PRODUCTION OF ENTEROTOXINS BY *STAPHYLOCOCCUS AUREUS* ISOLATED FROM SHEEP MILK

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Abstract

The production of staphylococcal enterotoxin A and staphylococcal enterotoxin B was investigated in *S. aureus* isolates obtained from milk of clinically healthy sheep. In comparison with valid standard (up to 1.0 x 10^3 CFU ml⁻¹), the *S. aureus* counts were higher in 36 of the total number of 46 positive milk samples (ranging from 1.2 x 10^3 to 8.2 x 10^3 CFU ml⁻¹). Six (13.0%) of the 46 isolates were enterotoxigenic and the production of SEA, SEB or SEA+SEB was recorded. SEA concentrations ranged from 7.0 ng ml⁻¹ to 16.0 ng ml⁻¹, SEB concentrations were 20.0 ng ml⁻¹ and 27.0 ng ml⁻¹. Milk and milk products could be foods at risk as concerns staphylococcal food poisoning, in case of enterotoxigenic *S. aureus* growth and toxin production.

Key words: ewes, milk, *Staphylococcus aureus*, enterotoxins

Ewe milk is a valuable product for the preparation of sheep lumpy cheese and Bryndza cheese. In Slovakia, these food products are traditionally prepared mostly from non-pasteurized milk (35). For this reason, the hygienic properties, particularly the absence of potentially pathogenic microorganisms such as *Staphylococcus aureus* is of special importance.

From a great number of *S. aureus* metabolites, low-molecular emetic proteins - staphylococcal enterotoxins (SEs), are thought to be the most risky for consumers. As a consequence of high heat resistance, the biological properties of the enterotoxins may remain unchanged after pasteurisation (4). If SE is ingested, very low concentrations can cause staphylococcal enterotoxiosis, one of the most frequent food-borne diseases in some countries (32, 37, 24).

*S. aureus* is an important causative agent of ovine mastitis. However, only limited data have been reported about enterotoxigenicity of *S. aureus* strains isolated from healthy ewes in Slovakia. The purpose of this study was to examine the staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) production of *S. aureus* isolates obtained from breed of clinically healthy sheep.

Material and Methods

Reference *S. aureus* strains. The reference strains used as positive controls were *S. aureus* FRI 722-SEA (Food Research Institute, University of Wisconsin, USA) and *S. aureus* CCM 5757-SEB (Czechoslovak Collection of Microorganisms, Brno, Czech Republic). As negative controls, non-enterotoxigenic strains of *S. aureus* CCM 2351-α-haemolysin and *S. aureus* CCM 6188-β-haemolysin were used (Czechoslovak Collection of Microorganisms, Brno, Czech Republic)

Sample collection and cultivation. 110 samples of raw sheep milk were obtained by technique of Phuetses et al. (30) in summer season. The milk samples were collected from sheep (Valaska breed) in the absence of clinical illness. Samples (0.1 ml) were inoculated on plates with selective Baird-Parker medium and cultivated for 24-48h at 37°C (34). Suspect colonies were transferred to BHI broth (Brain-Heart Infusion broth, Oxoid) (24h, 37°C) and on blood agar. Tube coagulase test (PK-Staphylo test, Imuna, Šarišské Michaľany, Slovak Republic) and haemolysin test were performed. The enumeration of colony forming units (CFU. ml⁻¹) was based on the valid methods for the determination of *S. aureus* counts in raw materials and foods (34).

Radioimmunoanalysis (RIA). Coagulase-positive *S. aureus* isolates were inoculated to 5ml BHI broth (Brain-Heart Infusion, Oxoid) and cultured for 24h
at 37°C. RIA (8) was used to examine the culture supernatants in order to determine the presence of enterotoxins. Tracers of $^{125}$I-SEA and $^{113}$I-SEB were prepared by the chloramine T method according to Greenwood et al. (19). Polyclonal antibodies against SEA and SEB were obtained from immunised rabbits (New Zealand White) (36). Relative concentrations of enterotoxin standards were 5.0; 10.0; 25.0; 50.0 and 100.0 ng. ml$^{-1}$. SEA and SEB concentrations were determined by means of the logit transformation of standard curve logit $b = \log[(b)/(100-b)]$, where $b$ is the proportion of tracer bound expressed as a percentage of that in the zero standard (12).

**Polymerase Chain Reaction (PCR).** *S. aureus* genomic DNA was isolated from BHIs by the method of Ausubel et al. (5). Lysates of colonies were prepared according to McLauchlin et al. (26). Primers (20-mer) were prepared as described by Johnson et al. (22). PCR was performed in thermocycler Genius (Techne, USA). The initial denaturation (94°C, 120s) was followed by 35 cycles of denaturation (94°C, 60s), annealing (55°C, 30s) and extension (72°C, 30s) and by one final extension cycle (72°C, 150s). The amplicons were detected on 2% agarose gel after ethidium bromide staining and visualisation by means of UV transilluminator. The size of fragments was 120 bp for *sea* and 476 bp for *seb*.

Results

In the present study, 46 (41.8%) coagulase-positive *S. aureus* were obtained from 110 raw milk samples. *S. aureus* counts in 1 ml of milk (CFU. ml$^{-1}$) ranged from 1.0. $10^3$ to 8.2. $10^8$. ml$^{-1}$. As shown in Table 1, 6 (13.0%) *S. aureus* isolates out of 46 were found to be enterotoxigenic by means of RIA testing of culture supernatants. Of these, 4 (8.7%) isolates produced SEA and 1 - SEB (2.2%). The synthesis of both SEA and SEB (SEA+SEB) was detected in 1 isolate (2.2%).

According to logit transformation of standard curve, the production of SEA ranged from 7.5 to 9.0 ng. ml$^{-1}$ in 3 isolates and was 16.0 ng. ml$^{-1}$ in one isolate. SEB have been found in the concentration of 27.0 ng. ml$^{-1}$. The contents of SEA and SEB in the supernatant of *S. aureus* isolates synthesizing both enterotoxins were 7.0 ng. ml$^{-1}$ and 20.0 ng. ml$^{-1}$, respectively. In the reference *S. aureus* strains (positive controls) was recorded to produce 125.0 ng. ml$^{-1}$ of SEA and 175.0 ng. ml$^{-1}$ of SEB. Negative controls were not found to be enterotoxigenic.

Isolated genomic DNA as well as lysates of colonies of all *S. aureus* isolates were examined for the presence of genes coding for the synthesis of SEA and SEB. The results of PCR were consistent with those of RIA. The presence of enterotoxin *sea* and/or *seb* genes was not found by means of PCR in isolates without SEA and/or SEB synthesis examined by RIA.

<table>
<thead>
<tr>
<th>Number of <em>S. aureus</em> isolates</th>
<th>CFU. ml$^{-1}$ in BHI medium</th>
<th>Enterotoxins in BHI medium</th>
<th>SEs concentration in BHI medium (ng. ml$^{-1}$)</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SEA</td>
<td>SEB</td>
<td>SEA</td>
<td>SEB</td>
</tr>
<tr>
<td>18</td>
<td>1.5.10$^3$</td>
<td>+</td>
<td>-</td>
<td>8.0</td>
</tr>
<tr>
<td>20</td>
<td>5.5.10$^3$</td>
<td>+</td>
<td>-</td>
<td>7.5</td>
</tr>
<tr>
<td>21</td>
<td>1.5.10$^3$</td>
<td>+</td>
<td>-</td>
<td>16.0</td>
</tr>
<tr>
<td>24</td>
<td>1.7.10$^3$</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>25</td>
<td>2.7.10$^3$</td>
<td>+</td>
<td>-</td>
<td>7.0</td>
</tr>
<tr>
<td>45</td>
<td>1.8.10$^3$</td>
<td>+</td>
<td>-</td>
<td>9.0</td>
</tr>
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<table>
<thead>
<tr>
<th>Reference SEA strain</th>
<th>CFU. ml$^{-1}$</th>
<th>Enterotoxins</th>
<th>SEs concentration</th>
<th>Genes</th>
</tr>
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<tbody>
<tr>
<td>Reference</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>

<table>
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<th>Reference SEB strain</th>
<th>CFU. ml$^{-1}$</th>
<th>Enterotoxins</th>
<th>SEs concentration</th>
<th>Genes</th>
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</thead>
<tbody>
<tr>
<td>Reference</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
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</table>

**Discussion**

Sheep milk is a suitable medium for growing of many groups of microorganisms (14), such as coagulase-negative staphylococci and *S. aureus*. In the present investigation, 41.8% of raw sheep milk samples were found to be positive for the presence of *S. aureus*. This figure is higher as compared with the study of Trávníček et al. (38), who referred, that *S. aureus* was isolated from 7.1% clinically healthy ewes from Slovak breeds as well as in comparison with data of Abo Elnaga et al. (1), who confirmed the presence of *S. aureus* only in 16.0% (n=50) of milk samples of healthy sheep. One of the frequent explanations of higher *S. aureus* incidence is subclinical mastitis, as was reported by Al Maljali and Jawabreh (2). In the present study, neither clinical nor subclinical mastitis were indicated in the breed examined. Therefore, one of the possible
explanation of the higher *S. aureus* incidence is the contamination of milk resulting from improper hand processing.

According to current standard (11, 13) *S. aureus* can be present up to 1. $10^5$ CFU. ml$^{-1}$ in raw sheep milk. The higher *S. aureus* counts were enumerated in 36 cases of a total number of 46 (41.8%) positive samples, ranging from 1.2. $10^3$ CFU. ml$^{-1}$ to 8.2. $10^4$ CFU. ml$^{-1}$. As mentioned earlier, the improper handling of milk account for the major cause of the contamination.

It is well documented, that raw milk can be more inhibiting for staphylococcal growth than pasteurised. On the other hand, the higher the initial number of staphylococci in the raw milk, the higher the probability that staphylococci will overcome the milk inhibitory environment and the competing microflora. It is known, that contamination of food product with *S. aureus* pathogens may result primarily from their presence in basic raw material-milk (8). In the absence of pasteurisation, all cheeses made from raw material should be subjected to strict period controls (23).

In our study, the production of SEA and SEB were tested in all of 46 coagulase-positive *S. aureus* isolates, because SEA and SEB have often been reported as the most common enterotoxins recovered from food-poisoning outbreaks (37, 31, 39). Moreover, as concerns the regulation of gene expression of enterotoxin genes by accessory gene regulator (agr) locus, it is interesting, that the production of SEB (agr$^+$ i.e. agr-dependent) is dependent on the ability of *S. aureus* to grow to a high cell density ($10^5$ CFU.ml$^{-1}$) (24). On the contrary, SEA (agr- i.e. agr-independent) is present in the medium after 180 min of cultivation, so SEA production is rather a function of the growth stage (31).

The percentage of enterotoxigenic strains in sheep milk was estimated to be 13.0 in this study. Of these isolates, 4 were SEA producers, 1 SEB and 1 isolate SEA+SEB producer. Little information is available about the incidence frequency of enterotoxigenic *S. aureus* in sheep milk (6, 28, 29), particularly in Slovak breeds. Futhemore, published data are very variable and percentage is different, particularly when newly described enterotoxins (SEG-SEI) are investigated in addition to classical enterotoxins (24). According to Grieger et al., (20), in bovine and other animal *S. aureus* biotypes, only low percentage of enterotoxigenic strains have been found (10.0-20.0%) in comparison with high percentage (70.0%) in human biotypes. On the contrary, Bautista et al. (6) detected synthesis of 4 enterotoxin types (SEA, SEB, SEC and SED) in 62.9% of staphylococcal strains isolated from sheep milk, with the highest production of SEA and SED. Bone et al. (10) referred the presence of the strains with production of SEA in bulk milk samples of clinically healthy sheep. In the present investigation, *S. aureus* isolates with synthesis of SEA and/or SEB were recorded. This may be partly explained by the fact that our samples had probably human origin (contamination). Furthermore, it has been shown, that for ovine strains the production of species specific SEC is more typical (16, 28, 29, 33).

Several studies have been published on the concentrations of enterotoxin produced, but the results differ mostly as concerns types of tested strains used, cultivation conditions (temperature, pH) and by substrates used (culture media, foods). Gomez-Lucia et al. (17) have determined the SEA production by *S. aureus* FRI 100-reference strain in cheese as 11.1 ng. g$^{-1}$ and 22. 2ng. g$^{-1}$. This study has shown the higher SEA production by *S. aureus* reference strain (125 ng. ml$^{-1}$), which could be caused by testing of different strain (*S. aureus* FRI 722) and by using of different culture conditions (BHI broth).

In the field *S. aureus* isolates, the production ranged from 7.0 ng. ml$^{-1}$ to 27.0 ng. ml$^{-1}$ of SEs. Although the obtained SEs amounts are lower as compared with data of Bergdoll (9) (wild *S. aureus* strains sea+, sed+ and see+ up to 10µg SE in ml of culture medium, wild *S. aureus* strains seb+ and sec+ about 100µg. ml$^{-1}$medium), these concentrations are sufficient to cause staphylococcal enterotoxiosis. Evenson et al. (15) suggested that 0.5 ng.ml$^{-1}$ of SEA could evoke consumers’ intoxication. In addition, Nedelkov et al. (27) referred, that it is necessary to detect nanogram amounts of SEs per gram of food. As reported by Homola et al. (21), 200ng of SEA in 100g of food is the minimum intoxication level for SEA. In general, a dose of 20-35 µg of toxin is sufficient to elicit vomiting in humans (3).

In the present study, PCR method for enterotoxigenicity testing of *S. aureus* isolates was also used. Mäntynen et al. (25) emphasized, that the rapid detection of genes responsible for intoxication serves as an early warning system and the products can then be tested further with immunological methods.

The results of this study have shown that the quality control of sheep milk and milk products is of special importance in relation to enterotoxigenic *S. aureus*.

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**References:**