PHENOTYPICAL IDENTIFICATION OF ATYPICAL STAPHYLOCOCCUS AUREUS STRAINS ISOLATED FROM MILK OF COWS FROM ONE HERD

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Abstract

The aim of the study was to characterise the free coagulase-negative Staphylococcus sp. strains isolated from normal (9.4%) and inflamed quarter milk samples of dairy cows in one herd localised in southern Poland. The isolates were examined phenotypically by cultural features, tube coagulase test, clumping factor (CF), and commercial agglutination test. Total number of 427 strains were cultivated on different media: Congo red agar, DNase medium, mannitol salt agar, and crystal violet agar. About 25% of the strains were examined by the disc diffusion method for their sensitivity to chosen antibiotics. All of the analysed strains showed α, β, α+β, or lh type of haemolysis on plate with 5% sheep blood agar, and had positive reaction in CF and agglutination tests, but were negative in tube coagulase test. These strains showed DNase activity and ability to mannitol fermentation; however, none of the strains produced slime. There were variations in growth on crystal violet agar: examined strains belonged to three types of growth (A, E, and C). The strains were highly susceptible in vitro to oxacillin (99.2%), cefoperazone (95.2%), and amoxicillin with clavulanic acid (93.6%), and highly resistant to neomycin (27.2%), tetracycline (21.6%), and streptomycin (20.8%). In conclusion, it can be stated that intramammary infammations in cows can be caused by atypical free coagulase-negative strains of Staphylococcus aureus.

Key words: dairy cows, intramammary infections, atypical Staphylococcus aureus, identification.

Mastitis is continuously the most frequent disease in dairy cows (17, 32). In majority of farms, predominant aetiological agents of both subclinical and clinical forms of udder inflammations are Staphylococcus aureus and coagulase-negative staphylococci (CNS) (11, 33). Infections and inflammations caused by S. aureus (the major mastitis pathogen) are the most difficult to treat and control (3, 4, 31, 35). Changes in udders and milk connected with CNS infection are less intensive compared to those caused by coagulase-positive staphylococci (29).

Staphylococci are identified by variety of culture or biochemical tests (27). One of the typical features that distinguishes the more pathogenic Staphylococcus strains from the less pathogenic is the ability to produce free coagulase and bound coagulase (clumping factor) by the strains (7, 14). This feature is also considered as one of the virulence factors (10, 41). Strains that produce coagulase (coagulase-positive staphylococci; CPS) are simply called S. aureus. S. intermedius is also coagulase-positive, similarly to some strains of S. hyicus. However, in a review of clinical papers, only the tube-coagulase test that detects the free coagulase is reported as being the first criterion that differentiates S. aureus from other species of staphylococci (7, 23, 31).

The aim of this study was to identify free coagulase-negative Staphylococcus sp. strains isolated from milk samples of cows from one herd.

Material and Methods

Bacterial isolates. The examined staphylococci were isolated from quarter milk samples of cows belonging to one herd. The samples were collected aseptically, cooled, and transported to the laboratory, as described earlier (24). Bacteriological examinations were performed according to the commonly accepted rules (22), and the somatic cell count (SCC) was measured with Fossomatic 90 (Foss Electric, Denmark). Staphylococci, which gave the negative result in test for free coagulase were placed on 5% sheep blood agar plates (Graso Biotech, Poland), and after overnight incubation were picked up from plates and put into 1 ml of brain-heart infusion broth (bioMérieux, France), frozen at -20°C, and stored until further laboratory examinations.
Diagnostic tests for identification of *S. aureus*. All bacterial isolates were tested for haemolysis type according to Boerlin *et al.* (6). The test for free coagulase (tube coagulase test) and bound coagulase (clumping factor) was performed with rabbit plasma following the manufacturer’s (Biomed S.A., Poland) recommendations (22). The isolates were tested using Staphytect Plus Test (Oxoid, UK) following the instructions of manufacturer. Agglutinations obtained with Staphytect Plus were recorded as the positive results. Slime production was examined on Congo red agar medium according to Türkylmaz and Eskiizmirliler (37). DNase activity was determined using commercial DNA Test Lab-Agar (Biocorp, Spain) according to Gündogan *et al.* (15). Mannitol fermentation ability was examined using commercial Chapmann mannitol salt agar medium (bioMérieux, France) (27). Growth on crystal violet agar was examined using a method described by Freeman *et al.* (12).

**Antibiotic sensitivity:** The antimicrobial sensitivity of 25% of the isolated staphylococci was tested by the disc diffusion method in the Mueller-Hinton agar using cefoxitin (30 µg). The interpretation of results was based on the same criteria as in antimicrobial sensitivity test.

### Results

All analysed *S. aureus* strains were isolated from 427 quarters (21%) of 244 cows (46.4% of all cows in the herd). The characteristics of the SCC in infected milk samples are presented in Table 1. The examined *S. aureus* were mostly isolated from quarter with a SCC from 501,000 to 1,000,000/mL. Clinical signs in udders and/or macroscopic changes in milk were observed in 77 infected quarters (18.0%). The average SCC in the quarters infected with these staphylococci was 1,130 ±1,425.9 x 10³/mL.

Apart from these bacteria, infections were also caused by: free coagulase-positive staphylococci (0.1% of quarters), free coagulase-negative, clumping factor-negative, non-haemolytic staphylococci (8.5%), *Streptococcus* sp. (1.5%), coliform bacteria (0.5%), *Corynebacterium* sp. (0.2%). The remaining quarters (68%) were bacteriologically negative, and 0.2% of quarter samples were contaminated (two or more bacteria species).

### Table 1

<table>
<thead>
<tr>
<th>Infected quarters</th>
<th>Somatic cell count (x10^3/ml)</th>
<th>Changes in udder and/or milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>427 of 100%</td>
<td>&lt;100</td>
<td>101-300</td>
</tr>
<tr>
<td>n</td>
<td>40</td>
<td>71</td>
</tr>
<tr>
<td>%</td>
<td>9.4</td>
<td>16.6</td>
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</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Examined features</th>
<th>Positive strains</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysis type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td></td>
<td>163</td>
<td>38.2</td>
</tr>
<tr>
<td>β</td>
<td></td>
<td>17</td>
<td>4.0</td>
</tr>
<tr>
<td>α+β</td>
<td></td>
<td>191</td>
<td>44.7</td>
</tr>
<tr>
<td>lh</td>
<td></td>
<td>56</td>
<td>13.1</td>
</tr>
<tr>
<td>Free coagulase (tube coagulase test)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Clumping factor (slide coagulase test)</td>
<td>427</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Staphytect Plus Test</td>
<td>427</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Congo red agar medium</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DNase medium</td>
<td></td>
<td>427</td>
<td>100</td>
</tr>
<tr>
<td>Mannitol salt agar medium</td>
<td>427</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Crystal violet agar medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type A</td>
<td></td>
<td>375</td>
<td>87.8</td>
</tr>
<tr>
<td>type C</td>
<td></td>
<td>17</td>
<td>4.0</td>
</tr>
<tr>
<td>type E</td>
<td></td>
<td>35</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Infected quarters:

- 427 quarters (21% of 244 cows)
- SCC range: 501,000 to 1,000,000/mL
- Clinical signs observed in 77 quarters (18.0%)
- Average SCC: 1,130 ±1,425.9 x 10³/mL

Characteristics of SCC in infected milk samples: 68% bacteriologically negative, 0.2% contaminated with two or more bacteria species.

**Antibiotic sensitivity**:

- 25% of isolates tested using disc diffusion method
- Cefoxitin (30 µg) as the antimicrobial sensitivity test

**Phenotypic characteristics** of 427 strains:

- Haemolysis type: α, β, α+β, lh
- Free coagulase test
- Clumping factor test
- Staphytect Plus Test
- Congo red agar medium
- DNase medium
- Mannitol salt agar medium
- Crystal violet agar medium:
  - type A
  - type C
  - type E
The analysed isolates mostly showed $\alpha+\beta$ and a zone of haemolysis (Table 2). All strains were Gram-positive cocci in clusters and catalase positive, also slide coagulase test and Staphytec Plus Test positive, but tube coagulase test negative. Total number of 427 bacterial strains (100%) appears as a positive on DNase and mannitol salt agar medium; however, negative on Congo red agar medium. Variations were observed in growth on crystal violet agar medium of types A, C, and E.

All analysed strains (Fig. 1) were especially sensitive in vitro to oxacillin (99.2%), cefoperazone (95.2%), and amoxicillin with clavulanic acid (93.6%). They were resistant mostly to neomycin (27.2%), tetracycline (21.6%) and streptomycin (20.8%). Moreover, 10% of 427 examined strains were sensitive to cefoxitin.

**Discussion**

Atypical staphylococci were the main reason of intramammary infections in the farm, causing problems with the production of an acceptable quality of milk. These pathogens caused both subclinical and clinical forms of mastitis. The changes in milk and udder quarters are usually more intensive for infections by *S. aureus* than by CNS (3, 4, 29, 31, 35).

All the analysed strains were positive for haemolysis. According to Leitner et al. (21) the most virulent are strains producing $\alpha$-haemolysin, followed by isolates producing $\alpha+\beta$-haemolysin and $\beta$-haemolysin. In the presented study, $\alpha+\beta$ type of haemolysis was the most common among analysed strains; but in Takeshige et al. (36) research, 95.4% of isolates from mastitic cows showed $\beta$-haemolysis.

The tube coagulase test (TCT) is still the “gold standard” in clinical microbiology laboratory (2). In this research total of 427 strains were negative in TCT; however, they were positive for clumping factor and commercial latex test (Staphytec Plus). Because of these characteristics, the analysed strains may be called atypical *S. aureus*.

DNase activity is important to distinguish between pathogenic and nonpathogenic staphylococci (30). In our results, DNase activity was found in 100% of analysed atypical *S. aureus* strains, which was similar to results of Mastunga et al. (25) and Takeshige et al. (36). However, other authors found the slightly lower DNase activity (94.5%) of *Staphylococcus* strains (15).

It has been thought that testing for biofilm formation could be a useful marker for the pathogenicity evaluation in staphylococci (9). Slime production by *S. aureus* strains helps in adherence, colonisation of the mammary gland and makes them more virulent (1). Strains examined in this study did not produce slime on CRA medium such as *S. aureus* strains reported in Blake and Metcalfe (5) study. However in a research of Ciftci et al. (8), 37.2% among tested *S. aureus* strains were slime positive. Many investigators have reported that the slime factor increases the resistance to antibiotics because of a decreased diffusion of antibiotics through the biofilm matrix (1, 16).

The sensitivity of the atypical *S. aureus* to antibiotics was very high and similar to results noted by Malinowski et al. (24). Watts and Salmon (39) suggested the need to identify MRSA (meticillin resistant *S. aureus*) because these strains are resistant to all compounds currently approved for treatment of bovine mastitis based on Clinical and Laboratory Standards Institute (CLSI) criteria. In veterinary medicine, MRSA *S. aureus* strains are reported occasionally (20). Atypical *S. aureus* in the presented study, as well as in the earlier one (24) did not belong to MRSA.

All examined strains fermented mannitol. Takeshige et al. (36) have reported 94.3% and Ishii et al. (18) 87.5% of *S. aureus* strains able to ferment mannitol. Only 4% of the analysed *S. aureus* strains (type C) showed a remarkable ability to bind to crystal violet stain. Earlier observations have shown that strains of *S. aureus* giving a purple reaction in the crystal violet test are significantly more pathogenic (12). Crystal violet
binding property of S. aureus has been suggested as an extrachromosomal character closely linked to the penicillin resistance. Interestingly, loss in crystal violet binding ability also results in loss of penicillinase production by S. aureus (28). This statement was confirmed in the present study.

In recent years, the atypical S. aureus strains, which do not produce free or bound coagulase, are much more frequently isolated from patients in hospitals (26, 34, 38). In Garbacz et al. (13) research, over 12.3% and 4.2% of S. aureus strains isolated from patients in hospitals in Poland have been coagulase- or clumping factor-negative, respectively. Other Polish authors (24, 40) also have noted the presence of atypical S. aureus strains in milk of cows and patients in hospitals, respectively. Moreover, Malinowski et al. (24), Wiśniewska et al. (40), and Lævens et al. (19) have used molecular methods to confirm the presence of genes characteristic for S. aureus in TCT negative strains.

Lack of free coagulase activity can cause a false identification of S. aureus, because TCT is still the most essential method for microbiological diagnostics. In case of intramammary infections in cows, which may be caused by atypical strains, apart from coagulase tube test, clumping factor should be also examined. Other phenotypic characteristics can be defined for recognition S. aureus; however for precise identification it is necessary to use molecular methods.

References
22. Malinowski E., Kłossowska A.: Diagnosis typical zakażeń i zapalen gruczołu mlekowego krów. Published by National Veterinary Research Institute, Pulawy, Poland, 2002.


