Antibiotic-Resistance *Staphylococcus Aureus* Strains Isolated from The Milk of Dairy Cows with Subclinical Mastitis

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Abstract*—Staphylococcus aureus* is the most common worldwide cause of subclinical mastitis. The main problems of subclinical mastitis are higher incidence, prolonged persistence, higher speed of spread in the herd and decreased production and quality of milk. *S. aureus* could be resistant to β-lactam antibiotics, while the long-term use of various antibiotics leads to an increased prevalence of multiresistant strains. *S. aureus* is one of the most common foodborne pathogen in the world, and milk represents a potential source of its spread. The aim of the work was to confirm the clinical strains of *S. aureus* isolated from bovine mastitis by the multiplex PCR method (mPCR) at the gene level, to determine the minimum inhibitory concentration (MIC) of antibiotics and to detect the genes of resistance to methicillin. From the total number (n=11) of morphologically and biochemically identified *S. aureus*, six isolates were confirmed by confirmatory mPCR. *S. aureus* isolates were mostly sensitive to all measured antibiotics. Two isolates were penicillins- and carboxypenicillins-resistant, and in one isolate was identified inducible MLSB type of resistance. No methicillin resistance genes (*mecA* and *mecC*) were confirmed by PCR. This result correlates with the results of MIC of antibiotics.

***Index Terms*—**subclinical mastitis, *Staphylococcus aureus*, multiplex PCR, MIC.

I. Introduction

Bovine mastitis is a serious multifactorial disease. It is defined as inflammation of the mammary gland and one of the main causes of its occurrence is a bacterial infection. It occurs in a clinical and asymptomatic subclinical form, resulting in a decrease in milk production and quality [1]. The major problem with the subclinical form of mastitis is its higher incidence, longer duration of occurrence during which it persists and the speed of spread in dairy farming [2]. The diagnostic method used to detect the subclinical form of mastitis is a test to determine the number of somatic cells in milk.

The most common worldwide cause of subclinical mastitis is *Staphylococcus aureus* [3]. During bacterial infection, the expression of virulence factors is upregulated, leading to an increase in resistance to phagocytosis, and upregulation of genes through which host tissue destruction occurs [4], [1]. *S. aureus* is able to evade the immune of the host [5], to resist the effect of β-lactam antibiotics, while the long-term use of various antibiotics leads to an increased prevalence of multiresistant strains resistant to different groups of antimicrobial substances.

Methicillin-resistant *S. aureus* (MRSA) was initially detected as a hospital-acquired infection (HA-MRSA), later in the human community (CA-MRSA) and more recently in livestock (LA-MRSA) [6]. In *S. aureus*, methicillin resistance is conferred by the expression of the *mecA* gene or homolog *mecC* gene, which encodes PBP2a, a protein with low affinity for β-lactam antibiotics, conferring resistance to methicillin, nafcillin, oxacillin, and cephalosporins. Both genes are structural components of the *mec* gene cassette, which is inserted into the larger staphylococcal *mec* chromosome cassette (SCC*mec*) [7]. Several structural variants of SCC*mec* have been described, which differ in their genetic content, size, and structural organization. Strains containing different elements of SCC*mec* differ in different sensitivity to antibiotics, which is significant from the point of view of clinical consequences.

The aim of this study was to confirm clinical isolates of *S. aureus* obtained from bovine mastitis by the multiplex PCR (mPCR) method. In the confirmed strains, the sensitivity to selected antimicrobial substances was subsequently determined in order to evaluate the frequency of occurrence of multiresistant strains and the detection of genes responsible for resistance to methicillin.

II. Materials and Methods

A. Sample collection, isolation and identification of *S. aureus*

Milk samples were taken from dairy cows from various Slovak farms with a subclinical form of mastitis with a positive NK-test. Isolation and identification of *S. aureus* was performed by classical microbiological methods, and then the isolates were subjected to confirmation at the molecular level using mPCR.

B. DNA extraction, simplex PCR and multiplex PCR (mPCR) amplification

Genomic DNA was extracted from overnight culture of isolates in modified Brain heart infusion broth (mBHI, HiMedia Laboratories, Mumbai, India) with 1% glucose and

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2% NaCl using the High Pure PCR Template Preparation Kit (Roche Molecular Systems, Inc.). The amount and purity of DNA was determined using an ND-8000 spectrophotometer (ThermoFisher SCIENTIFIC, USA).

For genus specification, primers amplifying the *16S rRNA* gene segment specific for the genus *Staphylococcus* and species-specific primers for *S. aureus* detecting the *eap* (extracellular adhesion protein) and *nuc* (thermostable nuclease) genes were used in mPCR. The presence of genes responsible for antibiotic resistance was monitored by PCR using primers amplifying sections of the *mecA* and *mecC* genes. The sequences of the primers used in this study are listed in Table 1.

The PCR and mPCR reaction mixture contained in the resulting volume 0.5 ng of isolated DNA, 1x DreamTaq Green PCR Master Mix (ThermoFisher SCIENTIFIC, USA), 0.125 pmol (*16S rRNA*), 0.5 pmol (*eap*, *mecA*, *mecC*), 0,25 pmol (*nuc*) of each primer ("forward, reverse") and water. The PCR and mPCR reaction took place on a Mastercycler® nexus X2 thermocycler (Eppendorf, Germany). The cycling conditions used for each of protocols followed are: conventional PCR (95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 15s. Followed by a final elongation at 72°C, for 10 min.) and mPCR (95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 20s, followed by 10 cycles 95°C for 30 s, 61°C for 30 s, and 72°C for 20s and final elongation at 72°C, for 10 min.

TABLE I: PRIMERS USED IN THIS STUDY

Gene	Primer sequence (5´-3´)	Amplicon size (bp)	Reference
16S rRNA	CTACAATGGACAATACAAAGGGC TCACCGTAGCATGCTGATCT	141	This study
eap	TACTAACGAAGCATCTGCC TTAAATCGATATCACTAATACCTC	230	[Hussain et al. 2007]
nuc	ACCTGCGACATTAATTAAAGCG TGTTTCAGGTGTATCAACCAATAATAG	103	This study
mecA	TGGAAGTTAGATTGGGATCATAGC CGATGCCTATCTCATATGCTGTT	154	This study
mecC	GACGATGGATCTGGTACAGCA CATTCATGAATGGATAAACATCGTA	94	This study

C. Antibiotic susceptibility testing

S. aureus isolates confirmed by PCR were analyzed for antibiotic susceptibility. Minimum inhibitory concentrations (MIC) were determined according to CLSI VET01-S2 [8] and EUCAST [9], using the Miditech system (Bratislava, Slovakia) with interpretive MIC reading. The following antibiotics were tested: ampicillin (AMP), ampicillin + sulbactam (SAM), oxacillin (OXA), cefoxitin (FOX), piperacillin + tazobactam (TZP), erythromycin (ERY), clindamycin (CLI), linezolid (LNZ), rifampicin (RIF), gentamicin (GEN), teicoplanin (TEC), vancomycin (VAN), trimethoprim (TMP), chloramphenicol (CHL), tigecycline (TGC), moxifloxacin (MFX), ciprofloxacin (CIP), tetracycline (TET), trimethoprim + sulfonamide (COT), nitrofurantoin (NIT).

III. RESULTS AND DISCUSSION

In milk samples obtained from dairy cows (n=215) with a subclinical form of mastitis, *S. aureus* isolates (n=11) were identified by classic microbiological methods, and to verify the correctness of the determination, they were subjected to confirmation using the mPCR method, in which the presence of specific staphylococcal genes was detected *16S rRNA* (141 bp), *nuc* (103 bp) and *eap* (230 bp). The *Staphylococcus* genus-specific *16S rRNA* gene was positively detected in all field isolates identified by standard microbiological procedures. The presence of species-specific genes for *S. aureus nuc* and *eap* was confirmed in 6 isolates (isolates 7 to 12) (Fig. 1).

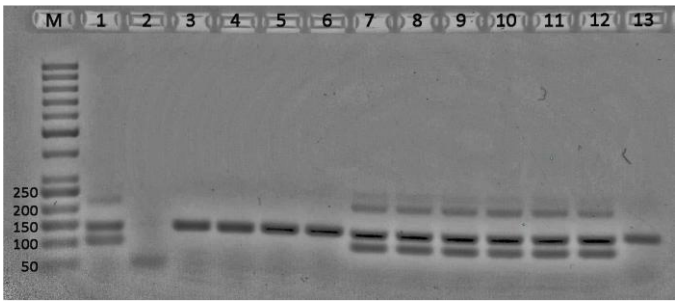

Fig. 1. Detection of *16S rRNA*, *eap*, *nuc* genes in *S. aureus* isolates using mPCR
M – standart GeneRuler 50 bp DNA ladder; 1 – control stain *S. aureus* CCM 4223 (Czech Collection of Microorganisms, Brno); 2 – negative control without DNA; 3 to 13 – isolates positive for *16S rRNA* (141 bp); 7 to 12 – isolates positive for *eap* (230 bp) and *nuc* (103 bp) gene.

Hussain et al. [10] based on a study conducted on a sample of 813 isolates including *S. aureus* and other species of the genus *Staphylococcus* demonstrated that the *eap* gene was detected in all 597 isolates of *S. aureus* and at the same time was not detected in any of 216 other staphylococcal isolates including 47 various species and subspecies of coagulase negative and other coagulase positive or coagulase variable staphylococci. They demonstrated that isolates other than *S. aureus* do not express Eap homologues. Therefore, they determined the sensitivity and specificity of the newly developed PCR aimed at detecting the *eap* gene as 100%. For the molecular detection of *S. aureus* through the amplification of part of the *nuc* gene, Gonzales-Dominguez and the team [11] also used it in their work. As in our study, they used specific primers designed to identify a segment within sequence homologies shared between the *nuc* gene of *S. aureus* species. The authors of the study state that despite the high specificity of the Staph-API kit (92.49%) to diagnose *Staphylococcus* species, the identification by means of phenotyping of staphylococci obtained from clinical samples does not reach the specified specificity, which may be one of the explanations for the results we obtained when we used the PCR method confirmed *S. aureus* in 6 out of 11 isolates, which represents 54.5%. Similar results were obtained by Qolbaini and the collective [12], who also detected the *nuc* gene in 49 (57%) of 86 *S. aureus* isolates identified by classical microbiological methods. To explain the differences between the mentioned methods of identification, it will be necessary to perform a sequence analysis of the isolates in which the *eap* and *nuc* genes were not detected.

In six confirmed isolates of *S. aureus*, sensitivity to antibiotics was subsequently determined by determining the MIC, while the resistance results are shown in Table 2.

TABLE II: MIC RESULTS OF S. AUREUS ISOLATES AND RESISTANCE ASSESSMENT

Sample	7		8		9		10		11		12	
ATB	MIC (mg/L)	Sensitive	MIC (mg/L)	Sensitive	MIC (mg/L)	Sensitive	MIC (mg/L)	Sensitive	MIC (mg/L)	Sensitive	MIC (mg/L)	Sensitive
AMP	0.25	S	0.25	S	0.25	S	2	R	0.25	S	>32	R
SAM	0.25	S	0.25	S	0.25	S	0.5	S	0.25	S	>32	R
OXA	0.25	S	0.25	S	0.25	S	0.5	S	0.25	S	0.25	S
FOX	2	S	2	S	2	S	2	S	2	S	2	S
TZP	0.5	S	0.5	S	0.5	S	2	S	0.5	S	0.5	S
ERY	0.25	S	0.25	S	0.25	S	>8	R	0.25	S	0.12	S
CU	0.12	S	0.12	S	0.12	S	>4	R	0.12	S	0.06	S
LNZ	4	S	4	S	4	S	2	S	4	S	2	S
RIF	0.03	S	0.03	S	0.03	S	0.03	S	0.03	S	0.03	S
GEN	0.5	S	0.5	S	1	S	1	S	0.5	S	0.5	S
TEC	1	S	2	S	2	S	2	S	1	S	1	S
VAN	1	S	2	S	1	S	1	S	1	S	1	S
TMP	2	S	4	S	4	S	2	S	1	S	1	S
CHL	8	S	8	S	8	S	16	R	8	S	8	S
TGC	0.12	S	0.06	S	0.06	S	0.12	S	0.06	S	0.06	S
MFX	0.12	S	0.06	S	0.06	S	0.06	S	0.03	S	0.03	S
CIP	0.5	I	0.25	I	0.5	I	0.5	I	0.25	I	0.25	I
TET	1	S	0.5	S	0.25	S	0.5	S	0.25	S	0.25	S
COT	0.25	S	0.25	S	0.25	S	0.25	S	0.25	S	0.25	S
NIT	16	S	32	S	32	S	16	S	16	S	16	S

S – sensitive; I – intermediate; R – resistant

S. aureus isolates were mostly sensitive to the monitored antibiotics. Two isolates were resistant to penicillins and carboxypenicillins, one of which was phenotypically identified as an inducible type of MLSB resistance (Fig. 2). The inducible resistance type MLSB (macrolide-lincosamide-streptogramin B) in *S. aureus* poses a danger due to failure of treatment with antibiotics such as clindamycin and erythromycin, which are used in the treatment of skin and soft tissue infections caused by staphylococci [13]. None of the confirmed *S. aureus* isolates isolated from subclinical mastitis showed resistance to cefoxitin and oxacillin, which is important for animal health because β-lactam antibiotics are still among the most common antimicrobial therapeutics used to treat mastitis and alternative treatment with non-β-lactam antibiotics is limited [14].

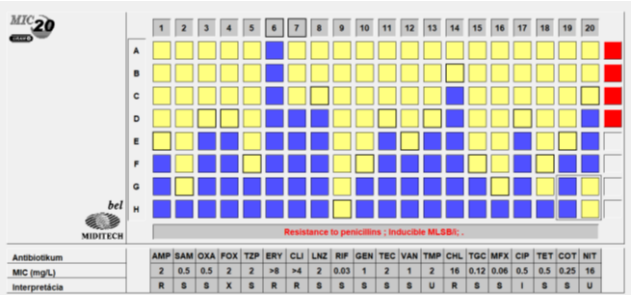

Fig. 2. MIC result of *S. aureus* isolate and analysis of resistance mechanisms using the MIDITECH system.

Most of the published studies aimed at monitoring the susceptibility of *S. aureus* strains to antibiotics report results obtained only through phenotypic resistance testing without demonstrating the presence of the *mecA* and *mecC* genes responsible for the production of an altered penicillin-binding protein 2a (PBP2a), which has a lower affinity for β-lactam antimicrobials substances as normal PBP. Phenotypic testing has been shown to lead to false negatives as well as false positives [14]. To confirm the obtained results of the sensitivity of *S. aureus* to antibiotics by determining the MIC, the presence of *mecA* and *mecC* genes was detected in the confirmed isolates using PCR, while their presence was not detected in any of them, which correlates with the MIC results.

IV. CONCLUSION

Staphylococcus aureus is generally ranked among the most common pathogens responsible for mastitis. The overall prevalence of *S. aureus* is variable and varies between farms and regions. From the results of isolation and identification from milk obtained from dairy cows with a subclinical form of mastitis, it can be concluded that *S. aureus* is not among the most common pathogens of the mammary gland. Inaccuracies in the identification of *S. aureus* in clinical samples based on classic microbiological procedures point to the need to include in the diagnostic procedure also methods for the detection of species-specific *nuc* and *eap* genes. The overall prevalence rate of MRSA in dairy farms is low, but there is still a risk of its increasing. By monitoring sensitivity to antibiotics, no MRSA strain was recorded, which is a positive fact, since the antibiotics of first choice in the treatment of staphylococcal mastitis are beta-lactams. Continuous monitoring of antibiotic resistance is essential because resistant strains of *S. aureus* (MLSB and penicillin-resistant strains) are emerging, which poses a problem in the treatment of bovine mastitis. The obtained results of the detection of MRSA strains were also confirmed by the detection of *mecA*, *mecC* genes, which were not present in any *S. aureus* isolate.

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