Detection of classical genes and enterotoxins of *Staphylococcus aureus* isolated from raw milk in the south-east region of Poland

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Abstract

The aim of the study was to investigate if the enterotoxigenic strains of *S. aureus* isolated from raw milk are able to produce staphylococcal enterotoxins (SEs) A – E. A total of 168 of *S. aureus* isolates from raw milk collected in the south – east region of Poland (Lubelskie Province) were tested for SE production by the ELFA, while multiplex PCR was applied for detection of enterotoxin genes (*sea, seb, sec, sed, see*). It was found that 20 (11.9%) out of 168 strains were positive for one or more classical SE markers and 19 of them produced a detectable level of enterotoxins. The results obtained by mPCR and ELFA were in agreement, when the presence of A, B, and D toxin types was tested; whereas SEC was not found by the ELFA although the *S. aureus* was positive for the respective gene. The results of the two methods showed that mPCR identified one more strain potentially producing enterotoxin than the ELFA, which may suggest that the enterotoxigenic *S. aureus* are not always able to express the toxin protein.

Keywords: raw milk, *S. aureus*, staphylococcal enterotoxins, ELFA, PCR, Poland.

Introduction

An increasing number of people are consuming raw milk and raw milk products. Such foods are considered to be ecological and more natural than those made in dairies and to have enhanced nutritional quality and taste. However, raw milk may be contaminated with bacteria, including *Staphylococcus aureus*. These microorganisms are present on the skin and mucosa of food-producing animals, such as ruminants, and are frequently associated with subclinical mastitis leading to contamination of milk (12). There are also other possible sources of contamination of bulk milk by *S. aureus*, such as poor hygiene during milking and processing.

*S. aureus* produces a wide range of extracellular proteins such as heat stable enterotoxins, which cause staphylococcal food poisoning (SFP), being considered as an important cause of gastroenteritis worldwide (9). Several staphylococcal enterotoxin types were recognised (*SEA, SEB, SEC, SED, and SEE*) (9); however, during the 1990’s “new” SEs were also reported and their encoding genes described (23). Some of the new SEs had no emetic activity and a defining property of SEs (19, 20). It is known that about 95% of staphylococcal food poisoning outbreaks are caused by SE types from A to E. The remaining 5% of infections may be associated with newly identified SEs (7). Growth of enterotoxigenic *S. aureus* up to 10⁶ or more cells per gram of food enables them to produce a sufficient amount of enterotoxins to cause intoxication (2, 11). As little as 20 ng of SE can induce nausea, violent vomiting, abdominal cramps, and diarrhoea between 1 to 8 h after food consumption (11, 15). According to the recent EFSA report, foodborne outbreaks caused by staphylococcal enterotoxins represented 6.4% of all outbreaks identified in 2012 in the European Union (10). However, the real incidence of infections is probably underestimated because SFP is mild, self-limited illness with a low mortality rate (5).

The aim of this study was to detect genes encoding classical enterotoxins among *S. aureus* isolated from raw milk in the south-east region of Poland and to verify if the isolates are able to produce detectable amounts of staphylococcal enterotoxins.
CPS strains. A total of 432 samples of bulk tank milk were collected from individual producers delivering milk to dairies in Lubelskie Province in Poland. Coagulase positive staphylococci (CPS) were isolated using Baird – Parker agar with a rabbit plasma fibrinogen supplement (bioMérieux, France) after incubation at 37°C for 24-48 h. One CPS isolate from each milk sample was chosen for further analysis.

DNA extraction. DNA was isolated using the Genomic Mini isolation kit (A&A Biotechnology, Poland) following the instructions provided by the manufacturer. One millilitre of the S. aureus culture incubated in BHI broth for 24 h at 37°C was used.

Identification of S. aureus. To confirm the presence of the S. aureus, a multiplex PCR was used to identify the specific 23S rRNA (1250 bp) and the coa (polymorphic) genes. The PCR mixture consisted of: 5 mM MgCl₂, dNTP in concentration 200 µM, 10 x Taq Buffer, 2 U of Taq polymerase (Fermentas, EU); primers: staur 4 and staur 6, coa 1 and coa 2, both in concentration of 0.2 µM, and 5 µL of DNA. The reactions were conducted 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 above 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).

Detection of sea, seb, sec, sed, and see genes. After DNA isolation, amplification of the sea, seb, sec, sed, and see genes was performed using five sets of primers in one reaction mixture as described previously (16).

Detection of SEA, SEB, SEC, SED, and SEE enterotoxins. The presence of five SEs (A - E) in enterotoxigenic S. aureus cultured in BHI broth for 24 h at 37°C was performed using the enzyme-linked fluorescent immunoassay (ELFA) VIDAS SET 2 (bioMérieux).

Reference strains. As a positive control, three enterotoxic S. aureus strains were used: FRI 913 (sea, sec, see), 50325 (sed) both obtained from the Norwegian Veterinary Institute, and CCM 5757 (seb) supplied by the University of Veterinary Medicine in Kosice, Slovakia.

Results

Identification of sea, seb, sec, sed, and see genes. One hundred and seventy (39.4%) out of 432 milk samples were contaminated by coagulase positive staphylococci. The vast majority (168; 98.8%) of the isolates were identified as S. aureus. It was found that 20 (11.9%) strains were positive for one or more enterotoxin genes tested. Eighteen (90.0%) S. aureus harboured one of five classical enterotoxin markers while the remaining two (10.0%) isolates possessed sea and seb genes (Table 1). The genes encoding enterotoxins C and A were most frequently found among S. aureus strains tested, i.e. in nine (45.0%) and six (30.0%) isolates respectively. None of the analysed strains harboured the see gene.

Detection of enterotoxins. The results obtained using the ELFA technique revealed that only one (5%) out of 20 strains harbouring enterotoxin genes (sec) did not produce staphylococcal toxins (Table 1). For the remaining 19 isolates, a 100% correlation between the presence of SE genes and expression of the corresponding proteins was observed.

Table 1. The correlation between the presence of enterotoxin genes and SE production

<table>
<thead>
<tr>
<th>SE type</th>
<th>Number (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6 (30) 6 (30)</td>
</tr>
<tr>
<td>B</td>
<td>2 (10) 2 (10)</td>
</tr>
<tr>
<td>C</td>
<td>9 (45) 8 (40)</td>
</tr>
<tr>
<td>D</td>
<td>1 (5) 1 (5)</td>
</tr>
<tr>
<td>E</td>
<td>0 0</td>
</tr>
<tr>
<td>A + B</td>
<td>2 (10) 2 (10)</td>
</tr>
<tr>
<td>Total</td>
<td>20 (100) 19 (95)</td>
</tr>
</tbody>
</table>

Discussion

In the present study, 39.4% of the analysed raw milk samples were contaminated with CPS, which is similar to the results of the previous investigations (16). Similar results were obtained by D’Amico et al. (8), who found S. aureus in 46 (34.6%) out of 133 samples during analysis of milk quality and prevalence of four bacterial pathogens in raw milk for farmstead cheesemaking. Schlegelova et al. (24) also isolated S. aureus from bulk milk at the level of 34.2% (24). On the other hand, Jørgensen et al. (14) identified 165 S. aureus strains among 220 (75%) samples of bulk milk and raw milk products (13). As found in this study, almost all CPS were identified as S. aureus although other staphylococci, e.g. S. intermedius and S. hyicus, have been previously described by other authors as enterotoxigenic (1, 3). In the current study, two of such non - S. aureus isolates were non - enterotoxigenic as tested by PCR and ELFA. However, S. hyicus and S. intermedius strains were found by Bendahou et al. (2) among CPS isolated from raw milk and the traditional Moroccan milk products. None of them harboured the genes encoding classical SEs or newly described markers such as seg, sei, sej, sek, sel, sem, sen, and seo (2). On the other hand, Borelli et al. (4) identified S. intermedius and S. hyicus isolated from milk and curd, which produced enterotoxins B and C.
The prevalence of enterotoxin S. aureus strains in bovine raw milk varied considerably among studies. In the present study, 11.9% of isolates carried genes encoding classical SEs; however, the previous study revealed a lower (6.5%) occurrence of enterotoxigenic S. aureus isolated from the same source. Other studies conducted in Poland revealed the contamination of bovine unprocessed milk with enterotoxigenic S. aureus at levels of 68.8% and 4.2% respectively (5, 6). Most of the enterotoxin-positive isolates carried the sec or sea genes (6). In Brasil, Rall et al. (22) found that 57.9% bulk tank milk samples were positive for S. aureus harbouring one or more genes encoding the classical enterotoxins. Furthermore, in the studies from Portugal, 20.0% of raw cow’s milk samples were contaminated with enterotoxigenic S. aureus, which had the sec gene alone or together with the seg marker (21). A 75% correlation was observed between the expression of SEs and the presence of respective toxin genes as tested by the ELFA as in the present study (21). Similar results were obtained in Italy, where SEs production showed by the ELFA and the presence of enterotoxin genes corresponded to 80% of all S. aureus isolates (18). It seems, also on the basis of the present results, that PCR is able to identify a higher number of potentially enterotoxin producing strains than immunological tests. These findings may be explained by the fact that se gene detection does not necessarily indicate the production of toxins and their biological activity (17). The presence of enterotoxigenic S. aureus in raw milk poses a potential health risk to consumers. However, not only identification of such strains but also appropriate conditions for SE genes expression in S. aureus during production and storage of milk and milk products should be taken into account in risk analysis.

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References

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