ISSN 2347-6893

Multiplex PCR and RPLA detection of enterotoxins in Staphylococcus aureus strains isolated from milk, dairy products and human faecal samples

Maria Cristina Ossiprandi[1], Laura Zerbini[2]

Maria Cristina Ossiprandi[1], Department of Veterinary Medical Science - Unit of Infectious Diseases and Microbiology - Parma University, Via del Taglio n. 10 - 43126 Parma - Italy

mariacristina.ossiprandi@unipr.it

Laura Zerbini[2,], Department of Veterinary Medical Science - Unit of Infectious Diseases and Microbiology - Parma University, Via del Taglio n. 10 - 43126 Parma - Italy

laurazerbini@tiscali.it

ABSTRACT

Staphylococcus aureus is considered the world's third most important cause of food-borne illnesses, of which foods of animal origin, especially milk and dairy products, are responsible.

Many *S. aureus* strains can produce one or more of enterotoxins (SEs), that are recognized agents of intoxication staphylococcal food-borne syndrome but may be also involved in other types of infections with sequelae of shock in humans and animals.

The aim of this research was to detect classical staphylococcal SE genes (*sea* to *see*) by multiplex PCR and to reveal toxin *in vitro* production by reversed passive latex agglutination (RPLA) in 258 *S. aureus* isolates from milk and dairy products of mastitic bovines and from human diarrhoeic faecal samples.

Staphylococcal enterotoxin genes were detected in 84 isolates: 34 strains were *sea*+, 4 *seb*+, 13 *sec*+, 20 *sed*+, 11 *sea*+*sed*+, and 2 *sec*+*sed*+. Equivocal result was obtained by RPLA for 4 of the 84 strains tested, relatively to SEA and SED.

Our results prove that multiplex PCR is the preferential choose to characterize the toxigenicity of *S. aureus* strains and could complete efficaciously the conventional laboratory diagnosis by increasing its sensibility.

Indexing terms/Keywords

Staphylococcal enterotoxins, multiplex PCR, RPLA, milk and dairy products, human faecal samples

Academic Discipline And Sub-Disciplines

Microbiology

SUBJECT CLASSIFICATION

Diagnostic methods

TYPE (METHOD/APPROACH)

Survey

ISSN 2347-6893

INTRODUCTION

Staphylococcus aureus is one of the main determinants of clinical and subclinical bovine mastitis and its presence is frequently associated with enterotoxigenic strains representing one of the main pathogens causing food poisoning worldwide (1, 2).

Foods of animal origin, especially milk and dairy products, are associated with foodborne disease. Milk is a good substrate for *S. aureus* growth and enterotoxin production (3).

Many *S. aureus* strains can produce one or more enterotoxins (SEs), that are recognized agents of intoxication staphylococcal food-borne syndrome but may be also involved in other types of infections with sequelae of shock in humans and animals (4, 5).

The severity of the illness depends on the amount of food ingested, the amount of toxin in the ingested food and the general health of the victim. Staphylococcal food poisoning (SFP) can be caused by as little as 20-100 ng of enterotoxin. After ingestion, symptoms appear rapidly and abruptly, consistent with diseases caused by preformed toxins. The symptoms include copious vomiting, diarrhoea, abdominal pain or nausea (6).

To date, 21 SEs or enterotoxin-like proteins (SEls) have been identified (6). Staphylococcal enterotoxins, on the basis of antigenic characters are classified into five main types serological characterized by the initials SEA, SEB, SEC (with the SEC1, SEC2 and SEC3, SEC ovine and bovine variants), SED and SEE (enterotoxins “classic”) (2, 6). However, in recent years has been reported in the literature, the existence of “new types” of SE (SEIG, SEIH, SEI, SEIJ, SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, SEIQ, SER, SES, SET, SEIU and SEIV) (6). TSST-1, the toxic shock staphylococcal toxin, initially designated as SEF, lacks emetic activity (7).

While SEs are the toxins that induce emesis, the related SEls either lack emetic activity or have not yet been tested for this.

SEA, that is toxic in low concentration (0.6 ng/ml) (8), is the enterotoxin most commonly reported in foods, and is also considered as the main cause of SFP, probably due to its extraordinarily high resistance to proteolytic enzymes (>75% of outbreaks), followed by SEB, SEC and SED (9). The predominance of SEA is well documented in different countries (9). The fifth classical enterotoxin, SEE, has been infrequently reported in foods and food-producing animals, and its involvement in SFP outbreaks has only been demonstrated in rare occasions (9, 10).

The laboratory diagnosis, in addition to traditional methods of bacteriology, involve the use of serological tests (reversed passive latex agglutination - RPLA), immunoassays (ELISA) and immunodiffusion. These methods require an average of 3-24 hours and are able to trace values of toxin range from 0.25-1 ng/ml, although frequent instances of false positivity and possible interference due to the matrix same sample (food or faecal components) as well as to the protein A of *S. aureus* also exist, and with some frequency, false negatives attributable to any chemical factors, particularly at low concentrations of enterotoxin. Differences in the production of enterotoxins by *S. aureus* grown in natural substrate and in laboratory media have been also reported (11, 12).

Therefore, these methods are, to date, time consumption, expensive and designed only to detect the classical SEs, although ELISA has been described for SEH and for SEG and SEI (9).

An efficient control plan and monitoring of bovine mastitis and of SFP can not be separated by use of a diagnostic protocol quickly and with good sensitivity and specificity.

The purpose of this study was to be to detect the gene sequences encoding the classical SEs by multiplex PCR and to reveal toxin *in vitro* production by RPLA in jambs of *S. aureus* isolated from mastitis milk and dairy products and from human faecal samples, belonging to patients with diarrhoea.

MATERIALS AND METHODS

A total of 258 frozen and fresh *S. aureus* strains (190 isolated from human diarrhoeic faeces and 68 from milk and by-products of mastitic bovine samples, kindly provided from Istituto Zooprofilattico Sperimentale in Parma, Italy) were analyzed.

For *S. aureus* isolation, samples were plated on Chapman (Mannitol Salt) and/or Baird-Parker agar with 5% egg yolk tellurite emulsion and incubated at 35°C for 48 h. Characteristic colonies were tested for catalase and coagulase production using Staphylase test (Oxoid, Basingstoke Hampshire, England). All strains were identified as *S. aureus* by API-Staph (bioMérieux sa).

For DNA extraction, one or two colonies of *S. aureus* isolates were suspended in 100 μL of sterile distilled water and the suspension was heated at 100°C for about 10 min. After a centrifugation at 13,000 g for 2 min, 5 μL of the supernatant (DNA template) were tested by multiplex PCR, utilizing five pairs of primers, described in previous studies (13).

S. aureus reference strains were used: ATCC 13565, (SEA and SED) (Oxoid, Inghilterra), ATCC 43300 (SEC) (kindly provided by Istituto Superiore of Sanità in Rome), ATCC 14458 (SEB) and ATCC 27664 (SEE) (kindly provided by Istituto Zooprofilattico Sperimentale in Sassari).

S. aureus-positive multiplex PCR isolates and reference strains were tested for their ability to produce *in vitro* enterotoxins (SEA to SED) by reversed passive agglutination assay (SET-RPLA Staphylococcal Enterotoxin test kit, Oxoid), according to the manufacturer's instructions. The sensitivitiy of the test has been reported to be 0.5-1 ng/ml.

RESULTS AND DISCUSSION

The results of the multiplex PCR analysis of all the 258 strains of *S. aureus* are shown in Table 1.

Of the 68 strains of *S. aureus* isolated from milk and by-products of mastitic bovine samples, 16 (23.5%) were positive for one or more SE genes. On the other hand, of the 190 *S. aureus* isolates from human diarrhoeic faeces, 68 (35.8%) tested positive.

One or more *se* genes were carried by 32.6% of all isolates; 84.5% of these isolates possessed one kind of *se* gene, and the remaining 15.5% more than one *se* gene. Six different genotypes were observed.

Overall, 34 strains were *sea*+, 4 *seb*+, 13 *sec*+, 20 *sed*+, 11 *sea*+*sed*+ and 2 *sec*+*sed*+ (see Table 1 and Figure 1).

Table 1. Toxin genotype of 258 *Staphylococcus aureus* isolates by multiplex PCR

Staphylococcus aureus No.	Multiplex PCR (toxin genotype)				
	sea	***seb***	***sec***	***sed***	***see***
34	**+**	-	-	-	-
4	-	**+**	-	-	-
13	-	-	**+**	-	-
20	-	-	-	**+**	-
11	**+**	-	-	**+**	-
2	-	-	+	**+**	-
174	-	-	-	-	-

+: positive result; -: negative result

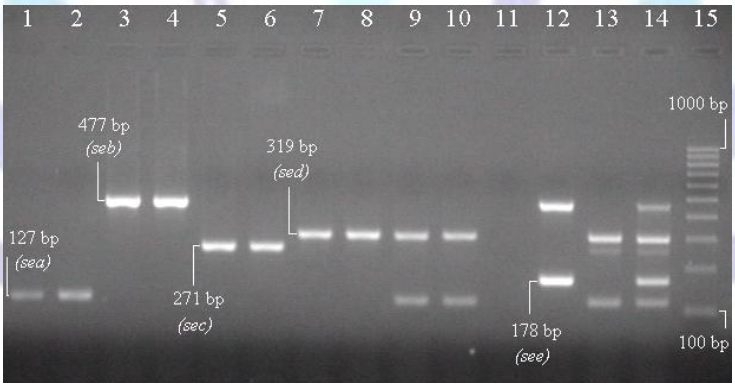

Figure 1. Agarose gel electrophoresis patterns showing typical amplification products in multiplex PCR for the staphylococcal enterotoxin genes. Lanes 1-10: *Staphylococcus aureus* isolates; lane 11: negative control ("0 DNA"); lane 12: positive control *seb*+/*see*+; lane 13: positive control *sea*+/*sec*+/*sed*+; lane 14: positive control *sea*+/*seb*+/*sec*+/*sed*+/*see*+; lane 15: molecular size markers (100 pb Molecular Ruler, Biorad).

The genes most commonly detected were *sea* and *sed*: *sea* was revealed in 45 (53.6%) and *sed* in 33 isolates (39.3%), respectively.

The strains collected from milk and by-products of mastitic bovine samples showed a lower incidence of *se* genes (16 out of 84, 19.0%): 2 isolates were *sea*+, 9 *sed*+ and 5 *sea*+*sed*+.

On the other hand, the isolates obtained from human faecal samples showed higher incidence (81.0%) of types of enterotoxins. The isolates *seb*+, *sec*+ and *sec*+*sed*+ were only from human faeces.

The results obtained for SET-RPLA methodology and for *se* genes detection were compared according to the presence of *sea-see* genotypes determined by PCR. There was a correlation of 95.9% between the toxin types and the presence of respective genes. Particularly, a correlation of 97.8% for SEA (44 vs 45), 90.9% for SED (30 vs 33) and 100% for SEB and SEC was observed. Equivocal results were obtained by SET-RPLA for 4 of the 84 strains tested, relatively to SEA and SED (see Table 2).

Table 2. Enterotoxigenicity characterization of *Staphylococcus aureus* isolates by multiplex PCR and Reversed Passive Latex Agglutination test (SET-RPLA)

Enterotoxin phenotype	*Staphylococcus aureus* isolates		
	SET-RPLA		Multiplex PCR
	No. positive (%)	No. negative (%)	No. positive
SEA	44	1	45
SEB	4	0	4
SEC	15	0	15
SED	30	3	33
TOTAL	93 (95.9%)	4 (4.1 %)	97

Milk and dairy products are considered to be a primary source of human exposure to *S aureus* enterotoxins, that is a major human pathogen and causes a variety of nosocomial and community-acquired infections.

SEA is the most common enterotoxin recovered from food poisoning outbreaks followed by SED and SEB, and it has been shown that 95% of staphylococcal food poisoning outbreaks are caused by enterotoxins SEA through SEE (14). In humans, symptoms can occur after the ingestion of very small quantities of toxin (0.5 ng/mL) (15).

For the determination of SEs, the SET-RPLA is the most commonly used method. However, SET-RPLA limited the selection of enterotoxins to SEA to SED, takes 2.5 days to get results and its efficiency depends on various factor, including culture conditions. It detects a protein only if it is expressed *in vitro* and depends on sufficient amounts of toxin being produced. For these reason, several PCR assays have been developed to identify specific gene sequences for SEs (11). These methods are more sensitive and less time-consuming than the immunological assays.

In this study we utilized a multiplex PCR to reveal the “classic” enteroxin genes (*sea-see*) in *S. aureus* isolates and SET-RPLA test to verify enterotoxin *in vitro* production.

With regard to the genes encoding enterotoxins, 84 (32.6%) out of 258 strains tested were positive for at least one enterotoxin gene: 16 (19.0%) of 84 *S. aureus* strains were isolated from milk and dairy products of mastitic bovine samples. Overall, the most frequently observed gene was *sea*, observed in 45 (53.6%) isolates, followed by *sed* (33 strains, 39.3%), *sec* (15 strains, 17.8%), and *seb* (4 strains, 4.8%).

Several researchers have reported variability in the prevalence of *S aureus* enterotoxin genotypes among farms and countries. In spite of the great discrepancy in data concerning the prevalence of enterotoxigenic *S. aureus* isolates found in the literature, in studies conducted in Germany, Brazil, Japan, and the United States, the prevalence of enterotoxin genes in *S aureus* isolates has been reported to range from 10% to 70% (7). This variability may be related to differences in severity of mastitis, geographic location of herds, laboratory methods, and the identification of different enterotoxins among studies.

In Italy, Morandi et al. (2007) (16) found a frequency value of 67% of the *S. aureus* strains isolated from milk and dairy products positive for the presence of toxin genes. Our lower frequency (23.5%: 16 out of 68) is probably linked to restricted sample number of dairy products analyzed.

Our data shows the only SEs observed in cow dairy products were SED, observed in 14 samples (87.5%), and SEA (7 samples, 43.7%). These findings are in agreement with Normanno et al. (2005) (14), Morandi et al. (2007) (16), and Serraino et al. (2004) (17), who reported frequent findings of enterotoxins A and D in many cow dairy products.

However, some of our findings differed from those reported by other researchers in other countries. Such studies have indicated that SEC was the enterotoxin most frequently produced by the tested cow strains while SEA was usually typical of human isolates (18)**.** In cow dairy products, we didn’t observed SEC, but in human faecal samples SEC was revealed in 15 samples (22.1%).

A comparison of the RPLA test for the detection of enterotoxins A-D with the presence of the corresponding gene on PCR revealed a correspondence of 95.9%: complete correspondence for SEB and SEC was observed; on the contrary, 97.8% and 90.9% of correlation was revealed for SEA and SED, respectively. This discrepancy could be explained by the production of enterotoxin in a quantity that was below the limit of detection of the RPLA test or its non-expression. In summary, for the toxins detectable using RPLA, a good level of correlation was observed between the two methods, as also reported from other authors (19).

Our study revealed the widespread presence of enterotoxigenic *S. aureus* strains. The use of PCR for the identification of genes coding for the production of enterotoxin, along with the traditional detection methods would enable the identification of strains that carry genes that might, in suitable conditions, produce as yet unknown toxins potentially capable of producing sickness in humans.

S. aureus is well established as a clinical and epidemiological pathogen; in this study it was demonstrated that the potentially pathogenic role of *S. aureus* as a food-borne pathogen should not be neglected.

Further epidemiological studies are needed to determine levels of isolated enterotoxigenic *S. aureus* or their toxins in milk and dairy products and in human samples, and investigations should also be performed to find the relationship between the presence of this pathogen or SEs in food in general and the ability to cause disease in humans.

In conclusion, our findings proved that multiplex PCR resulted more sensitive and time-saving than SET-RPLA, but PCR should be associated to *in vitro* detection of SEs, that, if revealed directly in the sample, can establish the illness aetiology. Moreover, they highlight the high potential risk for consumers in the absence of strict hygienic and preventative measures to avoid the presence of *S. aureus* isolates and SEs production in foods, emphasising the need for the implementation of specific monitoring programmes designed to reduce subclinical mastitis, which is often the cause of milk contamination, and for improved hygiene practices during food processing and also during the distribution and consumption of the final food products. Efforts from the entire production chain are required to improve consumer safety.

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Cinzia Reverberi and Mr. Roberto Lurisi for their technical support, the Istituto Zooprofilattico Sperimentale in Parma and in Sassari, and the Istituto Superiore of Sanità in Rome (Italy) for the *Staphylococcus aureus* isolates and the reference strains.

REFERENCES

[1] Genigeorgis, C. A. 1989. Present state of knowledge on staphylococcal intoxication. Int. J. Food Microbiol. 9, 327-360.

[2] Loncarevic, S., Jørgensen, H. J., Løvseth, A., Mathisen, T., and Rørvik, L.M. 2005. Diversity of *Staphylococcus aureus* enterotoxin types within single samples of raw milk and raw milk products. J. Appl. Microbiol. 98, 344-350.

[3] Rall, V. L. M., Vieira, F.P., Rall, R., Vieitis, R. L., Fernandes, A. Jr, Candeias, J. M. G., Cardoso, K. F. G., and Arau´jo, J. P. Jr. 2008. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Vet. Microbiol. 132, 408-413.

[4] Bergdoll, M. S. 1983. Enterotoxins. Staphylococci and staphylococcal infections. In: Easton CSF, Adlam C (eds.). Academic Press, London, United Kingdom, 559-598.

[5] Johnson, W. M., Tyler, S. D., et al. 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J. Clin. Microbiol. 29, 426-430.

[6] Schelin, J., Wallin-Carlquist, N., Thorup Cohn, M., Lindqvist, R., Barker, G. C., and Rådström, P. 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Virulence. 2, 580-592.

[7] Oliveira, L., Rodrigues, A. C., Hulland, C., and Ruegg, P. L. 2011. Enterotoxin production, enterotoxin gene distribution, and genetic diversity of *Staphylococcus aureus* recovered from milk of cows with subclinical mastitis. Am. J. Vet. Res. 72, 1361-1368.

[8] Holečkovà, B., Holoda, E., et al. 2002. Occurrence of enterotoxigenic *Staphylococcus aureus* in food. Ann. Agric .Environ. Med. 9, 179-182.

[9] Argudín, M. Á., Mendoza, M. C., and Rodicio, M. R. 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins. 2, 1751-1773.

[10] Vernozy-Rozand, C., Mazuy-Cruchaudet, C., Bavai, C., and Richard, Y. 2004. Comparison of three immunological methods for detecting staphylococcal enterotoxins from food. Lett. Appl. Microbiol. 39, 490-494.

[11] Klotz, M., Opper, S., Heeg, K., and Zimmermann, S. 2003. Detection of *Staphylococcus aureus* enterotoxins A to D by Real-Time Fluorescence PCR assay. J. Clin. Microbiol. 41, 4683-4687.

[12] Wilson, I. G., Cooper, J. E., and Gilmour, A. 1991. Detection of enterotoxigenic *Staphylococcus aureus* in dried skimmed milk: use of the polymerase chain reaction for amplification and detection of staphylococcal enterotoxin genes *entB* and *entC1* and the thermonuclease gene *nuc*. Appl. Environ. Microbiol. 57, 1793-1798.

[13] Becker, K., Roth, R., and Peters, G. 1998. Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. J. Clin. Microbiol. 36, 2548-2553.

[14] Normanno, G., Firinu, A., Virgilio, S., Mula, G., Dambrosio, A., Poggiu, A., Decastelli, L., Mioni, R., Sucuota, S., Bolzoni, G., Di Giannatale, E., Salinetti, A. P., La Calandra, G., Batoli, M., Zuccon, F., Pirino, T., Sias, S., Parisi, A., Quaglia, N. C., and Celano, G. V. 2005. Coagulase-positive staphylococci and *Staphylococcus aureus* in foods products marketed in Italy. Food Microbiol. 98, 73-79.

[15] Balaban, N., and Rasooly, A. 2000. Staphylococcal enterotoxins. Int. J. Food Microbiol. 61, 1-10.

[16] Morandi, S., Brasca, M., Lodi, R., Cremonesi, P., and Castiglioni, B. 2007. Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. Vet. Microbiol. 124, 66-72.

[17] Serraino, A., Alberghino, L., Fontana, M. C., Annemuller, C., and Rosmini, R. 2004. Occurrence of enterotoxin genes and macrorestriction analysis of *Staphylococcus aureus* isolated from bovine mastitis and bulk-tank milk samples in Italy, An epidemiological study. Italian J. Anim. Sci. 3, 47-53.

[18] Rahim, E., Mommtaz, H., Shakerian, A., and Kavyani, H. R. 2012. The detection of classical enterotoxins of *Staphylococcus aureus* in raw cow milk using the ELISA method. Turk J. Vet. Anim. Sci. 36, 319-322.

[19] Zschöck, M., Botzelr, D., Blöcher, S., Sommerhäuser, J., and Hamman, H. P. 2000. Detection of genes for enterotoxins (*ent*) and toxic shock syndrome toxin-1 (*tst*) in mammary isolates of *Staphylococcus aureus* by polymerase chain reaction. Int. Dairy J. 10, 569-574.