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Original article

Production of slime by coagulase-negative staphylococci (CNS) isolated from clinical and subclinical mastitis in cows

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Abstract

The aim of the study was to evaluate the slime-producing ability of coagulase-negative staphylococci (CNS) isolated from clinical and subclinical mastitis in cows. The study was carried out on 100 isolates of CNS obtained from milk of 86 cows from farms located in the Lublin region (Poland). Slime-producing ability was observed in over half of coagulase-negative staphylococci (54.0% of isolated CNS), including 19 isolates of methicillin-resistant staphylococci (95.5% of all MRCNS). Of 22 isolates of CNS responsible for the clinical form of mastitis, 20 isolates (90.9%) produced slime: *S. xylosus* (7 isolates), *S. haemolyticus* (6 isolates), *S. chromogenes* (4 isolates), and *S. sciuri* (3 isolates), including 9 isolates of MRCNS (45.0%). The remaining 34 isolates of CNS (43.6%) with the ability to produce this exopolysaccharide were isolated from the milk of cows with subclinical form of mastitis: *S. xylosus* (12 isolates), *S. sciuri* (9 isolates), *S. chromogenes* (6 isolates), *S. haemolyticus* (3 isolates), *S. warneri* (3 isolates) and *S. saprophyticus* (1 isolate), including 10 isolates of MRCNS (12.8%).

Key words: cows, mastitis, coagulase-negative staphylococci (CNS)

Introduction

Coagulase-negative staphylococci (CNS), which are widespread in the natural environment, colonizing the skin and mucous membranes of animals and humans, were for decades considered to be non-pathogenic microorganisms, but have currently become the predominant aetiological factor of bovine mastitis in many countries (Chaffer et al. 1999, Makovec and Ruegg 2003, Pitkälä et al. 2004, Rajala-Schultz et al. 2004, Taponen et al. 2007,

Malinowski and Kłossowska 2010, Waller et al. 2011, Bochniarz et al. 2013). CNS are considered minor pathogens, as in the majority of cases they cause subclinical mastitis (Taponen et al. 2006, Persson Waller et al. 2011). Nevertheless, they can also cause clinical mastitis, characterised by mild symptoms, but associated with increased somatic cell count (Taponen et al. 2007) and reduced milk production, leading to economic losses.

The major problem in the therapy of infections caused by CNS is their ability to form the bacterial

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biofilm and the mechanisms of acquiring the drug-resistance (Łopaciuk and Dzierżanowska 2002, Bartoszewicz-Potyrała and Przondo-Mordarska 2002). Recently a significant increase has been observed in the number of isolated methicillin resistance CNS (*mecA*-gene-positive) which are resistant to all groups of β -lactam antibiotics (Moon et al. 2007, Bochniarz and Wawron 2011, Persson Waller et al. 2011, Bochniarz et al. 2013).

The first stage of the pathogenesis of infection is adhesion of the bacteria to tissue surfaces (Ishak et al. 1985, Opdebeeck et al. 1988, Matthews et al. 1991, Iturralde et al. 1993, Krzeminski et al. 1993, Aguilar et al. 2001, Arciola et al. 2001). Adhesion is particularly important for the survival of staphylococci in cows' udder, as exfoliation of the epithelium and milk flow during milking can lead to mechanical removal of these bacteria. According to many authors, the adhesion ability of staphylococci is determined by the presence of surface adhesins and by the production of extracellular slime, described as a loose, shapeless, sticky material (Tylewska et al. 1985, Buxton et al. 1987, Matthews et al. 1991, Timmerman et al. 1991, Bartoszewicz-Potyrała and Przondo-Mordarska 2002). The main element of the slime pseudocapsule is a polysaccharide molecule (100,000 kDa), composed mainly of glucose and N-acetylglucosamine, which is resistant to the enzyme activity of the host organism (Bartoszewicz-Potyrała and Przondo-Mordarska 2002).

The aim of the study was to evaluate the slime-producing ability of coagulase-negative staphylococci (CNS) isolated from clinical and subclinical cases of mastitis in cows.

Materials and Methods

The study material consisted of 100 CNS isolated from the milk of 86 cows on farms located in the Lublin region (Poland).

Clinical examination of cows, i.e. determination of general symptoms and changes within the mammary gland, as well as a macroscopic evaluation of the milk, was performed prior to collection of samples for bacteriological testing. Milk samples were collected according to established procedure. After the skin of the udder had been cleaned, washed, and dried, and the teat orifices disinfected with 70% alcohol solution, milk was collected into sterile, labelled test tubes, chilled to 4°C and delivered to the laboratory of the Department and Clinic of Reproduction in Lublin.

Bacteriological examination of the milk was carried out according to standard procedures: milk

culture on agar medium (BTL, Łódź, Poland) supplemented with sterile, defibrinated sheep blood (5% of the agar solution volume), incubation for 24 h under aerobic conditions at 37°C; evaluation of the morphology of the bacterial colonies and Gram-stained microscopic specimens; evaluation of susceptibility to lysostaphin (Sigma, USA) and a tube coagulase test using rabbit plasma (Biomed, Kraków, Poland). Identification of coagulase-negative staphylococcus species was performed using the commercial API STAPH test (bioMérieux, France). The procedure followed the manufacturer's recommendations.

Evaluation of phenotypic methicillin resistance of coagulase-negative staphylococci

The test was conducted using Oxacillin Resistance Screening Agar Base (Oxoid, England) with the addition of ORSAB Selective Supplement. The medium solution, at a concentration of 51.75 g, was prepared in 500 ml of distilled water and sterilised in an autoclave at 121°C for 15 minutes; one vial of ORSAB Selective Supplement was added to the medium cooled to 50°C (oxacillin concentration in the solution was 0.5 µg/ml) and poured onto Petri plates, 9-10 cm in diameter. The CNS isolates were cultured on this agar. The plates were incubated at 37°C for 24-48 h. The reference *Staphylococcus aureus*, ATCC 43300, was used as a positive control.

Evaluation of genetically conditioned methicillin resistance of coagulase-negative staphylococci

DNA was isolated using the enzymatic digestion method with CTAB. The isolated DNA of individual methicillin-resistant CNS isolates was tested for the *mecA* gene using the PCR method. For amplification, primers complementary to the conserved region within the *mecA* gene were used, to amplify a 533 bp fragment. Primers for PCR were synthesized in Oligo-PAN (Warsaw, Poland). The following primer sequences for the *mecA* gene were used:

mec1: 5, AAA ATC GAT GGT AAA GGT TGG C 3,
mec2: 5, AGT TCT GCA GTA CCG GAT TTG C 3.

The reaction mixture consisted of the following: 1 U Taq Polymerase (Fermentas, Lithuania), 2.5 µl 10x Taq Polymerase Buffer, 2.5 µl dNTPs, 1 µl mec1 primer, 1 µl mec 2 primer, 3 µl MgCl₂, and 9.9 µl distilled water. The amplification products were analysed by electrophoresis on 1.5% agar gel (Sigma, USA) in the presence of a molecular weight standard (100 bp DNA, Fermentas, Lithuania).

Table 1. Percentage of coagulase-negative staphylococci isolates producing slime in clinical mastitis in cows.

Phenotypic feature	CNS species									
	<i>S. xylosus</i> (8)		<i>S. haemolyticus</i> (7)		<i>S. chromogenes</i> (4)		<i>S. sciuri</i> (3)		Total (22)	
	n	%	n	%	n	%	n	%	n	%
Slime production	7	87.5	6	85.7	4	100.0	3	100.0	20	90.9*

Table 2. Percentage of coagulase-negative staphylococci isolates producing slime in subclinical form of mastitis in cows.

Phenotypic feature	CNS species															
	<i>S. chromogenes</i> (22)		<i>S. xylosus</i> (20)		<i>S. haemolyticus</i> (18)		<i>S. sciuri</i> (11)		<i>S. warneri</i> (4)		<i>S. hominis</i> (2)		<i>S. saprophyticus</i> (1)		Total (78)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Slime production	6	27.3	12	60.0	3	16.7	9	81.8	3	75.0	0	0.0	1	100.0	34	43.6*

Legend for Tables 1 and 2: *statistically significant difference at $p \leq 0.001$ between percentages of slime-producing CNS isolated from the milk of cows with clinical and subclinical mastitis
n – number of isolates

Table 3. Percentage of slime-producing methicillin resistant coagulase-negative staphylococci (MRCNS) isolates in clinical and subclinical forms of mastitis.

Type of mastitis	Clinical mastitis (22)				Subclinical mastitis (78)			
	n		%		n		%	
Slime production by MRCNS	9		40.9**		10		12.8**	

Legend for Table 3: ** statistically significant difference at $p \leq 0.001$ between the percentages of slime-producing MRCNS isolated from the milk of cows with clinical and subclinical mastitis.

Evaluation of slime-producing ability

The slime-producing ability of CNS was evaluated by testing adhesion to polystyrene surfaces according to Christensen et al. (1983). Colonies of each CNS isolate collected from the solid medium (agar enriched with 5% sheep blood) were placed in sterile test tubes containing 4 ml tryptic soy broth – TSB (Oxoid, England). After incubation for 24 h at 37°C, the contents of the test tubes were removed by aspiration, washed with distilled water, and stained with crystal violet. A blue film covering the inner surface of the test tube, indicating the presence of slime, was considered to be a positive result.

Slime-producing ability was also tested using Congo Red Agar (Sigma) (Freeman et al. 1989). Overnight cultures in TSB were inoculated onto CRA plates and incubated at 37°C for 24h. Biofilm formation was detected based on the presence of black or nearly black colonies on the agar.

The type of mastitis was established based on the result of the bacterial culture of the milk and the somatic cell count (SCC), determined using a Fossomatic cell counter, together with the previously conducted clinical examination of the cows. $SCC > 200,000/\text{ml}$ of milk and the presence of bacteria in the milk culture without general symptoms were considered subclinical inflammation. Visible macroscopic changes in the milk and/or local changes within the mammary gland, occasional general symptoms and an elevated $SCC > 200,000/\text{ml}$ of milk, indicated clinical mastitis (Gentilini et al. 2002, Moon et al. 2007).

Statistical analysis

Significance of differences in percentages of slime-producing CNS isolated from clinical and subclinical forms of mastitis in cows was determined

using Statistica 6.0 software. Significance of differences between the features tested was determined at $p \leq 0.01$ and $p \leq 0.001$.

Results

Coagulase-negative staphylococci isolated from the milk of cows with the clinical form of mastitis (22 isolates) were classified into 4 species: *S. xylosus* (8 isolates), *S. haemolyticus* (7 isolates), *S. chromogenes* (4 isolates), and *S. sciuri* (3 isolates), while the subclinical mastitis was caused by 78 CNS belonging to 7 species: *S. chromogenes* (22 isolates), *S. xylosus* (20 isolates), *S. haemolyticus* (18 isolates), *S. sciuri* (11 isolates), *S. warneri* (4 isolates), *S. hominis* (2 isolates), and *S. saprophyticus* (1 isolate). Methicillin-resistant CNS (20 isolates) accounted for 20.0% of all CNS isolated from the clinical and subclinical forms of mastitis.

Slime-producing ability was observed in over half of the coagulase-negative staphylococci (54.0% of isolated CNS), including 19 isolates of methicillin-resistant staphylococci (95.5% of all MRCNS).

A positive result for the reaction was obtained for CNS from the milk of cows with both clinical and subclinical form of mastitis: In total, of 22 CNS responsible for the clinical form of mastitis, 20 isolates (90.9%) produced slime: *S. xylosus* (7 isolates), *S. haemolyticus* (6 isolates), *S. chromogenes* (4 isolates), and *S. sciuri* (3 isolates); including 9 MRCNS isolates (45.0%) (Table 1, 3). The remaining 34 CNS isolates (43.6%) with the ability to secrete this exopolysaccharide obtained from cows with subclinical form of mastitis: *S. xylosus* (12 isolates), *S. sciuri* (9 isolates), *S. chromogenes* (6 isolates), *S. haemolyticus* (3 isolates), *S. warneri* (3 isolates) and *S. saprophyticus* (1 isolate); including 10 isolates of MRCNS (12.8%). (Table 2, 3).

Discussion

Many authors studying factors responsible for the pathogenicity of coagulase-negative staphylococci suggest that slime production and the ability to adhere to surfaces, enabling the formation of a biofilm, is one of the most important elements of the invasiveness of these bacteria (Christensen et al. 1982, Ishak et al. 1985, Christensen et al. 1985, Opdebeek et al. 1988, Bedidi-Madani et al. 1998, Aguilar et al. 2001, Arciola et al. 2001, de Silva et al. 2002).

The ability of coagulase-negative staphylococci to adhere to tissue surfaces was described in both humans and animals as early as the 1990s. When Mat-

thews et al. (1991) tested 206 isolates representing 14 species of coagulase-negative staphylococci from cows' milk, they observed slime production in 85.0% of isolates. This result confirmed the authors' supposition about the importance of this feature as a virulence factor. Bedidi-Madani et al. (1998), in a study on goats' milk, found slime production in 70 of 165 CNS isolates (42.0%). The greatest numbers of isolates reacting positively in this experiment belonged to 4 species: *S. caprae* (30 isolates), *S. xylosus* (isolates), *S. simulans* (7 isolates) and *S. warneri* (6 isolates).

Krzeminski et al. (1993) tested 255 isolates of coagulase-negative staphylococci from humans and found 222 isolates capable of slime production. Christensen et al. (1982) observed this property in 63.0% of *S. epidermidis* isolates obtained from clinical cases of sepsis in humans. A study by Ishaka et al. (1985) demonstrated the presence of slime in a significantly higher percentage of CNS isolated from various infections in humans than in saprophytic strains of these staphylococci (91.0% and 14.8%). Younger et al. (1974) and Davenport et al. (1986) observed a link between slime production and chronic infections caused by CNS. Younger et al. (1987) and Diaz-Mitoma et al. (1987) found that antibiotics exerted a weak therapeutic effect in the case of infections caused by slime-producing CNS.

The antyphagocytic activity of slime was described by Lee and Lee (2006). This extracellular polysaccharide, produced by many *Staphylococcus* strains, masks the C3b complement protein on the surface of bacterial cells, thus preventing their recognition by phagocyte receptors. Moreover, slime-producing *Staphylococcus* strains have a greater ability to colonize tissues (Baselga et al. 1993), as the slime connects a layer of parent cells and newly-formed cells, thus forming a bacterial biofilm (Hussain and Wilcox 1993). The biofilm allows the bacteria not only to survive in unfavourable conditions, but also to multiply and grow. It also protects against antibiotics, disinfectants and the host's immune system (Costerton et al. 1999, Melchior et al. 2006).

Biofilm formation is thus one of the significant virulence factors in bacteria causing mastitis in cows, such as *Staphylococcus aureus* or *E. coli*, which has been demonstrated in numerous studies (Opdebeek et al. 1988, Baselga et al. 1993, Iturralde et al. 1993, Aguilar et al. 2001, Melchior et al. 2011). However, there is limited information on biofilm formation by CNS isolated from the milk of cows with mastitis.

In a study carried out by Tremblay et al. (2013), the ability to form a biofilm was observed in 85% of CNS responsible for mastitis in cows. The ability to form biofilms was found to vary among coagulase-negative *Staphylococcus* species; *S. xylosus* and *S.*

haemolyticus were strong biofilm formers, while *S. simulans* and *S. epidermidis* were weak biofilm formers.

Simojoki et al. (2012) found far fewer coagulase-negative staphylococci (31% of all CNS) forming a biofilm in the mammary gland of cows. The percentage of slime-producing CNS isolates was also small in this study: 14% produced slime on Congo Red Agar (CRA) with glucose, and 8.9% on CRA with lactose. The highest percentage of isolates with slime-producing ability belonged to the species *S. epidermidis*. The study cited found no association between the intensity of inflammation, measured by milk NAGase activity, and slime production. However, a higher percentage of isolates from IMIs was shown to have slime-producing ability than isolates from chronic udder infections.

In the present study, over half (54.0%) of the coagulase-negative staphylococci tested exhibited slime-producing ability. It is important to note that the presence of this exopolysaccharide was observed in 90.9% of CNS responsible for the clinical form of mastitis, including cases of acute mastitis. A statistically significantly lower percentage of CNS isolated from the milk of cows with subclinical mastitis had this property (43.6%). It is also statistically significant that 95.5% of the coagulase-negative staphylococci resistant to methicillin had slime-producing ability, including 40.9% of MRCNS responsible for the clinical form of mastitis with visible disease symptoms.

In conclusion, slime production, the element of the adhesion ability, is highly significant for the pathogenicity of staphylococci, therefore further research is necessary to improve our better understanding of it.

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