SPECIFIC DETECTION OF *STAPHYLOCOCCUS AUREUS* BY PCR IN INTRAMAMMARY INFECTION

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Forty milk samples from mastitic cows were examined bacteriologically and by PCR. Staphylococcus aureus strains were isolated from 29 samples, other bacteria from 5 samples and 6 samples were bacteriologically negative. Crude DNA from the isolated bacteria and from milk was extracted by rapid boiling and lysis with lysostaphin and proteinase K. The expected PCR product, amplicon of 270 bp was found in all *S. aureus* isolates and in 14, 18, 21, 13, 6 out of 29 *S. aureus* milk samples, depending on DNA template volume in the PCR mixture (0.25, 0.5, 1, 2, and 5 µl, respectively, in a final volume of 50 µl). Inhibitory effect of mastitic milk (1-5 µl of template) and inadequate amounts of microbial DNA (0.25 and 0.5 µl of template) were probably reasons of a low sensitivity of the PCR test. The sensitivity of the method can be increased by choice of suitable DNA extraction method and a DNA polymerase less sensitive to inhibitors.

Key words: cows, mastitis, *Staphylococcus aureus*, PCR.

*Staphylococcus aureus* is the causative agent of many opportunistic infections in humans and animals. *S. aureus* is increasingly recognized as etiologic agent of bovine mastitis and apart from streptococci and coliforms remains the most frequent bacterium isolated from clinical and subclinical forms of udder infections in Poland and other European countries (12, 28). In the last decade, a number of mastitis cases caused by *S. aureus* increased 3 times in Poland (12) and it is the main problem in herds with high bulk milk somatic cell count (BMSCC) (3, 14). A treatment efficacy of *S. aureus* mastitis is usually disappointing because the disease causes great damages in the udder and drugs are not able to penetrate to all infected sites (14).

On the other hand, *S. aureus* suppresses phagocytosis and cell mediated immunity (27) and produces an enzyme that inactivates most penicillin based treatments (16, 17). Once established, *S. aureus* usually does not respond to antibiotic treatment. Control of *S. aureus* mastitis can be achieved through the correct diagnosis, segregation of infected animals, dry cow therapy, treatment during lactation and culling program (25). The results of the analysis by Zecconi (28) showed that the incidence of new infection peaked in the first 30 d of lactation. Antibiotic treatment before calving applied to heifers and dry cow therapy to multiparous has been suggested to decrease infection rate after calving (15). This method could decrease the sensitivity of
bacteriological examination after calving by lowering the concentration of bacteria in milk, as well as by inhibition of bacterial growth in vitro. On the other hand, the sensitivity of bacteriological tests depends on shedding pattern of S. aureus (21). An unidentified infected cattle act as reservoirs of infection, since they are not selected for treatment, segregation or culling (28).

Recently, molecular methods, such as PCR have been used successfully for the identification of mastitis pathogens (7, 13, 18). The purpose of the study was an attempt to diagnose S. aureus intramammary natural infections by the PCR analysis. The nuc gene, which encodes thermonuclease was used as a target DNA to identify S. aureus.

Material and Methods

The study was carried out on a total 30 quarter milk samples from the 24 lactating cows with a previous history of S. aureus mastitis and 10 quarter milk samples from 9 cows without this infection. The examined cows belonged to 3 herds. The samples were tested for the presence of S. aureus by methods culture and PCR.

Bacteriological examination was performed as described previously (11). The number of bacteria per milliliter in milk samples was determined by the plate method. Isolates were identified as S. aureus on the basis of haemolysis, typical S. aureus colony morphology, the detection of coagulase production and by using a commercial identification system (API Staph; Biomerieux). The SCC of milk was measured by Fossomatic 90 (Foss Electric Denmark). Chromosomal DNAs from the S. aureus strains and other bacteria isolated from milk samples were extracted as described by Pozza et al. (19) with some modifications. Briefly, 1-2 colonies were collected from blood agar plates, resuspended in 300 µl of 10 mM Tris, 1 mM EDTA buffer, pH 8.0 and incubated at 100°C for 15 min and then at 37°C for 30 min with 80 µg/ml final concentration of lysostaphin, and subsequently treated with proteinase K, 100 µg/ml for 10 min at 55°C. Proteinase K was then inactivated by heating at 95°C for 15 min (all the reagents from Sigma – Aldrich Co).

To apply the PCR test for detection of S. aureus from cases of intramammary infection, DNA was extracted from all the milk samples. One milliliter of milk was centrifuged at 13 600 x g for 5 min. The cream was removed by use of a sterile cotton swab, supernatant was discarded and the pellet was resuspended in 1.0 ml of PBS, vortexed and centrifuged at 13 600 x g for 1 min. The last procedure was repeated for 5 times. Next, the pellet was resuspended in 300 µl of 0.1 mM Tris-HCl, 0.1 EDTA and then, the protocol mentioned above was applied. To select the optimal volume of genomic DNA: 5, 2, and 1 µl in the first series and 0.5, 0.25 µl of this suspension in the second series of the examination were used as a template for PCR amplification. One positive control containing S. aureus reference strain PCM 566 and one negative control containing water were included in each experiment. PCR amplifications were performed with a pair of primers specific for the nuc gene of S. aureus, synthesized (Integrated DNA Technologies, Inc., Coralville, Iowa) from the previously published sequences: primer 1: 5'-GCG ATT GAT GGT GAT ACG GTT-3', primer 2: 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3' (4). PCR amplification was carried out in 0.5ml tubes in a final reaction volume of 50 µl. The PCR mixture consisted of 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton®X-100, 200 µM (each)
deoxynucleotide triphosphate, 0.2 μM of the respective primers, and 0.625 U Taq polymerase (all the reagents from Promega Corp., Madison, Wis.). The amplification was performed with an automated thermocycler T-1 (Biometra). The PCR cycles consisted of pre-heating at 95°C for 10 min, denaturation at 94°C for 1 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1.5 min. The amplification was performed for 37 cycles with a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing 0.5 mg of ethidium bromide per ml (all the reagents from Promega Corp., Madison), visualized and photographed with Image Master VDS (Pharmacia Biotech). The sizes of the amplification products were estimated by comparison with a 50bp DNA step ladder (Promega Corp., Madison, Wis.). The specificity of this PCR was evaluated with the reference strain of *S. aureus*–PCM 566 and with all bacteria (*Streptococcus* spp., *Micrococcus* spp., coagulase negative *Staphylococcus*, *E. coli*) strains isolated from the milk samples. Student's t-test was applied to compare somatic cells count in PCR positive and negative samples.

**Results**

*Staphylococcus aureus* was isolated from 29 mastitic milk samples. Bacteria other than *S. aureus* were isolated from 5 samples (*Streptococcus* sp.-2, *Micrococcus* sp.-1, coagulase negative *Staphylococcus*1, *E. coli*1) and 6 samples were negative by culture. The number of *S. aureus* bacteria per ml ranged from 80 to 18 200 CFU. Somatic cell count in the milk samples from quarters infected with *S. aureus* ranged from 853 x 10^3 to 9558 x 10^3 /ml.

The PCR product, a single DNA band of approximately 270 bp (Fig. 1), was detected in all 29 *S. aureus* strains isolated from milk samples and *S. aureus* reference strain. Volume of DNA template in the PCR mixture had no effect on the number of positive results (data not shown) and on the amount of PCR product. There were no differences between the intensity of ethidium bromide- stain bands obtained from the 5 concentrations of the template (from 0.25 to 5 µl) in a PCR mixture of the final volume of 50 µl (Fig. 2). With the primers for the *nuc* gene we were not able to amplify and detect DNA from bacteria other than *S. aureus*. Table 1 presents the effect of various amount of extracted DNA from *S. aureus* culture positive milk samples in a final volume of 50 µl of the PCR mixture on the PCR results. Out of 29 *S. aureus* positive samples 6 (20.7%) with 5 µl, 13 (44.8%) with 2 µl and 21 (72%) with 1 µl of DNA template could be amplified. With 0.5 and 0.25 µl of DNA template, there were 18 (62.1%) and 14 (48.3%) PCR positive results, respectively. The bands produced with 5 µl of the template were weak or no product was detected. The strongest PCR reactions were obtained either with 0.5, 1, or 2 µl of the template. However, these results were highly cow (sample) specific. Different results were obtained with the same concentration of DNA template in PCR mixture (Fig. 2). Most of the positive results (21 of 29) were achieved with 1 µl of extracted DNA in the PCR mixture. At greater and lower amount of DNA template, number of positive results were lower (Table 1). *S. aureus* negative by culture milk samples were also negative by PCR. The lowest number of *S. aureus* organisms in milk detected by PCR was 500 CFU/ml. But there were two PCR negative samples with a higher number of *S. aureus* cells (1000 and 1800 CFU/ml) than the above-mentioned. The number of somatic cells in PCR
negative samples was $1557 \times 10^3 \pm 446.9 \times 10^3$/ml and in positive samples $8223 \times 10^3 \pm 759.1 \times 10^3$ /ml. The difference was not significant ($P >0.05$).

**Table 1**
Number of positive results of PCR analysis depending on the amount of template DNA in a final volume 50 µl of PCR mixture and sensitivity of the test compared to culture results

<table>
<thead>
<tr>
<th>Amount of DNA extraction in the PCR mixture, µl</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of <em>S. aureus</em> positive results of amplification</td>
<td>14</td>
<td>18</td>
<td>21</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Sensitivity compared to positive culture results (N=29), %</td>
<td>48.3</td>
<td>62.1</td>
<td>72.4</td>
<td>44.8</td>
<td>20.7</td>
</tr>
</tbody>
</table>

**Fig. 1.** Agarose gel electrophoresis of PCR amplification products from *S. aureus* chromosomal DNAs recovered from agar culture– lanes 3-4; mastitic milk – lanes 6-9; lane 10- reference strain *S. aureus* Wood 46-PCM 566 as a positive control; lane 1- DNA molecular weight markers; lane 2- negative control.
Discussion

The PCR method used to detect *S. aureus* in milk from cows with *S. aureus* mastitis was compared with bacterial culture as the standard method. In this study we were able to identify all *S. aureus* strains isolated from intramammary infection and the reference strain by amplification of the *nuc* gene (4). With the primers used we were not able to amplify and detect DNA from milk samples infected with bacteria other than *S. aureus* and the samples negative by culture. The lack of the false-positive results with 5 other than *S. aureus* species isolated from the milk samples and with DNA of milk somatic cells, indicated that the target genomic sequences were specific to *S. aureus*. The high specificity of the method was described by several authors (8, 9).

*S. aureus* DNA was detected in 20.7% of samples culture positive, when the PCR mixture contained 5 µl of DNA template in a total volume of 50 µl. That means that there were 79.3% of false negative results. Decreasing amount of the template to 2

![Agarose gel electrophoresis of specific dose-dependent amplification of *S. aureus* DNA recovered from mastitic milk from cow 7 (A, lanes 1-5), 8 (A lanes 6-10), 12 (B, lanes 1-5) and *S. aureus* DNA recovered from agar culture. As the amount of template increased from 0.25 to 2.0 µl (cows 7 and 12) and from 0.25 to 1.0 µl (cow 8) the product became gradually stronger, but at the amount 2.0 µl and 5.0 µl (cow 8) and only at the amount 5.0 µl (cow 12) of template bands became weaker. These differences were not seen in electrophoresis of PCR products from *S. aureus* DNA recovered from agar culture.](image-url)
and to 1 µl increased the number of PCR positive results, and decreased the number of false negative results 1.6 and 3.5 times, respectively. Inhibitors of PCR are common in clinical specimens. Milk (9), blood (1) and other biological fluids (10, 24) contain a variety of substances that inhibit the polymerase chain reaction and they are the reason of false negative results. Bacterial cells, and thermonuclease enzyme were found to be inhibitory factors in milk (26). In human blood, all its fractions obtained by centrifugation: buffy coat, plasma, platelets, and erythrocytes were found to be highly inhibitory to PCR (1). Immunoglobulin G was found to be the major inhibitor of PCR in human plasma (1). During intramammary infection, leukocytes are attracted to the area of inflammation, so the high somatic cell count in milk is frequently associated with *S. aureus* mastitis (22). The level of antibodies in milk increases during inflammation together with increasing of the somatic cell number (5, 6) and they can reduce the sensitivity of PCR amplification. Many studies have shown that endogenous amplification inhibitors can be removal by dilution, freezing and thawing, heating, or prolonged storage of samples (1, 10, 23, 24). In our study decreasing concentrate of the template in a final volume of PCR mixture can be compared with dilution procedure. Khan et al. (8) were able to remove the inhibitors and increase the sensitivity of the method (from 52.6% to 90%) by increasing the number of washes with PBS from 2 to 5. Despite of the pelleted bacteria were washed with PBS 5 times, our results were worse. Inadequate amounts of the microbial DNA were probably the reason of a lower number of positive results after decreasing the amount of template to 0.5 and 0.25 µl in the PCR mixture. An internal amplification control included for each sample could explain this result. False-negative reactions can occur through a number of mechanisms. Some factors can inhibit PCR by inactivation of DNA polymerase. In a study by Abu Al-Soud and Radström (2) it was noted that some commercially available polymerases are more resistant to inhibition by biological samples. Our Taq-PCR positive results (72%) are similar to Taq-PCR positive results (65%) obtained by Kim et al. (9), despite of the different DNA extraction methods used. These authors (9) have increased the sensitivity of the test to 80% by replacing Taq DNA polymerase with *Thermus thermophilus* (Tth) DNA polymerase, and they further raised it to 100% by purification of crude DNA extract with Chelex-100 before adding it to the PCR mixture.

False-negative results may influence the therapeutic decision and have medical consequences. Increased levels of sensitivity of the PCR are required for detection of *S. aureus* directly from mastitic milk in which the number of target cells can be low. Sensitivity of the PCR in diagnosis of *S. aureus* infection can be improved by choice of a suitable DNA extraction method (9, 1, 24), selection of DNA polymerase less sensitive to inhibitors (2, 9, 24), and an internal amplification control included for each sample (20).

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References