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Phenotypic and genotypic antimicrobial susceptibility pattern of *Streptococcus* spp. isolated from cases of clinical mastitis in dairy cattle in Poland

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ABSTRACT

Mastitis of dairy cattle is one of the most frequently diagnosed diseases worldwide. The main etiological agents of mastitis are bacteria of the genus *Streptococcus* spp., in which several antibiotic resistance mechanisms have been identified. However, detailed studies addressing this problem have not been conducted in north-eastern Poland. Therefore, the aim of our study was to analyze, on phenotypic and genotypic levels, the antibiotic resistance pattern of *Streptococcus* spp. isolated from clinical cases of mastitis from dairy cattle in this region of Poland. The research was conducted using 135 strains of *Streptococcus* (*Streptococcus uberis*, n = 53; *Streptococcus dysgalactiae*, n = 41; *Streptococcus agalactiae*, n = 27; other streptococci, n = 14). The investigation of the antimicrobial susceptibility to 8 active substances applied in therapy in the analyzed region, as well as a selected bacteriocin (nisin), was performed using the minimum inhibitory concentration method. The presence of selected resistance genes (n = 14) was determined via PCR. We also investigated the correlation between the presence of resistance genes and the antimicrobial susceptibility of the examined strains in vitro. The highest observed resistance of *Streptococcus* spp. was toward gentamicin, kanamycin, and tetracycline, whereas the highest susceptibility occurred toward penicillin, enrofloxacin, and marbofloxacin. Additionally, the tested bacteriocin showed high efficacy. The presence of 13 analyzed resistance genes was observed in the examined strains [gene *mef*(A) was not detected]. In most strains, at least one resistance gene, mainly responsible for resistance to tetracyclines [*tet*(M), *tet*(K), *tet*(L)], was observed. However, a relationship between the presence of a given resistance gene and antimicrobial susceptibility on the phenotypic level was not always observed.

Key words: *Streptococcus*, mastitis, resistance, nisin

INTRODUCTION

Mastitis of dairy cattle is a widespread disease. In both its clinical and subclinical form, mastitis causes enormous economic losses in the dairy industry due to decreased milk production in infected cows and costs associated with the implementation of appropriate treatment or complete elimination from herd animals with chronic mastitis (Rato et al., 2013). Healthy quarters are most often infected due to inadequate hygiene, mainly via contagious pathogens or an infection caused by environmental bacteria originating from the natural surroundings of dairy cows (Riffon et al., 2001). The bacteria of the genus *Streptococcus* include contagious pathogens and environmental bacteria that can cause both clinical and subclinical forms of mastitis (Heringstad et al., 2000; Bradley, 2002; Neiwert et al., 2014).

Some ways of preventing mastitis include providing balanced nutrition, reducing stress, and increasing hygiene on the farm. However, it is not always possible to avoid this disease. In this case, the most common weapon against inflammation of the udder is antimicrobial therapy (Denamiel et al., 2005). Identification of the strain responsible for the infection and determination of its antibiotic resistance profile can greatly improve the results of therapy. Unfortunately, identification is not always possible due to the cost and time required for the test (treatment is often implemented immediately; Guérin-Faublée et al., 2002). Misuse of antibiotics in nontargeted therapies or for economic purposes (growth promoters) led to an increase of and created new resistance mechanisms in bacteria. In dairy cattle

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and other farm animals produced for food, excessive use of antibiotics is associated with another risk, the creation of multiresistant foodborne pathogens, which are a hazard to human health (Rajala-Schultz et al., 2004; Pol and Ruegg, 2007; Thomas et al., 2015). To prevent this development, it is essential to monitor the trends of resistance among bacteria, in both human and veterinary medicine.

Several programs have been completed in Europe with the aim to monitor antibiotic resistance profiles [e.g., SVARM (Swedish Veterinary Antimicrobial Resistance Monitoring) 2001–2013 in Sweden, GERMAP 2008–2012 in Germany, and MARAN (Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals) 2002–2008 in the Netherlands (Thomas et al., 2015)]. Nevertheless, many countries have not undertaken such studies, which means that knowledge of the antibiotic resistance of bacteria, especially local, is limited (Denamiel et al., 2005).

Cases of multidrug resistance have forced researchers and investors to search for alternatives to antibiotics, for example, therapy with bacteriophages or bacteriocins (Gill et al., 2006; Pieterse and Todorov, 2010). For example, nisin, an antimicrobial peptide produced by *Lactococcus lactis*, has been approved in approximately 50 countries as an agent for food preservation. Because it shows a broad spectrum of antibacterial action against gram-positive bacteria, it is added to cheeses to prevent the growth of spores produced by *Clostridium tyrobutyricum* and to dairy products to protect against *Listeria monocytogenes* (Pieterse and Todorov, 2010). Another application of this bacteriocin is the prophylaxis of mastitis. In some countries, products containing nisin are used to disinfect the udder before and after milking. Furthermore, this bacteriocin is increasingly often considered an alternative to antibiotics. Studies conducted by Immucell Corporation (Portland, ME), in which nisin was administered to 139 cows with subclinical mastitis, have produced satisfying results. However, further research has not been performed (Pieterse and Todorov, 2010).

To the best of the authors' knowledge, few studies in Poland have focused on the mechanisms of antibiotic resistance in different pathogens, and none has focused on *Streptococcus* spp. isolated from cases of clinical mastitis (**CM**) in dairy cattle from north-eastern Poland, including their genotypic resistance. Hence, the objective of our study was to determine the profile of antibiotic resistance using the microdilution method (MIC) and molecular methods (PCR) in strains of *Streptococcus* spp. originating from clinical cases of mastitis in dairy cattle from north-eastern Poland and to test their sensitivity to nisin.

MATERIALS AND METHODS

Sample Collection

In total, 135 streptococcal isolates from cases of clinical bovine mastitis were collected from farms in north-eastern Poland from 2013 to 2015. Because the testing of epidemiologically related isolates should be avoided, only 1 isolate per dairy farm was included. Prior to milk sampling, all animals were tested using the California mastitis test. Only milk that came from quarters presenting the symptoms of infection was collected (slight thickening of the mixture; trace reaction seems to disappear with continued rotation of the paddle). After sampling, milk was kept in a container that was maintained a constant temperature of 6 to 8°C and was delivered to the laboratory in no more than 2 h.

Bacteriological Identification

Milk samples were transferred with a calibrated loop (0.01 mL) on Columbia agar (Oxoid, Basingstoke, UK) and Edwards medium (Oxoid), both supplemented with 5% of defibrinated sheep blood. The plates were incubated at 37°C for 48 ± 2 h in aerobic conditions. The grown cultures were examined microscopically after Gram staining; also the phenotypic traits were analyzed (type of hemolysis, esculin hydrolysis, production of catalase, Christie–Atkins–Munch-Petersen reaction). For further analysis, only G+, catalase-negative cocci were selected. The final identification was performed using the commercial latex agglutination test Streptococcal Grouping Kit (Oxoid), API strep (bioMérieux, Marcy l'Etoile, France), and PCR reaction.

Antimicrobial Susceptibility Testing. Minimum inhibitory concentrations were determined using the broth microdilution method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (**CLSI**, 2013). Antibiotics tested were enrofloxacin, erythromycin, gentamicin, lincomycin, marbofloxacin, penicillin G, and tetracycline (Sigma Aldrich, Taufkirchen, Germany) in a concentration range of 0.06 to 256 μg/mL. Additionally, nisin (Sigma Aldrich) was tested as an alternative for antibiotics in a concentration range of 9.76 to 20,000 IU/mL. All of the antimicrobials were filtered using a Millipore filter with a 0.22-μm pore size (Merck Millipore, Billerica, MA). *Streptococcus pneumoniae* ATCC 49619 was used as a reference strain for MIC quality controls. Reference MIC values of the selected active substances are summarized in Table 1.

DNA Isolation. Bacterial DNA was extracted using an ExtractMe DNA bacteria kit (Blirt, Gdańsk,