

SHORT REPORT

Multiplex PCR protocol for the diagnosis of cow udder infection with *Staphylococcus aureus**

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A total of 160 composite bovine milk samples were examined bacteriologically for the presence of *Staphylococcus aureus*. PCR method was used with two primer pairs, one corresponding to *Staphylococcus* specific chromosome region 16S rRNA, and the other to the gene *clfA* existing only in *S. aureus*. The proposed method is relatively rapid (4 hours) and very specific in relation to mastitis-causing *S. aureus* strains. *S. aureus* strains were identified on agarose gel on the basis of two PCR products: the 638 bp corresponding to *clfA* gene and the 791 bp corresponding to the region 16S rRNA, the presence of the latter indicating udder infection with *Staphylococcus* sp. other than *S. aureus*. When no PCR product on the gel was found, the infection was qualified as caused by organisms other than *Staphylococci*. All the *S. aureus* isolates identified by conventional methods were confirmed by the PCR test. Laboratory tests showed that the presented PCR diagnostic method can be helpful in identification of *S. aureus* infection of the bovine mammary gland.

KEY WORDS: mastitis / polymerase chain reaction / *Staphylococcus aureus* / udder infections

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Mastitis is the most important condition affecting dairy cattle [Shook and Schutz 1994]. The economic, international trade, animal's productivity and welfare issues associated with this disease consider it to be of great importance [Owen *et al.* 2000].

The bacteria responsible for bovine mastitis can be classified as environmental (*Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis*) or contagious (*Staphylococcus aureus* and *Streptococcus agalactiae*) depending on their primary reservoir [National Mastitis Council 1996]. Although the disease can be caused by several bacteria, *S. aureus* has emerged as one of the most prevalent pathogens which, once established in the mammary gland is very difficult to eradicate [Nickerson *et al.* 1995]. *S. aureus* represents a major agent of contagious mastitis in cattle [Phuektes *et al.* 2001].

Mastitis affects the total milk output and modifies its composition and technological quality. The largest contributing factor associated with mastitis is reduced milk yield, accounted for 69-80% of the total cost of mastitis [Janzen 1970].

Still the most common, but unspecific indicator of chronic infection of the udder is the somatic cell count (SCC). Currently, the mammary gland pathogen identification is based on an *in vitro* culture method. The method provides what is given a name of "gold standard", where the main clinically relevant factor is the differentiation between the contagious *S. aureus* and other *Staphylococci*. On one hand, selective agar-like modified Baird-Parker agar has successfully been used for the detection and identification of *S. aureus* and other coagulase-positive *Staphylococci* [Roberson *et al.* 1992]. However, these agars may not allow the detection of other microorganisms and can be used only for targeted search of *S. aureus*. On the other hand, several tests are currently used to differentiate *S. aureus* from the other *Staphylococci* in primary selective cultures. The treatment of mastitis caused by *S. aureus* is associated with poor success [Radositits *et al.* 2000], leading to a relatively high culling rate. Therefore, reliable and rapid methods for identification of *S. aureus* from mastitic milk are crucial for the control of the disease and for economically sound udder health management.

Nowadays, molecular methods allow to obtain more information regarding a dissemination of and contamination by microorganisms of different parts of the food chain. In most laboratories the accurate assessment of these issues depends on the identification of phenotypic traits of cultured bacteria. However, there are numerous reports describing the use of polymerase chain reaction (PCR) method for identification and characterization of staphylococcal isolates [Geha *et al.* 1994, Canvin *et al.* 1997, Gribaldo *et al.* 1997]. To maximize sensitivity, most protocols focused on amplification of conserved regions of eubacterial rRNA genes [Geha *et al.* 1994, Mariani *et al.* 1996, Gribaldo *et al.* 1997, Schmitz *et al.* 1997]. Other protocols were directed towards the specific detection of *S. aureus* and focused on amplification of genes found only in this species [William *et al.* 2001, Phuektes *et al.* 2003]. The PCR is suitable for rapid, efficient and cost-effective confirmation of results of phenotypic identification of *S. aureus* in milk. With continuously improving technologies and decreasing costs, genetic methods like identification of 16S rRNA and *clfA* gene will

soon find a place in routine veterinary diagnostics for *S. aureus* strains [Smeltzer *et al.* 1997].

The objective of this study was to establish multiplex PCR protocol for rapid identification of *Streptococci* strains in milk, enabling the diagnosis of infection of the udder with *S. aureus*.

Materials and methods

A total of 115 Polish Black-and-White (Polish Friesian) cows with a high share of Holstein blood and maintained at the Institute Farm, Kosów, were sampled once or twice during lactation by collecting milk from the four quarters and pooling it into a sterile bottle, as a composite milk sample. A total of 160 composite milk samples were examined. During sampling the first streams of milk were rejected since their cell and bacterial counts were likely to reflect the situation within the teat rather, than of the udder as a whole [Radositits *et al.* 2000]. Milk samples for bacteriological examinations were collected in the sterile bottles, placed on ice, and transported immediately to the laboratory.

One ml of milk from each sample was taken for isolation of *Staphylococci* on Baird-Parker agar (BPA, BIO-MERIEUX) plates with surface plating method and incubated at 37°C for 48 hours. Typical black or greyish-black colonies were gram-stained and catalase activity was determined. Presence or absence of a clearing or halo on BPA plates was recorded. Further identification of gram-positive and catalase-positive cocci was carried out using tube coagulase test performed with rabbit plasma, according to manufacturer's recommendations (BIO-MERIEUX). Bacterial colonies were classified as *Staphylococci* when their appearance on BPA was typical and were gram-, catalase- and coagulase-positive. *Mastitis* caused by *Staphylococci* was diagnosed when more than 500 *Staphylococcus* colonies/ml milk were grown up [Boddie and Nickerson 1986] and somatic cell count (SCC) exceeded 400,000/ml [Sender *et al.* 1987, Polish Standard 1999].

To identify the *S. aureus* and differentiate it from remaining *Staphylococci*, all the isolated gram-, catalase- and coagulase-positive bacteria were cultured in 9 ml of brain heart infusion broth (BHI - BIO-MERIEUX) at 37°C for 17 h and DNA from bacteria isolates was extracted. One ml of bacteria isolates were centrifuged at 8000 g for 7 min. The NucleoSpin® Tissue Kit was used to obtain bacterial DNA, according to the manufacturer's recommendations. The bacterial DNA was next frozen until used for the PCR.

The DNA amplification was performed using primers suggested by William *et al.* [2001]. Two genes were analysed. First corresponded to the region of the 16S rRNA gene conserved among *Staphylococci* and unique as compared to the other eubacterial species. The second corresponded to the *S. aureus clfA* gene which encodes a surface-exposed fibrinogen-binding protein [McDevitt *et al.* 1994]. The *clfA* was chosen based on the fact that it is characteristic of the *S. aureus* genome and appears exclusively in all *S. aureus* strains [Smeltzer *et al.* 1997].

The PCR reaction was conducted in a GenAmp PCR System 9600 Thermal Cycler (AB). The cycling parameters were: (1) 94°C for 10 min, (2) 94°C for 1.5min, (3) 55°C for 1 min, (4) 72°C for 1 min, 36 cycles of step 2 through 4 inclusive, and 72°C for 10 min. The PCR reaction was performed in a mix containing: 50-100 ng of genomic DNA, 200 μM of each dNTP, 3.0 mM MgCl₂, 20 pmol of each primer, 2.5 U Taq Gold Polymerase (AB). The PCR products were visualized using a 2.5% agarose gel containing 0.5 μg of ethidium bromide/ml in relation to the DNA mass ladder standard (DNA from pUC19, 11-1444 bp, BTL). Gene size was determined in base pairs (bp) comparing with the length of the PCR product. *S. aureus* strains were identified in presence of two PCR products on agarose gel: the 791 bp corresponding to the 16S rRNA regions of genes and the 638 bp corresponding to *clfA* gene. Detection of 791 bp PCR product on the gel indicated that *Staphylococci* strains found in milk of infected cows were different from *S. aureus*. Cows infected with other bacteria species were identified on the basis of total absence of any PCR product on the gel (Photo.1).

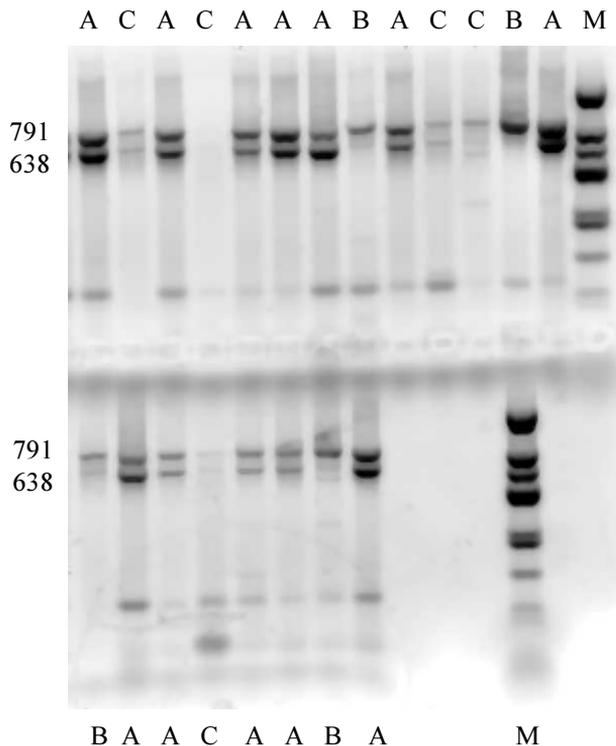


Photo. 1. Specificity of multiplex PCR. Line M – molecular size marker (11-1444 bp). Line A – sample containing *S. aureus* strain (791 bp). Line B – sample containing other *Staphylococcus* species (638 bp). Line C – other species.

Results and discussion

The PCR method has originally been used in human medicine, but currently the first attempts are reported at adopting it to diagnosis of *mastitis* in cattle [Phuektes *et al.* 2003]. Compared to conventional methods of identification of *S. aureus* isolates the PCR method is less laborious and cheaper. In the future it is likely to be predominant method of identification of pathogenic bacteria. In the present study the identification of *S. aureus* isolates was performed conventionally and with the PCR method. Using conventional method identified were about 14% of udders infected with *S. aureus*. (Tab. 1). All identifications of *S. aureus* isolates by conventional method were confirmed by the PCR test. In the present study PCR method was applied to isolated bacterial colonies grown on broth culture. In PCR, using isolated colonies is more specific and less time-consuming than working direct on clinical samples [Schmitz *et al.* 1997]; the latter requires additional steps (e.g. hybridization with species-specific probes) – Mariani *et al.* [1996]. PCR method with the use of two primer pairs (one corresponding to *Staphylococcus*-specific chromosome region 16S rRNA, and the other to the gene *clfA* existing exclusively in all *S. aureus* strains) consumes relatively little time (4 hours) and is very specific in relation to human strains of *S. aureus* [William *et al.* 2001]. The same criteria were successfully applied in the present study for preparing multiplex PCR protocol for diagnosis of bovine *S. aureus* strains causing *mastitis* (Photo 1).

Table 1. Number of udder infections as diagnosed with conventional method

Infection	No of udders
<i>S. aureus</i>	23 (14.38%)
Other types of bacteria	36 (22.50%)
Healthy udders	101 (63.12%)
Total	160 (100%)

The prevalence of subclinical infection is difficult to ascertain, as it can only be determined by isolation of bacteria from the gland [Leigh 1999]. Many organisms established in the cow's environment can cause *mastitis*. Even if they are isolated from mastitic milk it is always difficult to state whether their presence is really related to *mastitis*, or is an artefact resulting from simple contamination. Bacteriological investigations and cell counting are sometimes used in combination to determine the infection status of the mammary gland [Barkema *et al.* 1997]. However the interpretation of these combined results is not straightforward [Shoshani *et al.* 2000]. It can be concluded that PCR method of diagnosis of *S. aureus* in milk as an objective method seems to be helpful in recording infection status of the mammary gland.

Concluding, the *S. aureus* strain was identified on the basis of two PCR products on agarose gel: the 791 bp corresponding to the region of the 16S rRNA gene and the

638 corresponding to *clfA* gene. Detection of only one PCR product (791 bp) allowed to conclude that the cows were infected with *Staphylococcus* sp. other than *S. aureus*.

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Diagnozowanie metodą PCR stanu zapalnego wymienia krów wywołanego przez *Staphylococcus aureus*

Streszczenie

Bakteriologicznie zbadano 160 prób mleka pobranych z całego wymienia krów pod kątem występowania stanu zapalnego wywołanego przez *Staphylococcus aureus*. Celem pracy było opracowanie multipleksowej metody PCR pozwalającej na diagnozowanie infekcji wymienia wywołanych gronkowcem złocistym. W reakcji PCR amplifikacji poddano dwa geny: gen 16S rRNA występujący u wszystkich gronkowców oraz gen *clfA* występujący wyłącznie w chromosomie gronkowca złocistego. Gronkowca złocistego zidentyfikowano na podstawie obecności dwóch prążków na żelu agarozowym: jednego o długości 791 par zasad (gen 16S rRNA) i drugiego o długości 638 par zasad (gen *clfA*). Obecność jednego prążka na żelu o długości 791 par zasad świadczyła o wystąpieniu zapalenia wymienia spowodowanego gronkowcem innym niż gronkowiec złocisty. Wszystkie przypadki wystąpienia gronkowca złocistego zdiagnozowane metodą tradycyjną (płytkową) zostały następnie potwierdzone za pomocą metody multipleksowej PCR. Metoda ta jest stosunkowo szybka i charakteryzuje się wysoką specyficnością w stosunku do *Staphylococcus aureus* wywołującego *mastitis* krów.

