VIRULENCE FACTORS IN COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM COWS WITH SUBCLINICAL MASTITIS

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Abstract

The aim of this study was to investigate the presence of virulence genes in 46 coagulase-negative staphylococci (CNS) strains isolated from 46 udder quarters of 46 cows with subclinical mastitis. The isolates were examined phenotypically (cultural features, Gram stain, catalase, coagulase, API tests) and genotypically for: icaA, icaAB, icaD, atlE, bhp, agrA, sarA, mecA, and blaZ genes. These isolates had no nuc and coa genes, and were identified as S. warneri (11 strains), S. chromogenes (10), S. xylosus (seven), S. epidermidis (one), S. hyicus (one), and other Staphylococcus species (16). The single virulence genes or their combinations were detected in nine (19.6 %) of isolates. The following genes were found: blaZ (in four isolates), mecA (3), sarA (3), agrA (3), and atlE (three). None of the strains harboured the bhp, icaA, icaD, and icaAB genes. In conclusion, subclinical mastitis in cows can be caused by different species of CNS that harbour different virulence factors.

Key words: cow, mastitis, coagulase-negative staphylococci, virulence factors.

Material and Methods

Fourty-six coagulase-negative staphylococci were isolated from 46 quarters of 46 cows with subclinical form of mastitis in 15 farms (herds). Quarter milk samples were collected aseptically by a scientific personnel or by field veterinary practitioners. The teats were cleaned and dipped in a disinfectant and then teat ends were wiped with alcohol swabs and allowed to dry. The first few streams were discarded and then 2–4 ml of secretion were collected in sterile tubes. The samples were cooled and immediately transported to laboratory. Bacteriological examinations were performed according to commonly accepted procedures (14). Milk somatic cell count was determined by Fossomatic 90. Subclinical mastitis was diagnosed in quarters with positive bacteriologic result and SCC ranging between 1 x 10⁵/ml and 4 x 10⁵/ml without any clinical signs.

The isolates were examined phenotypically by cultural features, Gram stain, catalase, coagulase, API biochemical tests (14), and genotypically by PCR assays specific for regulatory genes: sarA and agrA, biofilm production: operon ica (icaA, icaAB, icaD), surface
proteins: bhp and atlE, and antibiotic resistance genes: mecA and blaZ.

Chromosomal DNA from bacterial strains was extracted as described by Dalla Pozza et al. (6) with some minor modification. In brief, the pellet of bacterial cells from 1 ml overnight culture in brain-heart infusion broth (bioMérieux) was first resuspended in 500 µl of PBS, and then resuspended in 100 µl of TE buffer (Promega). The samples were subsequently incubated at 100°C for 15 min. Further steps of DNA isolation were carried out according to Dalla Pozza et al. (6).

The staphylococci were subjected to PCR using the sets of primers that have been described previously by other authors. One pair of primers was specific to the Staphylococcus genus target gene (primers STA I, STA II) (7), one to the thermostable Staphylococcus aureus nuclease gene (nuc) (4), one to the Staphylococcus aureus coagulase gene (coa) (13), and eight sets of primers were specific to S. epidermidis (STAE-EpI, STAE-EpII) (7), S. xylosus (Xyl F, Xyl R) (17), S. saprophyticus (Sap 1, Sap 2) (17), S. hyicus (STAH-HyI, STAH-HyII) (7), S. warneri (Swa, Sta2) (3), S. simulans (STAS-SiI, STAS-SiII) (7), S. chromogenes (STAC-ChrI, STAC-ChrII) (7), and S. carnosus (Scal, ScII) (3) species genes. PCR primer pairs described by Frebourg et al. (9) were used to amplify sarA, agrA, icaA (biofilm), and atlE (adhesion) gene. The primers used for the amplification of icaA (biofilm) and icaD (biofilm) gene were described by Arciola et al. (1). The bhp (biofilm, adhesion) gene was amplified using the primers (bhp-P5, bhp-P3) described by Gu et al. (12), mecA gene by Geha et al. (10) (mecA1*, mecA2), and blaZ gene by Vesterholm–Nielsen et al. (28).

Amplifications were carried out in a final volume of 20 µl and performed in an automated thermocycler (Biometra). PCR mixture consisted of 0.4 µl of deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP): 10 mM of each; 20 µl of buffer (5 x Green GoTag®Flexi Buffer); 0.5 U of thermostable polymerase; 1.2 µl of 25 mM MgCl₂ (all the reagents from Promega); 0.1 µl of respective primer; 5.0 µl of DNA examined. S. epidermidis ATCC 35984 (sara, agrA, icaA, icaD, icaAB, bhp, atlE, blaZ, mecA) were used as positive controls, while S. epidermidis ATCC 12228 was used as a negative control. For blaZ gene the conditions were as follows: initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final elongation step at 72°C for 10 min. The samples tested for the presence of other genes were subjected to 30 cycles, each consisting of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of elongation at 72°C. A 2-min denaturation step at 94°C was included at the beginning of the cycling, and at the end a final 5-min extension step at 72°C was included. The PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.1 µl/ml), and visualised with the ImageMaster™ VDS System (Amersham Pharmacia Biotech).

**Results**

All strains were recognised as being the Staphylococcus genus, which did not possess the nuc and coa genes. Among them five species, which included 30 strains were genotypically identified, and 16 strains belonged to other CNS species, which were not identified by primers used in this work. Most frequently S. warneri (11 strains), S. chromogenes (10 strains), and S. xylosus (seven strains) were noted.

Among the isolates, six virulence gene patterns were found. The single virulence gene or a combination of genes were detected in nine (19.6%) isolates. The following genes were found: blaZ, mecA, sarA, agrA, and atlE. None of the examined strains harboured the bhp, icaA, icaD, and icaAB gene. Five isolates carried only single antibiotic resistance gene. The genotypes with two or more genes were connected with four strains: one of them harboured four genes, one strain three genes, and two strains harboured two genes.

The presence of identified genes in different CNS species is listed in Table 1. The strain, which harboured the most of the detected genes was S. epidermidis. The other strains that harboured virulence factor genes belonged to S. xylosus or unidentified Staphylococcus species. None of the virulence factor, mecA and blaZ genes were found in S. warneri, S. chromogenes, and S. hyicus strains. Regulatory genes (agrA, sarA) were detected in four strains, and surface-associated autolysin (atlE) in three strains. Beta-lactams (blaZ) and methicillin resistance genes (mecA) were found in four and three strains, respectively, but none of the strains harboured both genes simultaneously.

<table>
<thead>
<tr>
<th>Item</th>
<th>Genotype</th>
<th>No. of strains (%)</th>
<th>CNS species (no. of strains)</th>
<th>Herd symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sarA agrA atlE mecA</td>
<td>1 (11.1)</td>
<td>S. epidermidis</td>
<td>a</td>
</tr>
<tr>
<td>2</td>
<td>sarA agrA atlE</td>
<td>1 (11.1)</td>
<td>S. xylosus</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>agrA atlE</td>
<td>1 (11.1)</td>
<td>S. xylosus</td>
<td>b</td>
</tr>
<tr>
<td>4</td>
<td>sarA blaZ</td>
<td>1 (11.1)</td>
<td>Staphylococcus sp.</td>
<td>c</td>
</tr>
<tr>
<td>5</td>
<td>mecA</td>
<td>2 (22.2)</td>
<td>Staphylococcus sp.</td>
<td>d; e</td>
</tr>
<tr>
<td>6</td>
<td>blaZ</td>
<td>3 (33.3)</td>
<td>S. xylosus (1)</td>
<td>f</td>
</tr>
<tr>
<td>Total</td>
<td>9 (100.0)</td>
<td></td>
<td>Staphylococcus sp. (2)</td>
<td>e; f</td>
</tr>
</tbody>
</table>
The source of strains harbouring virulence factor and antibiotic resistance genes was also presented in Table 1. Strains harbouring an antibiotic resistance gene were isolated from five different farms, and a virulence factor gene from three farms; however, two isolates of S. xylosus harbouring biofilm-associated genes derived from cows within one herd. None of the strains with genes mentioned above was detected in nine farms.

**Discussion**

Coagulase-negative staphylococci are at present the main aetiological agents of bovine mastitis in a number of herds. Almost 20 species were identified by API Staph ID 32 (11, 21, 26). In the presented study 30 (65.2%) of CNS strains, which belonged to five species, were identified with the use of primers applied earlier by different authors. The species: S. warneri, S. chromogenes, S. xylosus, S. epidermidis, and S. hyicus occurred in order from the largest to the smallest percentage. The same species, or sometimes other ones, which occurred with similar or different frequency have been reported by other authors (11, 21).

The pathogenicity of CNS is connected with the presence or absence of certain genes, which are involved in the virulence process. These are ica gene (intercellular adhesion - operon - icaADBC), attE gene, encoding the vitronectin-binding cell surface protein involved in primary attachment, bap (biofilm-associated protein) or bhp (Bap homologue protein), sar (accessory gene regulator), ica (staphylococcal accessory regulator), and mecA gene, which controls the synthesis of the additional penicillin-binding protein PBP2a in methicillin-resistant staphylococcus (24). Though, many virulence factors are described, their effect on the ability of pathogens to form biofilm plays an clearly explained.

In pathogenesis of mastitis and in persistence of IMI the ability of pathogens to form biofilm plays an essential role (16, 19). Biofilm allows the persistence of infection by impairing the host cellular immune system and protection from antibiotic activity. From literature data (5, 8), it is known that biofilm formation by staphylococci from bovine mastitis is associated with the loci ica (intercellular adhesion), bap (biofilm-associated protein), agr (accessory gene regulator), and sar (staphylococcal accessory regulator). In the presented study, only small percentage of examined CNS species harbouring the genes involved (sarA, agrA, attE) and no strains with bhp, icaA, icaAB, icaD genes were detected. The bap gene has been identified in biofilm producing staphylococci isolated from mastitis, including S. epidermidis, S. chromogenes, S. xylosus, S. simulans, and S. hyicus (27). Both bap and ica genes have been identified in mucoid staphylococcal isolates involved in biofilm formation (5). However, icaADBC genes were also not found in CNS strains examined by Tormo et al. (27).

An important feature of staphylococci is their resistance to antibiotics. The genes mecA and blaZ were present in 6.5% and 8.7% of isolates, respectively. The resistance against β-lactam antibiotics genes were also found by other authors in similar or different percentage. The mecA was found by Moon et al. (18) in 12 CNS strains isolated from mastitis. The presence of mecA gene in combination with other resistance genes was found in 12 CNS isolates, and mecA as the single gene in 18 isolates and in combination with other genes in 13 isolates was found by Sawant et al. (22) among 168 strains isolated from bovine milk. Asfour and Darwish (2) found single mecA in 13 strains, single blaZ gene in six strains, and combination of both genes in next 14 strains among 70 isolated from clinical and subclinical mastitis in cows.

From our examinations and the literature data it can be seen that different species of CNS constitute aetiological factors of subclinical mastitis in cows. Some CNS strains that cause mastitis have virulence and resistance to β-lactam antibiotic genes. Various researchers have found, however, the presence of these genes in the different proportion in different strains. These differences may be related to the actual characteristics of microorganisms occurring in the different herds, and they can be the result of inadequate or inaccurate testing methods. This indicates the necessity for further studies. However, in the pathogenicity of CNS for the cow mammary gland, also enterotoxins, 34- to 36-kDa protein with cell-rounding cytotoxic activity, and many other virulence factors reviewed by Taponen and Pyörälä (24), should be taken into account.

In conclusion, mastitis in cows can be caused by different species of CNS that harbour various virulence factors.

**References**


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