IDENTIFICATION OF GENES ENCODING CLASSICAL STAPHYLOCOCCAL ENTEROTOXINS IN STAPHYLOCOCCUS AUREUS ISOLATED FROM RAW MILK

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Abstract

This study describes a multiplex PCR assay developed for the detection of Staphylococcus aureus enterotoxin types: SEA, SEB, SEC, SED, and SEE presented in the literature as classical. The method was then used to analyse the presence of genes encoding these enterotoxins in S. aureus strains isolated from raw milk. A total of 237 raw milk samples were used in the study and 77 (32.5%) of them were found to be contaminated with S. aureus. Among them, five isolates were harboring the genes encoding staphylococcal enterotoxins – type C (three strains) and type A (two strains). These results show that raw milk can potentially be a source of staphylococcal food poisoning.

Key words: raw milk, Staphylococcus aureus, enterotoxins, SE genes, multiplex PCR.

Staphylococcal food poisoning (SFP) is one of the most common foodborne illnesses resulting from ingestion of staphylococcal enterotoxins produced in food by enterotoxigenic strains of Staphylococcus aureus. Milk is considered to be a good substrate for S. aureus growth and enterotoxin production, and contaminated raw milk has been often involved in SFP (1). S. aureus mastitis is a serious problem in dairy production and infected animals may contaminate bulk milk. Additionally, human handlers, milking equipment, the environment, and udder and teat skin of dairy animals may be other possible sources of bulk milk contamination. From a food safety perspective, this is a concern because enterotoxigenic S. aureus may be a risk of SFP after consumption of raw milk products (14). SFP is suspected when the symptoms including nausea, violent vomiting, abdominal cramps, and diarrhea affect the patients between 1 and 8 h after food consumption. To date, over 20 SEs have been characterised and many S. aureus strains can synthesise more than one type of toxins (16, 17, 27). These toxins are low molecular weight proteins (MW 26,900–29,600), heat resistant, which means that their biological activity remains unchanged even after thermal processing of food (18, 19). More than 95% of staphylococcal food poisoning outbreaks are caused by SE types (SEA to SEE) defined as a classical enterotoxins. The remaining 5% of outbreaks are associated with newly described SE’s. It is difficult to obtain accurate estimates of the incidence of S. aureus intoxications because most cases are not reported. During the last few decades, SFP has been a third cause of foodborne illnesses in the world. In spite of being a mild, self-limited illness with low mortality rate, it is considered to be one of the most economically important diseases worldwide (10, 23, 25, 28).

The detection of S.aureus and SEs in foods is often difficult because food processing may inactivate the bacteria without destroying SEs, which are resistant to high temperatures and inactivation by gastrointestinal proteases such as pepsin (11). The currently available enzyme-linked immune-sorbent and fluorescent assay kits (ELISA and ELFA) detect only the presence of enterotoxins SEA – SEE; however, they do not give information about the presence and expression of genes encoding these toxins (17, 20). PCR is a rapid and sensitive tool, which can show the presence of enterotoxigenic S.aureus in food on the basis of specific gene sequences and detect the potential source of contamination before enterotoxins are produced (8).

The presented study aimed to identify the genes encoding the staphylococcal enterotoxins SEA, SEB, SEC, SED, and SEE in S. aureus strains isolated from raw cow milk.

Material and Methods

Samples. A total of 237 samples of cow’s raw milk were analysed for the presence of S. aureus. Coagulase positive staphylococci (CPS) were isolated using Baird Parker agar with a rabbit plasma fibrinogen
supplement (BP+RPF) (bioMérieux, France) after incubation at 37°C for 24–48 h. One typical colony from each milk sample was chosen for further analysis.

**DNA extraction.** DNA was extracted using the Genomic Mini isolation kit (A&A Biotechnology, Poland), following the instructions provided by the manufacturer. One milliliter of the culture, incubated in BHI broth overnight at 37°C was used.

**Multiplex PCR test.** The multiplex PCR was established using five pairs of primers (Table 1) allowing the detection of genes encoding staphylococcal enterotoxins A, B, C, D, and E. The amplifications were performed in 0.2 ml reaction tubes in a final reaction volume of 50 µl. The PCR mixture consisted of 5 mM MgCl2 (dATP, DTP, dGTP and dTTP in concentration 200 µM), buffer, 2 U of Taq polymerase (Fermentas, EU), primers in concentration described in Table 1, and 5 µl of DNA. PCR reactions were carried out in a thermocycler PTM – 100 (MJ Research, USA) using the following conditions: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and elongation (72°C for 1 min). A final step (55°C for 2 min and 72°C for 5 min) was performed after the completion of the cycles. The amplified PCR products were visualised by standard gel electrophoresis in a 1.5% agarose gel stained by ethidium bromide (5 µg/mL) for 2 min. The gels were photographed under ultraviolet light using the Gel-Doc 2000 system (Bio-Rad, USA).

The sensitivity of the mPCR reactions were carried out using serial dilutions of mixed DNA isolated from three reference S. aureus strains (FRI 913, CCM5757, 50325) starting from 2 ng to 3 pg. The specificity of the mPCR assay was tested by analysing the DNA preparations isolated from S. aureus FRI 913 (sea, sec, see), S. aureus VII/3 (sea) S. aureus CCM 5757 (seb), S. aureus 50109 (sec), S. aureus 50325 (sed), and S. aureus 1063 (see). Furthermore, strains of S. epidermidis, Escherichia coli, and Campylobacter jejuni obtained from the National Veterinary Research Institute culture collection, were used as negative controls for the PCR reactions (Fig. 2).

The sensitivity of multiplex PCR observed in this study is shown on Fig. 1, and is estimated at about 7.8 pg/µL. The reaction with each individual primer pairs combined in the multiplex PCR resulted in the amplification of single products when DNA from reference strains of S. aureus harbouring one of five targeted genes was used. Additionally, the amplification of three genes (sea, sec, see) from S. aureus FRI 913 was obtained. The ability of mPCR to detect five genes (sea, seb, sec, sed, and see) in one reaction was confirmed by using a mixture of DNA from three reference S. aureus strains – FRI 913 (sea, sec, see), CCM 5757 (seb), and 50325 (sed). The patterns for reactions described above are shown on Fig. 2. The sizes of the products obtained from control strains correspond to the predicted sizes.

**Identification of SE genes.** Out of 237 analysed milk samples, 77 (32.5%) were contaminated with coagulase positive staphylococci as shown by the culture method. In 153 (64.6%) samples growth of coagulase negative staphylococci occurred and in the remaining seven (2.9%) samples there was no growth of bacteria. Five (6.5%) strains of S. aureus appeared to have enterotoxigenic properties, as shown by the PCR analysis. Only genes encoding two types of staphylococcal enterotoxins were found - three strains harboured the sec gene and two strains had the see marker.

**Discussion**

S. aureus is often found in unprocessed milk and dairy products due to contamination caused by poor hygiene conditions, or the origin of the milk, which can come from mastitic cows. In our study, a PCR procedure was developed for simultaneous detection of five genes encoding staphylococcal enterotoxins.

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’ – 3’)</th>
<th>Gene</th>
<th>Size of amplified product (bp)</th>
<th>Primer concentration (µM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESA1</td>
<td>ACGATCAATTTTTTACAGC</td>
<td>sea</td>
<td>544</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>ESA2</td>
<td>TGCATGTTTTCAGAGTTAATC</td>
<td>sec</td>
<td>416</td>
<td>0.4</td>
<td>11</td>
</tr>
<tr>
<td>ESB1</td>
<td>GAATGATATTACATCGATC</td>
<td>seb</td>
<td>257</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>ESB2</td>
<td>TCTTTGTCGTAAGATAACCTC</td>
<td>sec</td>
<td>334</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>ESC1</td>
<td>GACATAAAAGCTAGGAAATT</td>
<td>sed</td>
<td>170</td>
<td>0.4</td>
<td>8</td>
</tr>
<tr>
<td>ESC2</td>
<td>AATCGGATTAACATTATTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESD1</td>
<td>TTACTAGTTTGGTATATCCCTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESD2</td>
<td>CCACACATAAACAATTAATGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESE1</td>
<td>ATAGATAAGTTAAAACAAAGCAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESE2</td>
<td>TAACTTACCGGTGGACCC</td>
<td></td>
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</table>
Out of 66 isolates of *S. aureus* recovered from raw cow’s milk, five (7.6%) had genes encoding classical staphylococcal enterotoxins (SEA – SEE). Bystron et al. (7) analysed 150 samples of raw milk and isolated 16 strains of *S. aureus*, among them 11 (68.8%) strains harboured the genes encoding staphylococcal enterotoxins. The same authors four years later described a study on 68 samples of bovine unprocessed milk and 35% strains of *S. aureus* had an enterotoxigenic potential (6). Studies on occurrence of enterotoxigenic *S. aureus* were also performed in other countries. In Hungary, 27.1% out of 59 strains isolated from unprocessed cow’s milk by Peles et al. (22), were shown to harbour one or more SE genes. Scherer et al. (26) analysed 172 samples of goat and sheep milk in Switzerland and found that 65.2% of 296 isolates had genes encoding enterotoxins.

In Japan, Katsuda et al. (15) observed that 183 (67.8%) out of 270 *S. aureus* isolates were positive for the presence of genes encoding one or more enterotoxins. In Italy, Morandi et al. (20) also found a very similar frequency value – 67% of the *S. aureus* strains isolated from milk and dairy products were positive for the presence of toxin genes. With the discovery of new enterotoxins other than SEA to SEE, the perceived percentage of potentially enterotoxigenic *S. aureus* strains increased. In our study only genes encoding the classical enterotoxins were identified. Rall et al. (24) found that 68.4% out of 57 strains isolated from raw or pasteurised bovine milk were positive for the presence of at least one SE gene, however that number dropped down to 52.5% when only the classical enterotoxins (SEA – SEE) were considered. Similar observations were made by Rosec and Gigaud (25), who detected 30% of the isolates with the genes encoding classical enterotoxins, but that frequency has increased to 57% when the new SE’s were taken into account. Other authors observed, that *S. aureus* strains isolated from animals produce mainly SEC, whereas among strains isolated from humans, SEA was most frequently identified (3, 12, 21). In agreement with these results, also in our study the genes encoding staphylococcal enterotoxin C was the most often observed. In spite of discrepancies in data concerning the prevalence of enterotoxigenic *S. aureus* isolated from different types of food, our study also confirmed, that SEC and SEA are the most often observed toxins in enterotoxigenic strains of *S. aureus*. According to the European legislation on food safety, cheeses, powdered milk, and whey are examined for the presence of staphylococcal enterotoxins A to E when high level of *S. aureus* is found in the sample. Despite the fact, that other than classical staphylococcal enterotoxins are responsible for 5%–10% of SFP, there is no obligation to examine the samples for the presence of these enterotoxins (6).

**Fig. 1.** Sensitivity of multiplex PCR: lane 1 - molecular marker; lane 2 - 2 ng/µL; lane 3 - 1 ng/µL; lane 4 - 500 pg/µL; lane 5 - 250 pg/µL; lane 6 - 125 pg/µL; lane 7 - 62.5 pg/µL; lane 8 - 31.3 pg/µL; lane 9 - 15.6 pg/µL; lane 10 - 7.8 pg/µL; lane 11 - 3.9 pg/µL; lane 12 - H2O.

**Fig. 2.** Specificity of multiplex PCR: lane 1 - molecular marker; lane 2 - *sea, seb, sec, sed, see* genes obtained after mixing DNA from *S. aureus* FRI 913, *S. aureus* CCM 5757, and *S. aureus* 50325; lane 3 - *S. aureus* FRI 913 (*sea, sec, see*); lane 4 - *S. aureus* CCM 5757 (*seb*); lane 5 - *S. aureus* 50109 (*sec*); lane 6 - *S. aureus* 50325 (*sed*); lane 7 - *S. aureus* 1063 (*see*); lane 8 - *S. epidermidis*; lane 9 - *E. coli*; lane 10 - *C. jejuni*; lane 12 - H2O.

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**References**