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Study of the antibacterial sensitivity of *Staphylococcus aureus* isolated from dairy cattle

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ABSTRACT

The aim of this work was to isolate and to identify *Staphylococcus aureus* from dairy cattle in the northern region of Kazakhstan and to determine phenotypic and genotypic resistance profiles of isolated strains. During 2018-2019, 1245 samples of cow milk were examined, from which 68 strains of *Staphylococcus aureus* were isolated and confirmed by detection of species-specific thermonuclease gene. The most common genes encoding antimicrobial resistance were as follows: blaZ was detected in 29 isolates; *aac(6)-aph2* in 5 isolates, *aph(3)* in 6 isolates *erm*C in 14 isolates, *erm*B - in 19 isolates, *tet*K gene in a single isolate and *tet*M gene in 2 isolates from 68 isolates tested. Phenotypical resistance to cefoxitin demonstrated high rate (28 isolates out of 68) of methicillin-resistant *S. aureus* however, these isolates did not harboured neither *mec*A nor *mec*C genes. According to the data obtained it may be presumed that methicillin-resistant *S. aureus* prevalent in cattle population in the Northern Region of Kazakhstan carries unknown resistance determinant encoding resistance to beta-lactam antibiotics.

Key words: *Staphylococcus aureus, Antibiotic resistance, MRSA, Genes*

Introduction

The uncontrolled use of antimicrobials, the widespread use of antibiotics in veterinary, livestock and poultry production, and in the production and storage of animal products raise the risk of increasing resistance to a global level (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2017).

The aim of this work was to isolate and to identify *Staphylococcus aureus* from dairy cattle in the northern region of Kazakhstan and to determine phenotypic and genotypic resistance profiles of isolated strains.

Materials and Methods

Place and sample collection

This work was performed at the Research Institute of Applied Biotechnology of Akhmet Baitursynov Kostanay Regional University and at the Institute of Microbiology and Virology of the Lithuanian University of Health Sciences.

During 2018-2019, 1245 samples of cow's milk were examined. The secretion of the mammary glands of cows was collected in sterile vial and sealed under aseptic conditions. Bacterial cultures were obtained by inoculation of samples on Yolk-Salt Agar (YSA) and Blood Base Agar with 5% defi-

brinated sheep blood (BA) (EO Laboratories, UK).

Identification of *S. aureus*

Initial identification of staphylococci was based on their cultural and physiological properties. Haemolytic activity was detected using BA, production of lecithinase using YSA and the DNase activity using the DNAse Test Agar (Hispanlab, Spain). Detection of clumping factor, Protein A and polysaccharides found in methicillin resistant *Staphylococcus aureus* (MRSA) was detected using STAPHYTEST PLUS latex agglutination test (Oxoid, UK).

Further identification with the aim to identify *Staphylococcus* species was based on biochemical testing using Staph test 24 test kit (Erbolachema, Czech Republic). *S. aureus* was confirmed by PCR using species-specific primers for the detection of thermonuclease (*nuc*) gene for *S. aureus* as described previously (Ruzauskas, Couto, Siugzdiniene, Belas, Klimiene and Virgailis 2014).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed using the disk diffusion method. Disks containing lincomycin (15 μg), tylosin (15 μg), norfloxacin (10 μg), tetracycline (30 μg), kanamycin (30 μg), benzylpenicillin (10 IU), furadonin (300 μg), erythromycin (15 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), streptomycin (10 μg), amoxicillin (25 μg), furazolidone (300 μg), gemifloxacin (5 μg), enrofloxacin (5 μg), gentamicin (120 μg), cefoxitin (30 μg), ampicillin (10 μg), sulfamethoxol with trimethoprim (1.25/23.75 μg), ofloxacin (5 μg), doxycycline (30 μg), neomycin (30 μg), cefoperazone (75 μg) were used. The results were interpreted according to the current recommendations provided by EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2019) and (in case of the absence of clinical breakpoints) CLSI (CLSI M100-ED29, 2019) standard, as well as in accordance with the Guidelines 4.2.1890 (MUK 4.2.1890-04 MU, 2004).

Detection of the genes encoding resistance

Identification of the genes, encoding antimicrobial resistance in *S. aureus* isolates was performed using the PCR. PCR methods, including the study of target genes, used in this study have been described previously (Klimiene 2016).

Results

In total, 230 *Staphylococcus* isolates were isolated from 1245 samples tested. The species identified were as follows: *S. aureus* – 68, *S. saprophyticus* – 46, *S. albus* – 29, *S. xylosus* – 27, *S. warneri* – 23, *S. chromogenes* –22 and *S. epidermidis* – 15.

As a result, the largest number of resistant isolates was detected to benzylpenicillin – 62, cefoperazone – 39, tylosin - 37, ofloxacin - 36, ampicillin and erythromycin - 30, enrofloxacin - 27, furazolidone - 21, amoxicillin and tetracycline 18, norfloxacin-17, levomycetinum - 16, neomycin - 15, doxycycline, ciprofloxacin, furadonin and lincomycin - 14, trimethoprim / sulfamethoxazole - 13, streptomycin and kanamycin - 11, cefoperazone - 9, gemifloxacin - 8, gentamicin - 4.

In S. aureus isolates, the genes encoding antimicrobial resistance of certain classes of antimicrobial drugs were isolated: beta-lactams - blaZ gene - 29 isolates; aminoglycosides - aac (6) -aph2 gene - 5 isolates, aph (3) gene - 6; macrolides - ermC gene - 14, ermB gene - 19; tetracyclines: tetK gene - 1, tetM gene - 2 isolates.

Discussion

A total of 68 isolates of *S. aureus* from mastitis bovine milk were isolated and identified. The highest resistance of the isolates is observed to benzylpenicillin (88.2%) and ofloxacin (54.4%) as well as to tylosin - 50%, enrofloxacin - 45.6%, cefoxitin - 44.1%, ampicillin - 42.6% and furazolidone - 26.5%. Resistance to two or more antibiotics was observed in 95.6% of isolates.

According to the state register of veterinary drugs and feed additives of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan as of March 19, 2020, the most commonly used in veterinary practice are antimicrobials of the â-lactam class. The intensive use of antibiotics of this class leads to the spread of resistant microorganisms, and also reduces their effectiveness.

All studied isolates of *S.aureus* were susceptible to lincomycin and gemifloxacin. This is primarily due to the fact that hemifloxacin, a 4th-generation fluoroquinolone drug, is mainly used to treat infections of the upper and lower respiratory tract, as well as infections of the skin and soft tissues. As for

lincomycin, according to the instructions for use, this drug is contraindicated in lactating animals, and animals with mature rumen digestion (adult cattle and small cattle older than 2-6 months) are not allowed.

In the studied strains of *S.aureus*, the *bla*Z gene encoding beta-lactomases is most common. This gene was found in 29 DNA samples. 27 of 29 strains had phenotypic resistance to benzylpenicillin, however, 2 strains were sensitive to the indicated antimicrobial preparation. This discrepancy may be due to the fact that traditional methods for testing penicillin susceptibility S. aureus may not reliably determine penicillin resistance in all isolates (El Feghaly, Stamm, Fritz and Burnham, 2014).

In this study, 33 *S. aureus* isolates resistant to benzylpenicillin didn't demonstrate genotypic resistance to penicillin, which is similar to data from previous studies by several authors (Frey, Rodriguez, *et al.,* 2013; Yang, 2015). In these isolates, phenotypic resistance could be caused by point mutations rather than gene acquisition. In addition, a number of authors (Frey *et al.,* 2013; Croes *et al.,* 2009) suggest that, with the exception of general resistance mechanisms (Pantosti *et al.,* 2007), other pathways, such as biofilm formation, can play a major role in antimicrobial resistance mechanisms. The *bla*Z gene in staphylococci isolated from mastitis milk is also often found in countries such as Lithuania, Switzerland (Klimiene, 2016).

No *mec*A and *mec*C genes encoding a penicillin-binding protein among the studied staphylococcal strains were detected in this study although even 28 *S. aureus* isolates were resistant to cefoxitin, which is a good standard for detection of phenotypical detection of MRSA (Anand *et al.,* 2009). Since 2001, the 30 μg cefoxitin disc test was recommended to predict methicillin resistance and cefoxitin-resistant *S. aureus* was accounted as *mec*A-positive in human resource MRSA (Swenson and Tenover, 2005), which has been approved by CLSI guidelines (CLSI M100-ED29, 2019). In this regard, it can be assumed that *mec*A and *mec*C negative strains of staphylococci may be a risk factor for the transmission of MRSA to people in close contact with infected animals (Wang, 2015). However, further research is needed for determination of the resistance mechanism in the isolates.

Among 30 strains of *S. aureus* phenotypically resistant to antimicrobial drugs of the aminoglycoside group, *aac*(6)-*aph*2 genes - 5 samples and *aph*(3) - 6 samples were found, which are manifested in the presence of resistance to 3rd generation aminoglycosides, to kanamycin and neomycin.

46 strains phenotypically resistant to macrolides, in particular to erythromycin and tylosin, were identified. Of the 5 genes responsible for resistance to macrolides and lincosamides, 2 genes were detected in bacterial DNA samples. The *erm*C gene was found in 14 strains and the *erm*B gene in 19 strains. The *erm* genes (erythromycin ribosome methylation) are responsible for methylation, which leads to a decrease in target affinity for macrolide, lincosamide and streptogramin antibiotics.

Twelve strains of staphylococci showed phenotypic resistance to tetracyclines (tetracycline, doxycycline). Two main mechanisms of tetracycline resistance in *S. aureus* are known: the first is active outflow, which is mediated by a plasmid encoding the *tet*K and *tet*L genes, and the second is the ribosomal defense, which is mediated by the transposon or chromosome determinants *tet*M and *tet*O (McCallum *et al.,* 2010; Khoramrooz *et al.,* 2017). Two genes *tet*K (1 isolate) and *tet*M (2 isolates) were found in our study. Therefore, we can conclude that the microorganisms *S. aureus* circulating in the studied dairy farms are capable of developing both mechanisms of tetracycline resistance. However, a low percentage of detection of resistance genes may suggest a different resistance mechanism (Pantosti *et al.,* 2007). The *tet*L gene was not detected in the studied isolates. As in studies of other authors (Schmitz *et al.,* 2001), the *tet*L gene was not detected, which indicates that this gene does not play a special role in tetracycline resistance.

Among the 4 strains of the studied bacteria phenotypically resistant to trimethoprim / sulfamethoxazole, the *dfr*G and *dfr*K genes associated with sulfanilamides resistance were not detected in DNA samples. According to published data, the *dfr*G gene is mainly found in isolates isolated from humans (Nurjadi, 2014), most often from Asian, African and European travelers, while the *dfr*K gene is mainly found in the multilocus sequence of *S. aureus* associated with livestock (Kadlec and Schwarz, 2009). The fact of the presence of relatively low phenotypic resistance to trimethoprim/ sulfamethoxazole suggests: 1) this drug is not particularly popular with veterinarians for treating cattle, 2) the presence of an alternative route of transmission of resistance between bacteria (Pantosti *et al.,* 2007).

Studies have established a high degree of phenotypic resistance of *S. aureus* isolated from mastitis cow milk, while a total of 7 genes encoding resistance to 4 groups of antimicrobial agents were detected by molecular genetic methods (PCR). This may be due to other resistance mechanisms.

Further monitoring of antimicrobial resistance by phenotypic and genotypic methods will make it possible to predict the emergence of resistance to various groups of antimicrobial agents, as well as to assess the spread of resistant strains at local and regional levels. The data obtained should be taken into account when conducting antimicrobial therapy and prevention of mastitis in cows.

Conclusion

The studied isolates of *S. aureus* demonstrated high resistance to beta-lactams and macrolides. Resistance to these antimicrobials was due to the presence of resistance genes *bla*Z, *erm*C and *erm*B. The mechanism of methicillin-resistance remains unclear in the isolates obtained in this study. The uncontrolled use of antibacterial drugs of beta-lactam and macrolide groups for the treatment of mastitis in cows leads to the spread of not only phenotypic, but also genotypic resistance among microorganisms of the *S. aureus* species.

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