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1    **DETECTION OF CLASSICAL ENTEROTOXINS AND IDENTIFICATION OF**  
2    **ENTEROTOXIN GENES IN *STAPHYLOCOCCUS AUREUS* FROM MILK AND**  
3    **DAIRY PRODUCTS**

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1 **Abstract**

2 Milk and dairy products are frequently contaminated with enterotoxigenic  
3 *Staphylococcus aureus*, which is often involved in staphylococcal food poisoning. The  
4 distribution of genes encoding staphylococcal enterotoxins (SE) in *S. aureus* isolated  
5 from bovine, goat, sheep and buffalo milk and dairy products was verified by the  
6 presence of the corresponding SE production. A total of 112 strains of *S. aureus* were  
7 tested for SE production by immuno-enzymatic (SEA-SEE) and reversed passive latex  
8 agglutination (SEA-SED) methods, while multiplex-PCR was applied for SE genes  
9 (*sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel*). Of the total strains studied, 67% were detected  
10 to have some SE genes (*se*), but only 52% produced a detectable amount of the classic  
11 antigenic SE types. The bovine isolates frequently had enterotoxin SEA, SED and *sej*,  
12 while SEC and *sel* predominated in the goat and sheep strains.  
13 The results demonstrated i) marked enterotoxigenic *S. aureus* strain variations, in  
14 accordance with strain origin, ii) the two methods resulted in different information but  
15 concurred on the risk of foodstuff infection by *S. aureus* .

16

17 **Keywords:** *Staphylococcus aureus*, staphylococcal enterotoxins, SE genes, milk,  
18 multiplex-PCR, reverse passive latex agglutination.

19

20

## 1 **1. Introduction**

2 *Staphylococcus aureus* is considered the world's third most important cause of food-  
3 borne illnesses (Tirado and Schimdt, 2001).

4 The ability of *S. aureus* to grow and produce SEs under a wide range of conditions is  
5 evident from the variety of foods implicated in staphylococcal food poisoning (Le Loir  
6 et al., 2003). Indeed, milk is a good substrate for *S. aureus* growth, and dairy products  
7 are a known source of intoxication (De Buyser et al., 2001; Jorgensen et al., 2005). *S.*  
8 *aureus* contamination can occur through the presence of *S. aureus* in the raw milk itself  
9 or during its processing (Brasca et al., 2005).

10 Although pasteurization kills *S. aureus* cells, thermostable SEs generally retain their  
11 biological activity (Evenson et al., 1988; Asao et al., 2003). Thus, because of the  
12 importance of these toxins in the public health and food sectors, an efficient screening  
13 to detect the prevalence of enterotoxigenic strains in foods is required. Indeed, not all  
14 staphylococci produce SE, and SE production may be insufficient for food intoxication.  
15 Traditionally, classic antigenic SE types have been recognized: SEA, SEB, SEC<sub>1</sub>, SEC<sub>2</sub>,  
16 SEC<sub>3</sub>, SED and SEE (Bergdoll et al., 1973). During the 1990s new SEs (SEG, SEH, SEI  
17 and SEJ) were reported, and their genes described (Su and Wong 1995; Munson et al.,  
18 1998; Zhang et al., 1998; Zschock et al., 2005). More recent data resulting from partial  
19 or complete genome sequence analyses have led to the description of further "new" *se*  
20 genes: *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser* and *seu* (Fitzgerald et al., 2001; Jarraud et al.,  
21 2001; Orwin et al., 2001; Omoe et al., 2002; Orwin et al., 2003; Letetre et al., 2003).  
22 The role these new SEs play in food poisoning has not yet been clarified (Vernozy-

1 Rozand et al., 2004, Boerema et al., 2006): some are reported to lack emetic activity  
2 while others still have to be tested. Consequently it has been proposed to designate them  
3 “staphylococcal enterotoxin-like” (SEL) (Lina et al. 2004).

4 The currently available enzyme-linked immunosorbent and fluorescent assay kits,  
5 ELISA and ELFA, detect only SEA, SEB, SEC, SED and SEE. However the advent of  
6 molecular techniques, together with the availability of DNA sequence information for  
7 all the described SEs, have led to the use of the Polymerase Chain Reaction (PCR)  
8 method to detect specific enterotoxin gene sequences. The present study used PCR to  
9 analyze the distribution of genes encoding *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel* in *S.*  
10 *aureus* isolated from bovine, goat, sheep and buffalo raw milk and milk products. The  
11 results of this multiplex PCR (mPCR) method were compared with the corresponding  
12 SE shown by reverse passive latex agglutination (SET-RPLA) assay.

13

## 14 **2. Materials and methods**

### 15 **2.1 Source and identification of *S. aureus* strains**

16 A total of 112 *S. aureus* strains were isolated from different dairy products (milk, curd,  
17 1–2 month old cheeses, butter and whey) from different Italian regions and animal  
18 species (cow, goat, sheep and buffalo). The strains were isolated from 86 raw milk  
19 samples and 26 samples of raw milk products. To highlight the possible differences in  
20 the isolates of different origin, only one strain for each sample was considered.

1 The strains were isolated using Baird Parker agar with a rabbit plasma fibrinogen  
2 supplement (BP + RPF) (Biolife, Milan, Italy) incubated at 37°C for 24-48 h to detect  
3 coagulase positive staphylococci (ISO 6888 1/2: 1999).

4 The *S. aureus* identification was done by Gram staining, catalase activity determination,  
5 heat stable nuclease (TNase) test using Toluidine blu agar (Oxoid, Milan Italy)  
6 according to ISO 8870:2006, and haemolysis on blood agar (Merck).

7 A miniaturized biochemical system (Biolog GP Microplate, Biolog, Inc., Hayward, CA,  
8 USA) was used to confirm the staphylococcal species. Strains were maintained and  
9 propagated in Brain Heart Infusion broth (Oxoid) and incubated at 37 °C overnight.

10 The identification, coagulase and TNase detection were also confirmed by mPCR  
11 analysis, as previously described by Cremonesi et al. (2005).

12

### 13 **2.2 Detection of *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel***

14 DNA was extracted according to Cremonesi et al. (2006) from one milliliter of  
15 overnight grown bacterial culture, incubated in BHI broth at 37 °C and containing  
16 approximately 1-3 billion cells (in parallel, total sample counts were obtained with  
17 BP+RPF agar plate). Approximately, an average of 10 µg of DNA was obtained from  
18 10<sup>8</sup> cfu/mL.

19 After DNA isolation, amplification of selected genes (enterotoxin genes *sea*, *sec*, *sed*,  
20 *seg*, *seh*, *sei*, *sej* and *sel*) was achieved using 11 primer sets in the one reaction mixture.

21 DNA was amplified in a thermocycler (Mastercycler® ep, Eppendorf, Hamburg,

1 Germany) followed by determination of the PCR product by electrophoresis, and  
2 visualization by UV-transilluminator (Uvitec, Cambridge, UK).

3 As several studies have described that none of the investigated strains isolated from  
4 bovine and goat milk, and related dairy products, harbor any of the *seb*, *see* and *sek*  
5 genes, the primer pairs related to these three genes were not used when the  
6 amplification protocol for mPCR assay was established (Cremonesi et al. 2005).

7

8

### 9 **2.3 Specificity testing**

10 The specificity of the primers was tested by individually analyzing each primer pair, and  
11 the primer pair combined in the Multiplex PCR. The positive controls used were *S.*  
12 *aureus* ATCC 19095 FRI 137 (*sec*, *seh*, *seg* and *sei*), ATCC 23235 (*sed*, *seg*, *sei* and  
13 *sej*), ATCC 700699 (*sea*, *sec*, *seg*, *sei*, *sel*) and ATCC 25923 (*seg*, *sei*). Furthermore,  
14 strains of *S. intermedius*, *S. hycus* and *S. epidermidis*, obtained from the CNR ISPA  
15 bacterial collection, were used as negative controls for the PCR reactions.

16

### 17 **2.4 Production and detection of SEA, SEB, SEC, SED and SEE**

18 The ability for enterotoxin production was evaluated on *S. aureus* strains incubated in  
19 BHI broth for 24 h at 37 °C, and containing approximately 1-3 billion cells. The  
20 enzyme-linked fluorescent immunoassay (ELFA) VIDAS SET Test (bioMérieux, RCS  
21 Lyon, France) was used to detect the enterotoxin production of SEA to SEE without  
22 distinguishing the individual toxins, while SET-RPLA (Oxoid) was used to distinguish

1 the SEA, SEB, SEC and SED enterotoxins when SEs were detected (Bennet and  
2 McClure, 1999; Orwin et al., 2003).

3

### 4 **3. Results**

5 All 112 isolates were identified by mPCR reaction as belonging to *Staphylococcus*  
6 *aureus*. The Biolog GP identification was always in agreement with that of the PCR  
7 reaction. For 21 strains, 16 isolated from cow, 4 from goat and 1 from buffalo, the  
8 Biolog system did not give any identification at the species level, only at the genus level  
9 (*Staphylococcus* spp.).

10 All the cultures were positive for catalase, haemolysis, coagulase and thermonuclease  
11 reactions, and for coagulase and thermonuclease the results of the microbiological  
12 methods were confirmed by genotype methods.

13

#### 14 **3.1 Detection of *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel***

15 The presence of *se* genes in *S. aureus* isolates is shown in Table 1.

16 Of the 112 *S. aureus* isolates examined, 75 (67%) were found to be positive for one or  
17 more toxin genes and demonstrated marked genotype variability, so much so that we  
18 subdivided them into 17 groups according to gene presence (Table 2).

19 Twenty strains possessed only one type of toxin gene (13 *sea*, 2 *sed*, 1 *seg*, 3 *seh* and 1  
20 *sei*), while the remaining 55 harbored more than one toxin gene.

21 With regard to the 75 *se* positive isolates, 19 (25%) *S. aureus* enterotoxin producers (17  
22 isolated from cow and 2 from goat) produced classical *se*, while only 8 strains (6 from

1 cow and 2 from sheep samples) were positive for one or more of the newly described *se*  
2 (*seg-sel*) without harboring classical *se*.

3 Classical and new *se* were found in combination in the major part of the strains (64%)  
4 (31 from cow, 10 from goat and 7 from sheep dairy products).

5 The enterotoxins *sea* and *sed*, found in 36 and 37 strains respectively, were the most  
6 frequent among the toxins looked for, while *sej* was the most widespread of the new  
7 enterotoxins (Table 1). In fact 31 strains possessed the gene for *sej*, and in 27 of these it  
8 was found associated with *sed*.

9 None of the *S. aureus* strains isolated from goat products had only new enterotoxin  
10 genes, while 6 of the cow isolates and 2 from sheep samples were positive only for *seg*,  
11 *seh*, *sei*, *sej* and *sel*. In 2 of the 6 isolates from cow we detected the presence of *seg* and  
12 *sei* genes, in 3 the presence of *seh* and in 1 the *sei* gene. Two strains coming from sheep  
13 products were found to have *seg* and *seg-i* genes.

14 In general, there was considerable variation in the *se* gene types among the isolates of  
15 different origin.

16 On comparing the data relative to the strains isolated from cow, goat, sheep and buffalo  
17 milk products, 54 of the 71 (76%) strains from cow were positive to *se*, and in these  
18 samples the enterotoxins *sea*, *sed* and *sej* were found more frequently. Only one strain  
19 proved to be a producer of the type C enterotoxin (Table 2).

20 Twelve of the 22 *S. aureus* (55%) isolated from goat dairy products harbored *se* genes,  
21 and the enterotoxins *sec* and *sel* predominated, each of these being found in 7 strains.

1 A similar toxin pattern was noticed in *S. aureus* isolated from sheep. In fact 53% of the  
2 isolates produced enterotoxins, and *sec* and *sel* were the most widespread. The two  
3 strains isolated from buffalo did not produce staphylococcal enterotoxins.

4

### 5 **3.2 Production and detection of SEA, SEB, SEC, SED and SEE**

6 The results obtained by the two immunoassay approaches were always in agreement and  
7 only 58 of the 112 tested strains were found to be enterotoxin producers (Table 4).

8 With this type of determination, the SEA enterotoxin was found to be the one most  
9 present (31 positive strains), followed by SED (24) and SEC (14). Furthermore,  
10 investigating the *S. aureus* isolates for toxin formation by SET RPLA assay and ELFA  
11 revealed that only one of the strains produced SEB and none the SEE toxin (Table 3).

12 The SE production shown by SET-RPLA, and the existence of *se* detected by multiplex  
13 PCR, corresponded to 80% of the all *S. aureus* isolates analyzed by both methods  
14 (Table 4).

15 Twenty-three strains showed a lack of correlation between the presence of enterotoxin  
16 genes and their expression. In fact, non-correspondence was observed in 12 *se*-positive  
17 for which no toxin production was verified, while for the other 8 strains there was only  
18 partial correspondence between the presence of the enterotoxin genes and their  
19 expression. Six isolates were SEA positive, but showed the presence of the genes *sea*  
20 and *sed*, one strain was *sec-sed* positive but produced only SEC, and one strain showed  
21 the presence of *sea* and *sed* genes but the commercial kit detected enterotoxin type D.  
22 Only 3 strains, all isolated from sheep products, were found to be toxin producers, but

1 the mPCR did not show the presence of corresponding genes. Two of these strains were  
2 SEA producers, but no *sea* genes were detected; only 1 isolate was SEC positive,  
3 harboring only *seg* and *sei* genes. Toxin B was produced by 1 strain, but the  
4 corresponding gene was not identified as it was not detected by the mPCR.

5

#### 6 **4 Discussion**

7 On comparing the results of the two methods, it can be seen that the mPCR technique  
8 revealed a higher number of potential enterotoxin-producing strains (Table 1,4). Indeed,  
9 in a high percentage of isolates where classical SE production was identified by SET-  
10 RPLA (with limitation to A, B, C and D toxins), the presence of *se* was confirmed by  
11 the mPCR technique.

12 The PCR assay results revealed good correlation with the results of the immunoassay  
13 methods, even if in 12 cases the strains were positive only on molecular analysis.

14 This finding may be explained by the lower SET-RPLA sensitivity, or by the fact that *se*  
15 detection does not necessarily indicate toxin production and biological activity  
16 (Loncarevic et al., 2004).

17 Instead, for what concerns SEA and SEC, the fact that these could not be detected by  
18 PCR in 3 isolates (all from sheep) could be an indication of the existence of sequence  
19 variations in *se* genes, or a not yet described toxin that cross reacts in an immunological  
20 way with SEA and SEC. Isolates, positive to a certain degree for SEA by SET-RPLA  
21 but negative for *sea* by PCR, have also been observed in animals other than sheep by  
22 Mc Lauchlin (2000) and Jorgensen (2005).

1 Furthermore, our data show that SEs and *se* genes are in close correlation with the *S.*  
2 *aureus* strain origin, a higher ratio of strains isolated from cow producing SEA, SED  
3 and *sej*, while the strains from goat and sheep produced mainly SEC and *sel* (Table 2).

4 These findings are in agreement with Normanno et al (2005) who reported frequent  
5 enterotoxin A and D findings in many Italian cow dairy products. With regard to goat  
6 and sheep *S. aureus* enterotoxin production, our results are again in full agreement with  
7 other statements, those of Scherrer et al. (2004), Loncarevic et al. (2004), Jorgensen et  
8 al.(2005), and da Silva et al. (2005).

9 However some of our findings do differ from what was shown in studies from other  
10 countries; such studies indicate SEC as the enterotoxin most frequently produced by the  
11 tested cow strains (Stephen et al., 2001; Loncarevic et al., 2004; Jorgensen et al., 2005;  
12 Katsuda et al 2005; da Silva et al., 2005), and SEA was usually typical of human  
13 isolates (Orden et al., 1992).

14 The results of our study, placed alongside and compared with those of other countries,  
15 confirm and extend the conclusion that there is very marked geographical variation in  
16 the distribution of the enterotoxigenic *S. aureus* strains (Larsen et al., 2000).

17

## 18 **5. Conclusion**

19 The strains of *S. aureus* isolated from cow, goat and sheep raw milk and dairy products  
20 have an heterogeneous enterotoxigenic potential.

1 The immunoassay methods used to detect SE in bacterial strains can detect only  
2 classical SE (from SEA to SEE), thus an underestimation of the potential SE producing  
3 isolates must be expected if only these methods are used.

4 However the availability of DNA sequence information on the described *se*, and  
5 developed PCR methods, now give the opportunity to evaluate which strains harbor the  
6 *seg-sel* toxin genes undetectable by immunoassay.

7 The question that still remains concerns the evaluation of the effective hazard these new  
8 toxins present, and whether the strains harboring *se* produce these toxins in sufficient  
9 amount to cause food intoxication.

10

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1 **Figure captions**

2

3 **Table 1:** Distribution of *se* genes and SE production in *S. aureus* according to the origin  
4 of the isolates.

5 **Table 2:** Distribution of *sea-sel* genes in 75 *S. aureus* strains according to the origin of  
6 the isolates.

7 **Table 3:** Reverse passive latex agglutination detection of the SEA, SEB, SEC and SED  
8 in *S. aureus* strains and enterotoxin genes by mPCR .

9 **Table 4:** Detection of *se* genes and SEs produced by pure cultures of *S. aureus* of  
10 different isolate origins.

11

12

Tab. 1

	Strains pos. (%)	Origin of isolates pos. (%)			
		Cow	Goat	Sheep	Buffalo
<i>se</i>	75 (67)	54 (76)	12 (55)	9 (53)	0 (0)
<b>none</b>	37 (33)	17 (24)	10 (45)	8 (47)	2 (100)
<b>SE</b>	58 (52)	39 (55)	10 (45)	9 (53)	0 (0)
<b>none</b>	54 (48)	32 (45)	12 (55)	8 (47)	2 (100)

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Tab 2

	N of Strains	Origin of isolates			Multiplex-PCR							
		Cow	Goat	Sheep	<i>sea</i>	<i>sec</i>	<i>sed</i>	<i>seg</i>	<i>seh</i>	<i>sei</i>	<i>sej</i>	<i>sel</i>
Classical toxins	13	11	2	-	+	-	-	-	-	-	-	-
	4	4	-	-	+	-	+	-	-	-	-	-
	2	2	-	-	-	-	+	-	-	-	-	-
Classical and new	14	13	1	-	+	-	+	-	-	-	+	-
	1	1	-	-	+	-	-	+	-	-	-	-
	1	1	-	-	+	-	+	+	-	+	+	-
	1	1	-	-	+	-	+	+	-	-	+	-
	1	-	1	-	+	-	-	+	-	+	-	-
	1	-	1	-	+	-	-	+	+	+	-	-
	13	13	-	-	-	-	+	-	-	-	+	-
	1	1	-	-	-	-	+	+	-	-	+	-
	14	-	7	7	-	+	-	-	-	-	-	+
	1	1	-	-	-	+	+	-	-	-	+	+
New toxins	1	-	-	1	-	-	-	+	-	-	-	-
	3	2	-	1	-	-	-	+	-	+	-	-
	3	3	-	-	-	-	-	-	+	-	-	-
	1	1	-	-	-	-	-	-	-	+	-	-
<b>total</b>	<b>75</b>	<b>54</b>	<b>12</b>	<b>9</b>	<b>36</b>	<b>15</b>	<b>37</b>	<b>10</b>	<b>4</b>	<b>7</b>	<b>31</b>	<b>15</b>

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Tab 3

Toxin	Number of isolates	
	Multiplex-PCR	SET-RPLA
A	36	31
B	nd	1
C	15	14
D	37	24

nd: not done

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Tab 4

Strains	Origin of isolates			SE	se
	Cow	Goat	Sheep		
35	17	10	8	-	-
9	9			A	<i>a</i>
<b>5</b>	<b>4</b>	<b>1</b>		<b>A</b>	<b><i>a,d,j</i></b>
<b>1</b>	<b>1</b>			<b>A</b>	<b><i>a,d,g,i,j</i></b>
1		1		A	<i>a,g,i</i>
1		1		A	<i>a,g,h,i</i>
4	4			A,D	<i>a,d</i>
1	1			A,D	<i>a,d,g,j</i>
8	8			A,D	<i>a,d,j</i>
13		7	6	C	<i>c,l</i>
<b>1</b>	<b>1</b>			<b>C</b>	<b><i>c,d,j,l</i></b>
1	1			D	<i>d</i>
<b>1</b>	<b>1</b>			<b>D</b>	<b><i>a,d,j</i></b>
8	8			D	<i>d,j</i>
1	1			D	<i>d,g,j</i>
4	2	2		-	<i>a</i>
<b>1</b>	<b>1</b>			-	<b><i>a,g</i></b>
<b>1</b>	<b>1</b>			-	<b><i>d</i></b>
<b>5</b>	<b>5</b>			-	<b><i>d,j</i></b>
2	2			-	<i>g,i</i>
1			1	-	<i>g</i>
3	3			-	<i>h</i>
1	1			-	<i>i</i>
<b>1</b>			<b>1</b>	-	<b><i>c,l</i></b>
1			1	C	<i>g,i</i>
1			1	A	-
1			1	A,B*	-

Bold characters: strains have *se* genes, but not all the corresponding toxins are produced

Grey areas: enterotoxin production without harbouring toxin genes

\* not analyzed by mPCR