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CHARACTERISTICS OF *STAPHYLOCOCCUS XYLOSUS* ISOLATED FROM SUBCLINICAL MASTITIS IN COWS*

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Abstract

The aim of the present study was to determine virulence factors and antibiotic susceptibility of *Staphylococcus xylosus* isolated from subclinical mastitis in cows. The material consisted of 42 isolates of *S. xylosus* obtained from 276 samples of milk collected from cows with subclinical mastitis. The isolates were obtained from the udder secretions of 33 cows from farms in the Lublin region (Poland). *S. xylosus* was found in 15.2% of tested milk samples. The study did not reveal any macroscopic changes in the milk or symptoms in the cow's body. The number of somatic cells in milk samples ranged from 245,000 to 416,000/ml of milk (on average 268,000/ml of milk). The ability to produce slime was observed in 42.9% of *S. xylosus* isolates. None of the tested isolates demonstrated the ability to produce protease or cause haemolysis. Five isolates of *S. xylosus* (11.9%) were classified to the methicillin-resistant group. The *mec*A gene was not found in any of these isolates. The enzyme β-lactamase was detected in 28.6% of *S. xylosus* isolates. The highest efficacy against *S. xylosus* was demonstrated for cephalosporin antibiotics: cefacetrile and cefoperazone (80.1% and 76.2% of susceptible isolates of *S. xylosus*, respectively). A significant quantity of isolates was resistant to streptomycin, linkomycin, penicillin and neomycin (approximately 10% of susceptible isolates of *S. xylosus*).

Key words: cows, subclinical mastitis, *S. xylosus*

Coagulase-negative staphylococci (CNS) occur widely in the natural environment and colonize both the skin and mucous membranes of animals and people. While they have been regarded for decades as non-pathogenic, they have currently become an etiological factor for cow mastitis in many countries (Honkanen-Buzalski et al., 1994; Myllys et al., 1998; Chaffer et al., 1999; Makovec and Ruegg, 2003; Pitkälä et al., 2004; Rajala-Schultz et al., 2004; Taponen et al., 2007; Malinowski

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and Klossowska, 2010). CNS may cause clinical mastitis, but in the majority of cases they cause subclinical form of inflammation (Taponen et al., 2007; Persson Waller et al., 2011). Subclinical infections in individual dairy cattle farms affect 40 to 80% of cows over the year (Czerw et al., 2007). Cows with subclinical mastitis pose a risk for the entire herd, because the absence of visible external symptoms of inflammation means that this condition remains unnoticed by the owner and untreated for a long period of time. As a result, it leads to development of clinical form of mastitis or the appearance of lesions typical of the chronic process (Aarestrup and Jensen, 1997; Chaffer et al., 1999; Taponen et al., 2006). Management of mastitis caused by CNS is difficult primarily due to the heterogenicity of this group of microorganisms. More than 15 species of coagulase-negative staphylococci have been identified as aetiological factors of mastitis (Thorberg et al., 2009; Persson Waller et al., 2011). The reference literature (Malinowski et al., 2006) and our own research (Bochniarz et al., 2013 a) suggest that *S. xylosus* is often predominant among CNS isolated from cow mastitis.

The aim of the present study was to determine virulence factors and antibiotic susceptibility of *Staphylococcus xylosus* isolated from subclinical mastitis in cows.

Material and methods

The study material consisted of 42 isolates of *Staphylococcus xylosus* from 276 samples of milk collected from cows with subclinical mastitis. The isolates were obtained from the udder secretion of 33 cows from farms located in the Lublin region (Poland). In 24 cows *S. xylosus* was isolated only from one quarter of the udder, while in 9 cows from two udder quarters. The cows with *S. xylosus* belonged to 4 free-stall herds. Clinical examination of cows and macroscopic evaluation of the milk were performed prior to collection of milk samples for bacteriological testing. The cows from which the milk samples were collected were not given any medications in the current lactation.

Bacteriological testing of the milk was carried out in compliance with the generally accepted procedures. Milk samples were brought to room temperature, thoroughly mixed and inoculated on agar medium (BTL, Łódź, Poland) supplemented with sterile, defibrinated sheep blood (5% of the agar solution volume). After 24 hours of incubation at 37°C under aerobic conditions, the microorganisms were initially identified based on the colony morphology, examination of microscopic preparations coloured with the use of the Gram method and the coagulase test. Afterwards, the coagulase-negative staphylococci were identified using the API STAPH commercial test (Biomerieux, France). This test was performed in accordance with the manufacturer's recommendations.

Evaluation of ability to produce haemolysins. A solution of Columbia Agar Base (Oxoid, England) with a concentration of 39 g in 1 litre of distilled water was sterilized at 121°C for 15 minutes. After cooling the medium to 50°C it was supplemented with defibrinated sheep blood (5% of the medium solution volume) and

poured on Petri plates. A Columbia Agar Base medium with the addition of 5% defibrinated rabbit blood was prepared in the same manner. After consolidation of the medium the CNS strains were inoculated. The plates were incubated for 24 hours at 37°C. The reference *Staphylococcus aureus* from the ATCC 25923 collection was used as a positive control.

Evaluation of ability to produce protease. A solution of Nutrient Gelatin (Oxoid, England) with the concentration of 128 g in 1 litre of distilled water was sterilized at 121°C for 15 minutes and spread on Petri plates. After consolidation of the medium the CNS strains were inoculated in the form of a thin line. The plates were incubated for 24 hours at 37°C. Interpretation of the result: a zone 4 times wider than the CNS strain growth line was considered a positive result. The reference *Staphylococcus aureus* from the ATCC 25923 collection was used as a positive control.

Evaluation of slime-producing ability. The slime-producing ability of *S. xylosus* was evaluated by testing adhesion to polystyrene surfaces according to Christensen et al. (1983). Colonies of each tested 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 24 h. Biofilm formation was detected based on the presence of black or nearly black colonies on the agar.

Evaluation of phenotypic resistance of coagulase-negative staphylococci to methicillin. The test was performed with the use of Oxacillin Resistance Screening Agar Base (Oxoid, England) with the addition of ORSAB Selective Supplement. In order to perform the test a medium solution at a concentration of 51.75 g in 500 ml of distilled water was sterilized at 121°C for 15 minutes. One vial of ORSAB Selective Supplement was added to the medium cooled to a temperature of 50°C (oxacillin concentration in the solution was 0.5 μg/ml) and then the result was poured onto Petri plates. The CNS isolates were inoculated after consolidation of the medium. The plates were incubated for 24–48 hours at 37°C. The reference *Staphylococcus aureus* ATCC 25923 was used as a positive control.

Evaluation of genetically conditioned resistance of coagulase-negative staphylococci to methicillin. Isolation of DNA was carried out with the use of the enzymatic digestion method using CTAB. Then the isolated DNA underwent testing with the use of the PCR method in order to determine the presence of the *mec*A gene. In the amplification reaction, complementary starters were used in the conservative region in the area of the mecA gene, limiting the fragment to a length of 533 bp. Starters for the PCR were synthesized in Oligo-PAN in Warsaw (Poland). The following sequences of starters were used for the mecA gene (Louie et al., 2002):

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: 1 U of Taq Polymerase (Fermentas, Lithuania), 2.5 µl of 10x concentrated buffer for Taq Polymerase, 2.5 µl of dNTPs, 1 µl of mec1 starter, 1 µl of mec2 starter, 3 µl of $MgCl_2$, and 9.9 µl of distilled water. The amplification products were analysed electrophoretically in 1.5% agar gel (Sigma, USA) with the addition of the mass standard (100bp DNA, Fermentas, Lithuania).

The tubes were placed in Biometra T3 (Germany) thermal cycler and were heated for 1 min at 95°C, followed by a 1-min denaturation at 94°C, 1-min annealing at 55°C and elongation for 1 min at 72°C. Denaturation, annealing and elongation were repeated for a total of 40 cycles. Next the samples were loaded onto a 1.5% agarose gel and were electrophoresed for 30 min, stained with ethidium bromide and viewed with UV light. The presence of a 533-bp band was considered a positive result.

Evaluation of capacity of MRCNS to produce β-lactamase. The determination was carried out using the β-Lactamase Test (Oxoid, England). The rod tip impregnated with nitrocefin was immersed in the staphylococci colony on a solid agar medium and a small mass of bacterial cells was collected. The result was noted after 5 minutes. The change in the colour of the impregnated stick tip to pink-red was interpreted as a positive result.

Antibiotic susceptibility of microorganisms was evaluated using the disc-diffusion method on Mueller-Hinton agar. *S. xylosus* was examined for sensitivity to the following antibiotics: amoxicillin with clavulanic acid (30 µg), ampicillin (10 µg), cefacetrile (30 µg), cephalexin (30 µg), cefoperazone (75 µg), ceftiofur (30 µg), linkomycin (15 µg), neomycin (30 µg), penicillin (10 u), streptomycin (10 µg) and tetracycline (30 µg). Isolates were classified as susceptible or resistant based on species-specific epidemiological cut-off values issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http:\\www.eucast.org).

The types of mastitis were classified according to the standard rules (Gentilini et al., 2002; De Vliegher et al., 2003; Moon et al., 2007) after taking into account the results of the clinical examination of cows, bacteriological test of milk and determination of SCC (somatic cell count) in milk samples with the use of a Fossomatic apparatus (Denmark). SCC > 200 000/ml of milk and the presence of bacteria in bacteriological cultures without general symptoms were considered indicative of subclinical mastitis.

Results

S. xylosus was found in 15.2% of tested milk samples. The study did not reveal any macroscopic changes in the milk or symptoms in the cow's body. SCC in milk samples ranged from 245,000 to 416,000/ml of milk (on average 268,000/ml of milk).

The test results related to the characteristics responsible for the pathogenicity of *S. xylosus* isolated from subclinical mastitis are presented in Table 1. The ability to

produce slime was observed in 18 isolates of *S. xylosus* (42.9%). None of the isolates demonstrated the ability to produce protease or cause haemolysis.

The five isolates of *S. xylosus* (11.9%) were classified to the methicillin-resistant group (Table 2). The *mec*A gene was not observed in any of these isolates. The ability to produce β-lactamase was detected in 12 isolates of *S. xylosus* (28.6%).

The data presented in Table 3 suggest that the most significant effectiveness was demonstrated by antibiotics from the group of cephalosporins: cefacetrile and cefoperazone (80.1% and 76.2% of susceptible isolates of *S. xylosus*, respectively). A large percentage of isolates susceptible to amoxicillin with clavulanic acid and ceftiofur (64.3% and 61.9% of susceptible isolates, respectively) was also observed. The percentage of *S. xylosus* isolates susceptible to cephalexin, ampicillin and tetracycline was 38.1%, 33.3% and 26.2%, respectively. A large proportion of CNS isolates was resistant to streptomycin, lincomycin, penicillin and neomycin (approximately 10% of susceptible isolates).

Table 1. Some virulence factors of *S. xylosus*

Virulence factor	*Staphylococcus xylosus* (42)	
	No. (%) of *S. xylosus* isolates producing virulence factors	
Slime	18	42.9
Proteases	0	0.0
Haemolysins	0	0.0
β-lactamase	12	28.6

Table 2. Phenotypic resistance to methicillin and *mec*A gene

	Staphylococcus xylosus (42)	
	No. (%) of *S. xylosus* isolates	
Phenotypic resistance to methicillin	5	11.9
*mec*A gene	0	0.0

Table 3. Antibiotic susceptibility of *S. xylosus*

Antibiotic	*Staphylococcus xylosus* (42)	
	No. (%) of sensitive isolates	
Ampicillin	14	33.3
Amoxicillin with clavulanic acid	27	64.3
Cephalexin	16	38.1
Cefacetrile	34	80.1
Cefoperazone	32	76.2
Ceftiofur	26	61.9
Lincomycin	5	11.9
Neomycin	4	9.5
Penicillin	4	9.5
Streptomycin	5	11.9
Tetracycline	11	26.2

Discussion

In a previous study (Bochniarz et al., 2013 a), *S. xylosus* was the most frequently isolated CNS species from cow mastitis. It is worth emphasizing that it was predominant in the clinical forms of mastitis which were accompanied by lesions in udder and macroscopic changes in milk. This result correlated with the data obtained by Malinowski et al. (2006) in the western part of Poland. The authors claim that *S. xylosus* was the most frequently observed species among CNS isolated from milk samples between 2003 and 2006. *S. xylosus* has also been isolated by other authors (Jarp, 1991; Birgersson et al., 1992; Davidson et al., 1992; Thorberg et al., 2009).

The reference literature suggests that coagulase-negative staphylococci may cause both transient and persistent udder infections (Taponen et al., 2007). In the study carried out by Thorberg et al. (2009), *S. xylosus* was one of the CNS species most frequently isolated from mastitis, but the percentage of persistent inflammations caused by this pathogen was below 20%. Similar results were obtained by Taponen et al. (2007).

In the study conducted by Jarp (1991), *S. xylosus* constituted 7.4% of all CNS. It was isolated with approximately the same frequency in both clinical and subclinical cases of mastitis. It caused mastitis accompanied by topical lesions in the udder. However, no general symptoms were observed in the cow's body. In the study conducted by Birgersson et al. (1992), a considerably larger percentage of *S. xylosus* isolates was obtained from clinical and subclinical mastitis (50.0% and 38.9%, respectively) than from milk of healthy cows (11.1% of all *S. xylosus* isolates). In addition, in this study, a capsule was detected only in 5 of 203 strains of CNS, and 3 of these strains belonged to *S. xylosus*. This suggests that production of a true capsule is probably not a major virulence determinant in the pathogenicity of CNS causing mastitis. However, it shall be stressed that all the encapsulated *S. xylosus* were isolated from clinical cases of bovine mastitis.

The authors claim that an important role in pathogenesis of mastitis is played by the staphylococcus slime capsule, which enables adherence of bacteria to the tissue surface and production of bacterial biofilm (Aguilar et al., 2001; Lee and Lee, 2006). In the study of Tremblay et al. (2013), *S. xylosus* had the greatest ability to form biofilms. In the study conducted by Bedidi-Madani et al. (1998), *S. xylosus* constituted 35.7% of all CNS producing slime, isolated from the milk of goats. In our own study, production of pseudocapsules was observed in 42.9% of *S. xylosus* isolates; whereas, all the isolates of *S. xylosus* were likewise protease-negative and haemolysin-negative during earlier research (Bochniarz and Wawron, 2012), regardless of whether they were isolated from clinical or subclinical mastitis. This correlates with the results obtained by other authors (Birgersson et al., 1992; Devriese et al., 1994).

The common use of antibiotics, mainly of the β-lactam type, in the treatment of cow mastitis resulted in multi-resistance of the bacteria isolated from milk (Pitkälä et al., 2004; Moon et al., 2007; Persson Waller et al., 2011; Bochniarz et al., 2013 b). In our research, a large percentage of *S. xylosus* isolates was resistant to penicillin, neomycin, lincomycin, tetracycline and ampicillin. Data from the reference literature

confirm that the number of CNS isolated from different locations and different clinical cases in people and animals, as well as from animal products, has increased and at the same time the number of strains of these staphylococci resistant to antibiotics has also increased (Myllys et al., 1998; Pitkälä et al., 2004). In the study carried out by Gundogan and Ataol (2013), 66.7% of the *S. xylosus* isolates obtained from meat, milk and cheese were resistant to at least 2 different antibiotics.

In addition, the number of CNS isolates producing β-lactamases and isolates with the mecA gene resistant to all the groups of β-lactam antibiotics has increased recently (Devriese et al., 2002; Gentilini et al., 2002; Moon et al., 2007). In our own study, β-lactamase was detected in approximately 30% of *S. xylosus* isolates. The *mec*A gene was not observed in any of these isolates, unlike earlier research (Bochniarz et al., 2013 b) in which the presence of this gene was observed in 2 isolates of *S. xylosus* isolated from the clinical form of mastitis. Analogical results were obtained by Kot et al. (2013). From among 91 strains of *S. xylosus*, only in two cases did the researchers observe the presence of the *mec*A gene (1 strain isolated from clinical mastitis and 1 strain from subclinical mastitis).

The results of the study demonstrate that, in spite of the significant variability of features, *S. xylosus* is potentially pathogenic and may cause mastitis with different intensities of clinical symptoms. These infections are often difficult to treat due to the large number of isolates resistant to antibiotics.

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