CHANGES IN ANTIBIOTIC SUSCEPTIBILITY PATTERN OF ATYPICAL *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM COWS OF THE SAME HERD IN 2008-2010

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Abstract

The isolates of *Staphylococcus aureus* strains were examined phenotypically by cultural features, tube coagulase test and clumping factor (CF), and genotypically by conventional PCR. The strains had positive reaction in CF test, but were negative in tube coagulase test. The analysed strains from the same cows in each year expressed also *nuc* and *coa* genes. About 25% of the strains were examined by the disc diffusion method for their sensitivity to antibiotics. During three years, the strains were highly susceptible *in vitro* to amoxicillin with clavulanic acid, oxacillin, bacitracin, and cefoperazone (more than 90%), and highly resistant to tetracycline, neomycin, and streptomycin. Forty randomly chosen strains, and eight strains from the same cows in each year were analysed for minimal inhibitory concentration of penicillin G using microdilution method. An increasing resistance to the penicillin was noted. Moreover, eight strains, the same in each year, were also examined for β -lactamase production and methicillin resistance. No β -lactamase producers and no methicillin resistant strains were found using phenotypic and genotypic methods. In conclusion, it can be stated that antimicrobial susceptibility can change from one year to another.

Key words: cows, mastitis, atypical *Staphylococcus aureus*, antibiotic susceptibility.

Staphylococcus aureus is considered to be a major pathogen that causes intramammary infections in dairy cows leading to severe economic losses in dairy industry (9). One of the typical features that distinguishes the more pathogenic *Staphylococcus aureus* strains from the less pathogenic staphylococci is the ability to produce coagulase (2, 6). Coagulase is an enzyme, which coagulates plasma and occurs in two forms: free and bound. Free coagulase (tube-coagulase) is produced extracellularly and acts on fibrinogen converting it to fibrin, which results in plasma clotting. Bound coagulase is an enzyme, which binds with cell's wall, acting directly on fibrinogen, and is detected in slide coagulase test (20, 21). The tube-coagulase test (TCT) is still the 'gold standard' in clinical microbiology laboratory (1). *S. aureus* is generally coagulase-positive although atypical coagulase-negative strains also occur (5).

It is important to determinate antimicrobial susceptibility of *S. aureus* strains not only for the purpose of therapy, but also to monitor the spread of resistant strains throughout the populations (4). Antimicrobial therapy plays a role in mastitis control by reducing the levels of herd infections and by preventing new infections (33). However, antibiotic treatment of mastitis leads to significant increase in milk quantity and

quality, lower somatic cell count, and is likely associated with reduction in prevalence of clinical mastitis among herds, which is economically beneficial (25).

The efficacy of bovine mastitis treatment depends on the cause, clinical manifestation, antibiotic susceptibility of aetiological agents, and the efficiency of immunological system (18). However, cure rates of *S. aureus* infections are poor after antibiotic treatment mainly because of resistance of the bacteria (11, 18) and capsule and slime formation (30). Therapy of *S. aureus* infections is usually made empirically and is often based on previous susceptibility information for the herd. Thus, information on susceptibility trends for bacterial species within a given herd is extremely important (26).

Atypical staphylococci, causing problems in the production of milk of acceptable quality, were the main reason of intramammary infections in the farm in 2008-2010. In the treatment of mastitis, the most often used drugs were those containing β -lactamic antibiotics, especially penicillin G and its half-synthetic derivatives. Because of this fact, the aim of the presented study was to characterise changes in susceptibility pattern of atypical *S. aureus* strains to antibiotics, especially penicillin G, in the examined herd.

Material and Methods

Bacterial isolates. The examined staphylococci were isolated from milk samples of cows belonging to the same herd. The samples were collected from 468 cows in 2008, 526 cows in 2009, and 314 cows in 2010, but because of a high culling rate in the herd during this time, only eight cows were the same in each year. Samples were collected, cooled, and transported to the laboratory, as described earlier (19). Bacteriological examinations were performed according to the commonly accepted rules (17).

Genetic examination. Strains from eight cows, the same in each year, were examined for *nuc*, *coa*, *mecA*, and *blaZ* genes by conventional PCR.

Antibiotic susceptibility. The antimicrobial sensitivity of 25% of the isolates from each year, was tested by the disc diffusion method in the Mueller-Hinton agar (Oxoid). The following discs with antibacterial agents (Oxoid) were used: amoxicillin with clavulanic acid (30 µg), penicillin G (10 i.u.); cephalexin (30 µg), cloxacillin (5 µg), bacitracin (10 i.u.), cefoperazone (30 µg), tetracycline (30 µg), neomycin (30 µg), streptomycin (10 µg), and oxacillin (1 µg). In case of eight strains, the same during three years, antimicrobial sensitivity to penicillin G (10 i.u.) and cefoxitin (30 i.u.; Oxoid) were tested by the disc diffusion method in the Mueller-Hinton agar (Oxoid). The interpretation of the test results: sensitive (S), resistant (R), was based on Clinical and Laboratory Standards Institute (CLSI) criteria (3).

Minimal inhibitory concentration (MIC). On 40 randomly chosen strains, the MICs of penicillin G was determined by the broth microdilution method as described in CLSI criteria (3). On eight strains from the same cows in each year, the MICs of penicillin G, oxacillin, and cefoxitin were determined by the broth microdilution method. Reference strains, *S. aureus* ATCC 29213 and *Escherichia coli* ATCC 25922, were used for quality control. The susceptibility of each strain to antimicrobial agents was then defined by comparing the results to those of the breakpoint values. The first dilution with no visible growth was considered as MIC for each isolate.

β-lactamase production. Eight strains from each year were analysed for β-lactamase production using nitrocefin sticks according to manufacturer's recommendation (Oxoid).

Results

Atypical *S. aureus* strains were isolated from 264 cows in 2008, from 244 cows in 2009, and from 184 cows in 2010 that comprised 56.4%, 46.4%, and 58.6% of all cows in the herd, respectively. Apart from these bacteria, infections were also caused by free coagulase-positive *S. aureus* strains, free coagulase-negative staphylococci (non-haemolytic, free coagulase-negative, and clumping factor-negative), *Streptococcus* sp., coliform bacteria, and *Corynebacterium* sp. (Table 1).

Table 1
Proportional participation of microorganisms in the same herd in 2008-2010

Microorganism	2008 (%)	2009 (%)	2010 (%)
<i>S. aureus</i> (free coagulase negative)	26.6	21.4	58.6
<i>S. aureus</i> (free coagulase positive)	0.1	0.1	0.0
CNS	18.8	8.1	23.9
<i>Streptococcus</i> sp.	0.8	1.5	1.0
Coliform bacteria	0.3	0.5	0.6
Coryneform bacteria	0.1	0.2	0.0
Bacteriologically negative samples	51.0	67.5	15.6
Contaminated samples	2.3	0.7	0.3

Table 2
Minimal inhibitory concentration (MIC) for penicillin G of 40 strains of atypical *S. aureus* isolated from cows of the same herd in 2008-2010

	MIC (µg/mL) breakpoints	Range (µg/mL)	Susceptible		Resistant	
			n	%	n	%
2008		0.03-0.25	39	97.5%	1	2.5%
2009	≤0.12 ≥0.25	0.03-0.5	34	85%	6	15%
2010		0.12-0.5	22	55%	18	45%

Table 3

Minimal inhibitory concentration (MIC) for penicillin G, oxacillin, and cefoxitin of atypical *S. aureus* strains isolated from the same cows of the same herd in 2008-2010

Antibiotic	MIC breakpoints (µg/mL)	Range (µg/mL)			Susceptible (%)			Resistant (%)		
		2008	2009	2010	2008	2009	2010	2008	2009	2010
Penicillin	≤0.12 ≥0.25	0.015- 0.12	0.03- 0.25	0.03- 1.0	100	87.5	62.5	0	12.5	37.5
Oxacillin	≤2 ≥4	0.25- 1.0	0.5- 1.0	0.5- 1.0	100	100	100	0	0	0
Cefoxitin	≤4 ≥8	1.0- 2.0	1.0- 4.0	2.0- 4.0	100	100	100	0	0	0

Fig. 1. Sensitivity to antibiotics of atypical *S. aureus* isolated from cows with mastitis in 2008;

S - sensitive, I - intermediate sensitive, R - resistant; Amc - amoxicillin with clavulanic acid, Cl - cephalexin, B - bacitracin, Cfp - cefoperazone, Ob - cloxacillin, Ox - oxacillin, P - penicillin, S - streptomycin, N - neomycin, Te - tetracycline.

Fig. 2. Sensitivity to antibiotics of atypical *S. aureus* isolated from cows with mastitis in 2009; symbols as in Fig. 1.

Fig. 3. Sensitivity to antibiotics of atypical *S. aureus* isolated from cows with mastitis in 2010; symbols as in Fig. 1.

The tested *S. aureus* strains gave positive results in the slide coagulase test, but the tube coagulase test was negative. Eight strains from the same cows in 2008-2010 expressed also the *nuc* and *coa* genes but none of them expressed *mecA* and *blaZ* genes.

All analysed strains in 2008 (Fig. 1) were especially *in vitro* sensitive to amoxicillin with clavulanic acid (100%), cephalexin (98.1%), bacitracin (98.1%), cefoperazone (98.1%), cloxacillin (98.1%), and oxacillin (98.1%), and mostly resistant to tetracycline (57.4%), neomycin (53.7%), and streptomycin (48.1%); in 2009 (Fig. 2) among the most active antibiotics against the strains were: oxacillin (97.8%), cefoperazone (95.6%), and amoxicillin with clavulanic acid (94.1%), and less active included: neomycin (25.2%), streptomycin (21.5%), and tetracycline (19.3%); in 2010 (Fig. 3) the highest effectiveness had amoxicillin with clavulanic acid, bacitracin, and cloxacillin (97.9%), and clearly lower effectiveness had penicillin G (18.7%), tetracycline (10.4%), and neomycin (6.3%).

However, eight strains isolated from the same cows in each year were sensitive to penicillin G in 100% in 2008, in 75% in 2009, and in 50% in 2010, and all strains were sensitive to cefoxitin.

Control strain value was within recommended ranges for microdilution method. During three years, higher number of resistant isolates was observed for penicillin G. In 2008, only 2.5% of 40 randomly chosen strains were resistant to this antibiotic, while in 2009, the percentage of resistant strains increased to 15% and reach even 45% in 2010 (Table 2). In case of eight strains from the same cows, in 2008 all analysed strains were sensitive to penicillin G, while in 2009, the percentage of resistant strains increased to 12.5% and reached 37.5% in 2010 (Table 3). In spite of increasing range of antibiotic dilution in time, resistance to oxacillin and cefoxitin was not observed.

By using identification sticks, no β -lactamase producers were found among eight strains of atypical *S. aureus* isolated in 2008-2010.

Discussion

All examined strains were coagulase-negative but clumping factor positive. The strains from the same cows in 2008-2010 expressed also *nuc* and *coa* genes,

which are characteristic for *S. aureus* (2, 6). Because of these features, the analysed staphylococci can be called atypical *S. aureus*.

Atypical staphylococci, causing problems with the production of an acceptable quality of milk, were the main reason of intramammary infections at the farm. They were found in 27% in 2008, 21% in 2009, and 58.6% in 2010 of all examined quarter milk samples. Similar results were noted by Gooraninejad *et al.* (10), Jánosi and Baltay (12), and Kumar *et al.* (13) who found *S. aureus* in 26.4%, 32.5%, and 54.9% milk samples, respectively.

Antimicrobial therapy is a primary tool for controlling staphylococcal mastitis. Antimicrobial susceptibility tests can guide the veterinarian in selecting the most appropriate antimicrobial agent for treatment of the disease caused by *S. aureus* (23). In fact, *S. aureus* pathogens have many characteristics that make them difficult targets for antimicrobial therapy (29). The previous studies regarding antibiotic resistance in *S. aureus* revealed that antibiotic resistance genes are often located on plasmids and transposons, which can easily pass from one staphylococcal species to another (15, 35).

Despite a variety of available antimicrobial agents, success in the treatment of *S. aureus* mastitis is still very low. In cases of mastitis, the incorrect or incomplete treatment of animals also contributes significantly to the development of bacterial resistance against antibiotics (31). The reason of decreasing sensitivity to antibiotics can be also the frequency of treatment in the analysed region, because frequent contact of bacteria with a specific antibiotic can determine an increase in resistance and decrease the effect of treatment (16). The indiscriminate use of antibiotics for the treatment of cows has led to development of multiple antibiotic resistant strains thereby rendering the antibiotic treatment ineffective (35). Some authors (7, 16) reported that *in vitro* antibiotic resistance of bacteria isolated in the same farm can change from one year to another. The statement was confirmed in our research where resistance to penicillin G, which was the most often used in antibiotic treatment in that herd, was higher in disc diffusion method as well as in microdilution method every year. In disc diffusion method, the resistance to penicillin G of randomly chosen strains increased from 11.1% in 2008 to 18.7% in 2010 and even more in case of eight strains from the same cows repeated in each year, where resistance to penicillin G increased from 0% to 50% during the time of experiment. Our findings are in agreement with the result obtained by Gooraninejad *et al.* (10), who reported that 57.4% of *S. aureus* isolates were resistant to penicillin G.

Moreover, in microdilution method, the number of resistant strains and range of resistance to penicillin G increased each year. In case of randomly chosen strains, proportional distribution of resistant strains increased from 2.5% in 2008 to 45% in 2010, and in case of eight strains from the same cows in each year, distribution of resistant strains increased from 0% in 2008 to 37.5% in 2010. In this study, the MIC varied from 0.015 to 1.0

µg/mL. Ünal *et al.* (32) and Russi *et al.* (27) noted 80.4% and 48.4% of *S. aureus* strains resistant to penicillin G in microdilution method, respectively. The range of minimal concentration of antibiotic, which stops growth of bacteria, differs among countries, for example: from 0.06 to 0.5 µg/mL in Norway (24), from 0.06 to 1.0 µg/mL in Denmark (28), and to 8.0, 16.0, and 64.0 µg/mL in the United States, Germany, or New Zealand, respectively (24, 28).

The β-lactams remain one of the most widely used classes of antimicrobial agents for treatment of bovine mastitis. Bacterial resistance mechanisms to this class of antibiotics include production of β-lactamases encoded by *blaZ* gene and low-affinity penicillin-binding protein 2a (PBP2a) determined by the presence of the chromosomal gene *mecA* (22). The detection of β-lactamase production in staphylococci is a useful and rapid method to detect penicillin resistance. Test for β-lactamase production should always be done to obtain the true picture of resistance to penicillin in staphylococci (8). In this research, among the same strains tested in 2008-2010, no β-lactamase producers were found using phenotypic (identification sticks) and genotypic (expression of *blaZ* gene) methods.

Strains expressing the *mecA* gene are referred to as methicillin resistant. Watts and Salmon (34) have emphasised the need to identify methicillin-resistant *Staphylococcus aureus* (MRSA) because these strains are resistant to all compounds currently approved for the treatment of bovine mastitis. Diagnosis of methicillin resistance in laboratory is based on the testing of oxacillin, where according to recommendations of the CLSI, oxacillin-resistant *Staphylococcus* isolates should be reported as resistant to other β-lactam antibiotics (3). In addition, the CLSI also recommends a cefoxitin disc diffusion test for the prediction of *mecA*-mediated resistance in staphylococci (3). In veterinary medicine, methicillin-resistant *S. aureus* strains are reported occasionally (14). Atypical *S. aureus* did not express the *mecA* gene, and all strains were sensitive to oxacillin in microdilution, as well to cefoxitin in both disc diffusion and microdilution methods.

The presented study demonstrated the existence of progressive level of resistance of *S. aureus* strains to commonly used antimicrobial agent in the studied farm. Therefore, to avoid the selection of resistant strains, it is very important to make a systematic application of an *in vitro* antibiotic susceptibility tests prior to the use of antibiotics in both treatment and prevention of intramammary infections.

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