caprine (77) milk were freeze-stored at −70°C in heart infusion broth with 15% glycerol before further investigation.

SE production test by SET-RPLA

Up to 10 isolates from each sample were tested for enterotoxin production (SEA to SED) by SET-RPLA assay (Oxoid). The isolates were grown aerobically on blood agar plates for 18–24 h, followed by inoculation into tryptic soya broth (Difco, Detroit, MI, USA) and incubation at 37°C for 18–24 h. Testing with SET-RPLA was there after performed according to the manufacturer's instructions.

se identification by multiplex PCR

Targeting *se* identification and characterization of the same isolates as tested with SET-RPLA were performed according to the method of Monday and Bohach (1999), with some modifications recommended by Løvseth *et al.* (2004). After the DNA isolation from overnight growth bacterial culture, amplification of selected *se* was obtained by use of 10 primer sets divided into two reaction mixtures (*sed*, *see*, *seg*, *sei* and *sea*, *seb-sec*, *sec*, *seh*, *sej*). DNA was amplified in a MJ Research thermocycler (MJ Research, Waltham, MA, USA) followed by determination of PCR product by electrophoresis and visualization on a UV-transilluminator (Syngene; Syoptics Ltd, Cambridge, UK). Product sizes were estimated using a pUC-mix molecular weight ladder (Fermentas, Vilnius,

Lithuania). 16S rRNA was used as a control for DNA isolation and presence of bacterial DNA in the PCR reaction.

REA with PFGE

Isolates tested by SET-RPLA and multiplex PCR from 20 samples were examined for clonal similarity by REA-PFGE. DNA from *in situ* lysed bacterial cells in chilled agarose-plugs was prepared according to the PulseNet protocol (McDougal *et al.* 2003). One colony of each *S. aureus* strain was incubated aerobically in brain heart infusion (BHI) broth (Difco) at 37°C for 18–24 h. The cells were harvested by centrifugation in a fixed angle rotor at 15 000 *g* for 3–4 min and after aspiration of the supernatant, the pellet was resuspended in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8·0). After incubation at 37°C in water bath for ≥10 min, 1 mg ml^{-1} Lysostaphin (Sigma-Aldrich) was added to the cell suspension and dispensed into slotformers. Solidified agarose plugs were incubated in a lysis buffer at 37°C for at least 4 h followed by rinsing of plugs with TE buffer for at least four times. Restriction enzyme digestion was carried out using 200 μl of digestion buffer containing 30 units of *Sma*I (Boehringer-Mannheim) enzyme. After enzyme incubation at 30°C for at least 3 h, the plugs were placed in 1% SeaKem Gold agarose (BioWhittaker, Molecular Applications, Rockland, ME, USA) and electrophoresed in a CHEF DR-III-System (Bio-Rad, Richmond, CA, USA). An 19 h migration period at 200 V with a pulse time of 5 s/40 s was used. After running, the gel was treated with ethidium bromide solution (final concentration 1 μg ml^{-1}) for 20 min and destained for 45 min in fresh distilled water. The patterns were visualized with shortwave u.v. light and documented (Syngene; Syoptics Ltd).

RESULTS

Staphylococcal enterotoxin production and presence of *se* in 386 isolates of *S. aureus* obtained from 18 samples of raw milk and 26 samples of raw milk products are shown in Table 1. SE or *se* were detected in isolates from 10 and 24 *S. aureus* positive samples, respectively, while no SE or *se* were detected from the remaining eight and two samples respectively. The diversity in SE production and presence of *se* among the isolates is shown in Tables 2 and 3.

Twenty-one of the 34 (61·7%) samples of raw milk and raw milk products producing SE and/or harbouring *se* contained a mixture of *S. aureus* isolates that were positive and negative for SE and/or *se*. The remaining 23 samples contained isolates that either were all negative for SE or *se* (10) or all positive (13).

The greatest diversity of SE types was shown in a sample of imported raw milk soft cheese sample no. 400 (Table 3; Fig. 1).

Table 1 Number of enterotoxin producing *Staphylococcus aureus* isolates found in raw milk and raw milk cheese by use of SET-RPLA and multiplex PCR

Origin	No. of samples	No. of isolates	SET-RPLA (no. of SE)		PCR (no. of *se*)	
			Samples	Isolates	Samples	Isolates
Caprine milk	8	77	6	51	6	51
Bovine milk	10	100	2	12	4	22
Raw milk products	26	209	13	70	24	142
Total	44	386	21	133	34	215

Table 2 SE production and *se* (SE genes) in *Staphylococcus aureus* isolates obtained from raw milk on BP-RPF and BA, detected by use of SET-RPLA and multiplex PCR

		No. of isolates		SET-RPLA		PCR						
				No. of positive isolates		No. of positive isolates						
				BP-RPF	BA	BP-RPF			BA			
Origin	Samples	BP-RPF	BA	SEC	SEC	*sec*	*seg*	*sei*	*seb*	*sec*	*seg*	*sei*
Caprine milk	2394	5	5	4		4						
	2396	5	5	5	5	5				5		
	2399	5	5	5	5	5				5		
	Sg213	5	5	5	5	5				5		
	Sg214	4	5	4	5	4				5		
	Sg215	5	3	5	3	5				3		
Bovine milk	2204	5	5	5	5	5				5		
	SK254	5	5				1	5			3	5
	SK262	5	5						1			
	SK265	5	5	2		2						

Table 3 SE production and *se* (SE genes) in *S. aureus* isolates obtained from raw milk cheese on BP-RPF and BA detected by use of SET-RPLA and multiplex PCR

	No. of isolates		SET-RPLA (no. of positive isolates)			PCR (no. of positive isolates)										
			BP-RPF		BA	BP-RPF						BA				
Cheese sample nos	BP-RPF	BA	SEC	SED	SEC	*sec*	*sed*	*seg*	*sei*	*sej*	*seh*	*seb*	*sec*	*seg*	*sei*	*seh*
29-1	5	5						5	5					1		
29-2	5	4						5	5							
29-3	5	5						5	4							
294	5	5						5	5					2		
29-5	5	5						5	5					1		
400	5	5		1			1	4	4	1		2		2		
882-1	5	5			1								1			
927-10	5	5	3		3	3							3			
29	3	5	2		4	2								4		
35	5	5							5				1		4	
37	3	2							3						2	
62	5	1						1	1							
64	5	5							2					3	3	
65	5	5	5		4	5							4			
66	1	3	1		2	1							2			
68	0	2			2								2			
69	5	3	5		2	5							2			
71	3	5	2		5	2							5			
74	2	5									2					5
75	5	5						3	3		2					3
79	1	5	1		5	1							5			
80	4	0	4			4										
82	0	6			6								6			
83	4	10	4		8	4							8			

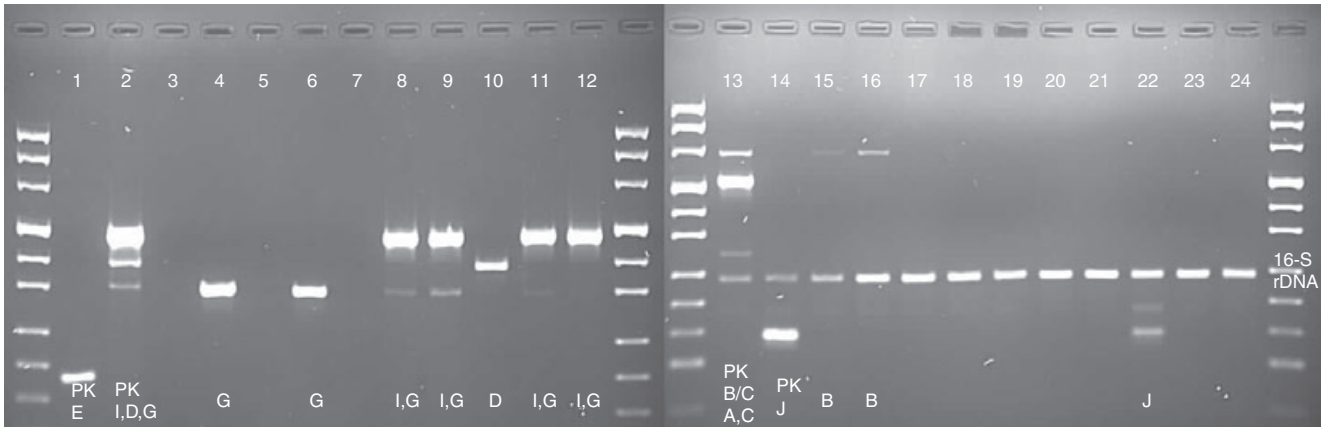

Fig. 1 *se* genes in *S. aureus* isolates (lines 3–24) from cheese sample no. 400 obtained by multiplex PCR. Lane M marker; lane 1, FRI913 (positive control) *see*; lane 2, R5010 (positive control) *sei*, *sed*, *seg*; lane 4, isolate nr.319, *seg*; lane 6, isolate no. 321, *seg*; lane 8, isolate no. 323, *sei*, *seg*; lane 9, isolate no. 324, *sei*, *seg*; lane 10, isolate no. 325, *sed*; lane 11, isolate no. 326, *sei*, *seg*; lane 12, isolate no. 327, *sei*, *seg*; lane 13, FRI913 (positive control) *seb/c*, *sea*, *sec*; lane 14, R5010 (positive control) *sej*; lane 15, isolate no. 318, *seb*; lane 16, isolate no. 319, *seb*; lane 22, isolate no. 325, *sej*. Lane 5, isolate no. 320, lane 7, isolate no. 322, no *see*, *sed*, *seg* or *sei* were detected. Lanes 17–21, 23 and 24, isolates nos 320–324, 326 and 327, no *sea*, *seb*, *sec* or *sej* were detected. 16S rDNA, positive control for presence of bacterial DNA in the PCR reaction

The multiplex PCR technique increased the number of potentially SE positive isolates, i.e. isolates harbouring *se* with 51% among the products and 48% among the raw bovine milk isolates. All except four isolates that harboured classical *se* also produced the corresponding SE. There was no difference observed between detection of SE and *se* in isolates from raw caprine milk isolates.

Isolates from 10 raw milk product samples were positive for one or more of the newly described *se* (*seg–sej*) without producing classical SEs or harbouring their genes. Among the 122 isolates harbouring new *se*, *seg* and *sei* were predominant (39·3 and 46·7%). *Seg* and *sei* in combination were found in 34·4% of these isolates.

Tables 2 and 3 show various abilities among isolates of *S. aureus* from the same sample to produce SEs or harbour *se*, related to the method used (SET-RPLA and multiplex PCR), and the agar (BA and BP-RPF) from they were recovered. All these SE positive isolates produced only one 'classical' toxin type, SEC, identified by SET-RPLA and confirmed by multiplex PCR technique (Table 3). Difference between the total number of SE-producing and *se*-harbouring isolates from all raw milk and raw milk products from BA and BP-RPF, was not observed. However, isolates with *seg* and *sei* in combination were found more often among the isolates obtained from BP-RPF (33) than from BA (10).

By examination of clonal similarity among isolates from 20 samples by REA-PFGE, 14 DNA restriction pattern groups were observed. From single sample, distinguishable patterns were usually identified in isolates containing and not containing SE and/or *se*. However, one of five isolates that contained SEC/*sec* obtained from BP-RPF from a raw caprine milk sample (no. 2396) showed a different DNA restriction pattern from the other four isolates. *S. aureus* isolates that produced SEC and contained *sec*, obtained from different samples of raw caprine milk (nos 213, 214, 215) and cheeses from raw caprine milk (nos 79, 82 and 83) showed identical DNA restriction patterns. The greatest diversity in DNA restriction patterns was shown among isolates from cheese sample no. 400. Characterization of these 10 isolates by REA-PFGE (Fig. 2) showed that two isolates harbouring *seg/seb* and *seg* had identical DNA restriction pattern (clone §). Four other *sei* and *seg* positive isolates, from the same cheese, also displayed identical pattern (clone £). The other isolates were different clones of *S. aureus*.

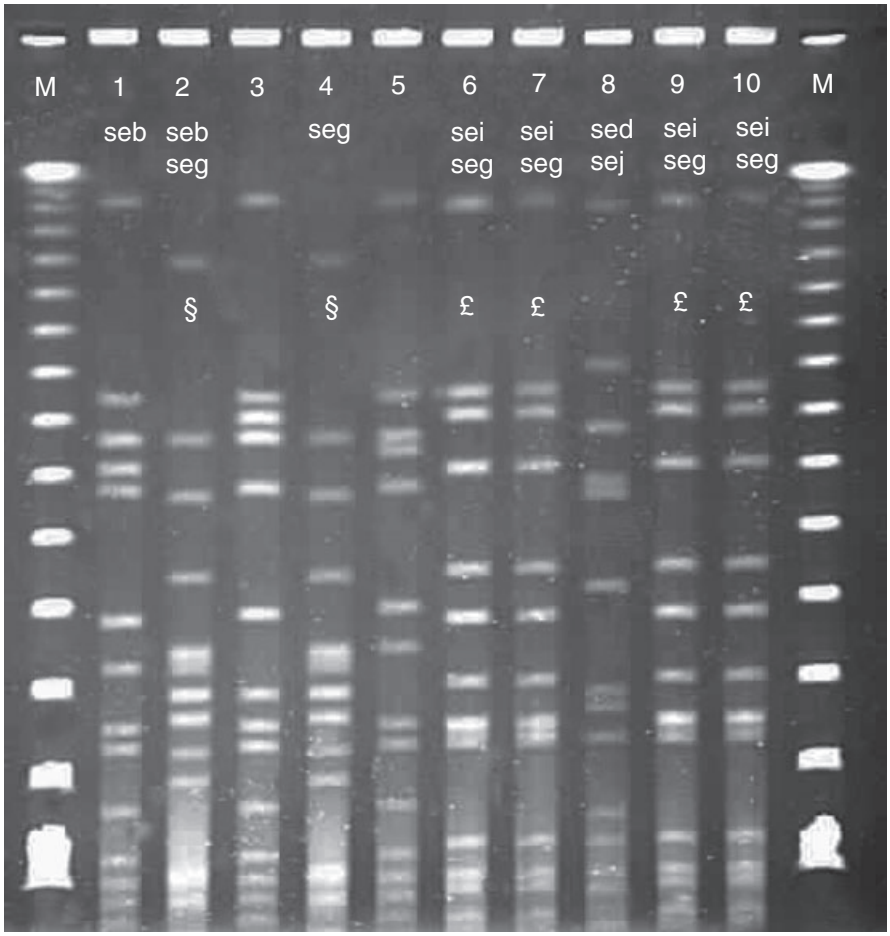

Fig. 2 PFGE profiles of *S. aureus* isolates obtained from cheese sample no. 400. Lane M, Lambda Ladder PFG Marker (BioLabs). Isolates no. 319 producing *seg* and *seb* (lane 2) and 321 producing *seg* (lane 4) had identical DNA restriction patterns (clone §). Isolates nos 323, 324, 326 and 327 producing *sei* and *seg* (lanes 6, 7, 9 and 10) displayed identical DNA restriction patterns (clone £)

DISCUSSION

Examination of SE production and the presence of *se* in *S. aureus* isolated from 44 samples of raw milk and raw milk products revealed considerable diversity in the *S. aureus* population both among and within single samples. Altogether 53% of the samples contained a mixture of SE and/or *se* positive and negative isolates. Testing of up to 10 colonies instead of only one from each food sample increased the chance of identifying a potential source of staphylococcal intoxication. In cases where the concentration of an enterotoxin-producing strain of *S. aureus* has been high enough to cause intoxication, the *S. aureus* population in the food item may be less diverse. Examination of only one 'wrong' isolate of *S. aureus* from a suspected food item can, however, lead to missing the source of intoxication. Determination of SE-production and presence of *se* in several colonies of *S. aureus* from the incriminated food is therefore recommended.

Considerable variation in SE production and presence of *se* was observed among the isolates from raw bovine milk and raw milk products. The multiplex PCR technique doubled the number of potentially enterotoxin-producing isolates from these samples compared with SET-RPLA. No difference was observed between SE and *se* positive isolates by the two methods among isolates from raw caprine milk, probably because all SE positive isolates produced only one classical toxin type, SEC.

In all isolates where production of classical SE was identified by SET-RPLA, the presence of *se* was confirmed by the multiplex PCR technique. In contrast, toxin production indicated by *seb* and *sec* in four isolates was not verified with SET-RPLA. This finding may be explained by lower sensitivity of the SET-RPLA, or by the fact that detection of *se* does not necessarily indicate production and biological activity of the toxin.

As SET-RPLA, which is the most common laboratory method for detection of SEs from bacterial strains, is designed to detect only classical SE (SEA-SED), underestimation of potentially SE producing isolates may be expected. Availability of DNA sequence information of all described *se* and development of PCR methods has, however, given the opportunity to overcome these problems. In a UK study the incidence of potentially SE-positive isolates increased from 54 to 79% when this technique was used (McLauchlin *et al.* 2000). Rosec and Gigaud (2002) observed that the percentage of enterotoxigenic *S. aureus* dramatically increased from 30 to 60% among the foodborne strains when new *se* were included in the investigation. As a number of sporadic cases and foodborne outbreaks of staphylococcal intoxication is unsolved, the possibility of *S. aureus* producing new SE in food should be taken in consideration. Other SE than A-D are not yet well characterized, but some of them (SEG, SEH, SEI and SEJ) produce emetic reaction in monkeys (Genigeorgis 1989; Bergdoll 1990; Ren *et al.* 1994) and SEH has already been involved in a food-poisoning case (Pereira *et al.* 1996).

Isolates from the samples of raw milk products harboured newly described *se* without producing classical SE types more often (13/24 samples) than isolates from raw milk (one of four samples). McLauchlin *et al.* (2000) detected *se* fragments from newly described *se* in 26% of 129 *se* positive isolates without revealing SEA-SED either by PCR or SET-RPLA.

A notable finding was that 27·9% of 215 PCR-positive isolates in our investigation harboured *seg* and *sei* genes, while 5·1 and 11·8% strains harboured *seg* and *sei* alone respectively. Rosec and Gigaud (2002) reported that 80·6% of 155 PCR-positive isolates harboured *seg* and *sei*. McLauchlin *et al.* (2000) observed that over 45 of 129 (35%) isolates harboured *seg* and/or *sei*, while *seg* alone was associated with 16% of incidents. Systematic association of *seg* and *sei* genes also observed in this and other studies suggests that they are reservoir or part of a reservoir for enterotoxin gene rearrangement in *S. aureus* (Rosec and Gigaud 2002), as has been hypothesized for the region where *sei* was identified and sequenced (Munson *et al.* 1998).

We know from previous studies that one food sample may yield different serovars and clones of the same bacterial species, depending on the method used (Loncarevic *et al.* 1996). In the present study, one selective (BP-RPF) and one nonselective medium (BA) were employed. The number of *S. aureus* isolates potentially producing classical types of SEs was much higher from BA both with SET-RPLA and PCR (42 and 46 respectively) than from BP-RPF (28 and 28). In contrast, the selective medium revealed a higher number of isolates harbouring new *se* (96) than BA (26). The indication that certain strains of a bacterial species may be suppressed or favoured by different media is in agreement with the statement of Lewis and Corry (1992) and Loncarevic *et al.* (1996). To prevent that a causative *S. aureus* is missed, it is important to examine colonies from different media in a case of staphylococcal intoxication.

In the present study, 10 samples contained *S. aureus* isolates with two to six different SE/*se* profiles and up to six different REA-PFGE patterns. In one raw milk product sample (no. 400) the 10 isolates showed six different *se* profiles (*seb*, *sed*, *sei*, *seg*, *sej* and combinations), while only one isolate produced SE (SED). Except for two isolates harbouring *seg*/*seb* and *seg* showing identical DNA restriction pattern, isolates with the same toxin profile shared identical PFGE patterns, and those with different toxin profiles also yielded different patterns. It was not surprising that a cheese sample showed the highest diversity of *S. aureus* as the potential sources of bacterial contamination

of raw milk cheese are multiple; raw milk possibly from several farms, the processing environment and personnel.

In conclusion, several isolates of *S. aureus* should be tested for enterotoxin production in cases where typical symptoms of SE intoxication are observed. Different plating media seem to suppress or favour different strains of *S. aureus*. Methods with ability to reveal new *se* (G–J), such as multiplex PCR, should be used in solving food poisoning cases. REA-PFGE mostly confirmed clonal similarity among isolates sharing SE/se profile and did not further differentiate between them.

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