OCCURRENCE OF RESISTANCE TO ANTIBIOTICS THERAPY IN COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM SHEEP´S MILK IN HOLDING IN SLOVAKIA

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ABSTRACT

The occurrence of bacteria *Staphylococcus* spp. was examined in a total of 3466 individuals and in 12 pool milk samples from 2017 to 2019. The experiment was carried out in two herds of the breed of sheep, Improved Valaska, in the Slovakia region. Eleven species of the genus *Staphylococcus* spp. (n = 431) were isolated and taxonomically identified. From the coagulase-positive staphylococci (CPS), *S. aureus* was isolated during the reporting period, however, most often in the third year (50). The incidence of *S. intermedius* and *S. hyicus* were irregular. The incidence of *S. schleiferi* was highest at the end of the follow-up duration. From the coagulase-negative staphylococci (CNS) (n = 158), were isolated *S. epidermidis* present in 20.4% (88) and *S. chromogenes* 11.4 % (49), *S. caprae, S. xylosus,* and other species rarely occurred. *S. aureus* (n = 133) showed maximum resistance to erythromycin 12.0%, novobiocin 10.5%, and neomycin 9.0%. The incidence of intermedial susceptibility was observed predominantly to a penicillin (16 strains), novobiocin (11 strains), erythromycin (14 strains), oxacillin, and cloxacillin (12 strains), neomycin (11 strains), and lincomycin (9 strains). Observantly, *S. schleiferi* (n = 101) showed the highest resistance to novobiocin (5.9%) and erythromycin (5.0%), however, a high incidence of intermediate susceptibility to erythromycin (9), amoxicillin, novobiocin (8), ampicillin, lincomycin (7), penicillin, methicilin and cefoperazone (5 strains) can be identified as adverse. The incidence of resistant and intermediate sensitive test strains *S. aureus* and *S. schleiferi*, especially for erythromycin, novobiocin, and neomycin, which are often used to treat udder inflammation in sheep, is relatively adverse.

Keywords: Sheep milk; coagulase-positive staphylococci; coagulase-negative staphylococci; antibiotics resistance; mastitis

INTRODUCTION

Mastitis is one of the most common diseases that affects dairy sheep. Mastitis leads to major economic loss, mainly due to discarded milk, reduced milk production, and quality, alteration of cheese-making properties, early culling, and increased health care costs **(Legarra et al., 2007)**.

Over 100 different microorganisms cause mastitis, particularly, coliform bacteria, staphylococci, and streptococci **(Smith and Hogan, 2001)**. In dairy sheep, the most important agents involved in clinical mastitis are bacterial infections, while the most frequently isolated pathogens are coagulase-negative staphylococci (CNS) present on and around the udder skin **(Leitner, Silanikove and Merin, 2008)**, with different pathogenicity causing clinical and subclinical mastitis **(Riggio and Portolano, 2015)**. Mastitis is of both extreme zoonotic and economic importance. It is the cause of multiple hazardous effects on human health and animal production.

Recently, it was recognized that the antimicrobial susceptibility of coagulase-negative staphylococci, which represents the majority of organisms isolated from ovine milk, is important for the early recognition of newly emerging resistant milk-borne bacterial agents **(Onni et al., 2011)**. Furthermore, with reference to specific conditions of ruminants, many authors in their works referred to these as the most frequently isolated: *S. epidermidis, S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. warneri,* and *S. sciuri* **(Fthenakis, 1994; Ergün et al., 2009)**.

Although, CNS does not have a comparable range for the virulence factors, such as *S. aureus*, one of the important factors of virulence is the ability to create resistance to an antibiotic, while some were described as multiresistant **(Moniri, Dastegholi, and Akramian, 2007)**. Multidrug resistance is defined as the resistance of three or more classes of antimicrobial agents **(Schwarz et al., 2010)**. The use of antibiotics is the most common treatment for these cases **(Gomes and Henriques, 2015)**, and β-lactams are the most frequently used classes for the treatment of mastitis. Additionally, mastitis therapy is usually started before the results of the antimicrobial susceptibility test of

pathogens **(Hendriksen et al., 2008)**, thus, representing one of the most important reasons for treatment failure. Moreover, this antibacterial strategy has many disadvantages including a low cure rate, increasing the presence of antibiotics residues, and the occurrence of antimicrobial resistance **(Minst et al., 2012)**. Mastitis resistance is a complex and multifactorial trait, and its expression depends on both genetic and environmental factors, including infection pressure **(Tolone et al., 2016)**. In the broadest sense, resistance can be defined as the ability to avoid any infection and/or the quick recovery from an infection **(Rupp and Boichard 2003; Rupp and Foucras, 2010)**. This involves different factors such as preventing the entry of the pathogen into the mammary gland, inducing an immune response capable of limiting pathogen development in the udder and recovery from the infection, as well as controlling the pathogenic effects of the infection, such as tissue damage **(Rupp et al., 2009)**. Resistance to antibiotics may be acquired by spontaneously occurring genetic mutations and, more commonly, by the horizontal transfer of mobile DNA elements from a donor cell to another bacterial species **(Chambers, 2001)**.

Over the years, extensive use of antimicrobials has led to increasingly resistant bacteria at an alarming rate and has become a serious concern worldwide. To ensure suitable antibiotic therapy, bacterial isolation, and evaluation of antibiotic susceptibility are essential. Also, milk produced from animals with subclinical mastitis poses serious veterinary and epidemiology risk since its rich nutrient composition and neutral pH make it a good vehicle for the survival and growth of bacteria. Resistant bacteria may contaminate food products, which could be transmitted to humans through the food chain. This underlines the importance of pathogen surveillance. Consequently, monitoring pathogens and their antimicrobial resistance patterns have become today's necessity **(Ceniti et al., 2017)**.

Scientific hypothesis

This work aimed to determine the occurrence and the most common types of staphylococci. Individual pools and sheep's milk samples were investigated, and a comparison of the incidences of antibiotic resistance of the most numerous tested species *S. aureus* and *S. schleiferi* was conducted.

MATERIALS AND METHODS
Characteristics of experimental breeds of sheep

One of the breeds of sheep with 350 Improved Valaska sheep and another farm with 280 sheep with a program of gradual crossing with the "Lacaune" breed were used for the experiment. Tracking the etiology in mastitis in the findings of the pool samples was carried out during the three seasons of the machine milking in the holdings, compliant with the technological standards of Slovakia. During the three seasons between April and September, a total of 12 complex examinations were repeatedly performed. A significant measure in the course of the experiment was to treat all cases of clinical mastitis solely based on proven susceptibility to a range of selected antibiotics.

Testing and sampling of the herds' sheep's milk

Clinical examination of the udder supplemented by a Californian Mastitis Test individual sheep's milk was conducted at the beginning and the end of each season, alongside a bacteriological examination of samples according to the principles posited by these authors **(Fthenakis, 1994; Vasil, 2004; Mørk et al., 2007)**. Emphasis was placed on aseptic sampling and transport of the mixed pools samples and the individual sheep's milk samples intended for bacteriological examination.

Bacteriological examination

The inoculum of each sample of milk was inoculated on the plates with 5% blood agar, incubated at 37 °C, and after 24 hours of reading. When the growth was more than 7 colonies from one type of colony, it was inoculated and cultured on selective nutrient soils. Identification of *Staphylococcus* spp. bacterial cultures were carried out by assessing the growth of suspected bacteria on nutrient agars (5% of blood Agar, N° 110, Baird-Parker agar, Brilliance UTI Clarity Agar (Oxoid Ltd., Basingstoke, Hants, UK). The pigment formation, hemolysis, catalase positivity, Gram positivity, creation of free or coupled coagulase, and other characters, were determined. The identification of each species was made by STAPHYtest 24 and evaluated by TNW ProAuto 7.0 (Erba-Lachema, Brno, Czech Republic) with a probability of correct designations of the kind above 90%. The functionality of the set was controlled using a strain of *Staphylococcus aureus* CCM 7113 (CCM, Masaryk University, Brno, Czech Republic).

Testing of the sensitivity of antibiotics on the most numerous species of Staphylococcus

Bacteria isolated from various forms of mastitis (n = 432) and pools milk samples (n = 12), were tested *in vitro* by disc method **(EUCAST, 2014)**, by evaluation of the zones of inhibition to grow on Mueller-Hinton agar after 24 h incubation at 37 °C. To test the sensitivity of staphylococci to fourteen antibiotics; ampicillin 10 μg (28 – 29 mm), amoxicillin 25 μg (28 – 29 mm), cefoperazone 30 μg (14 – 18 mm), cefoxitin 30 μg (23 – 29 mm), cloxacilin 5 μg (10 – 13 mm), erythromycin 10 μg (13 – 23 mm), lincomycin 15 μg (9 – 15 mm), neomycin 10 μg (12 – 17 mm), methicillin 10 μg (9 – 14 mm), novobiocin 5 μg (10 – 13 mm), oxacillin 5 μg (10 – 13 mm), penicillin 10U (28 – 29 mm), streptomycin 10 μg (11 – 15 mm), and tetracycline 10 μg (14 – 19 mm) test discs were used (Oxoid Ltd., Basingstoke, Hants, UK). The choice of antibiotics reflects the range contained in several intramammary products available for treating mastitis in Slovakia. The sensitivity or resistance of the bacteria tested was interpreted according to the reference zones in conformity with the instructions of **EUCAST (2014)**. The tribes *S. aureus* CCM 5973 and *S. epidermidis* 4418 were used as a control in the tests. In view of the abundance of the species of the CPS and CNS, it was only possible for the species *S. aureus* and *S. epidermidis* in practical terms, to evaluate resistance as a percentage: a negligible (<0.1%), very low (0.1 – 1%), low (1 – 10%), moderate (10 – 20%), high (20 – 50%) or very high (50 – 70%).

Statistical analyses

Statistical analysis was performed using the Microsoft Excel 2007 software. Chi-square test (χ^2 test) was used to compare the individual proportions **(Kabrt, 2013)**.

The dependence of the individual signs was tested at a significance level $\alpha = 0.05$, with critical value = 5.991.

RESULTS AND DISCUSSION

Table 1 gives an overview of the bacteria *Staphylococcus* spp., which was isolated from sheep's milk, during the three years (2017 – 2019) on holdings in Slovakia. In the reporting period, 273 coagulase-positive staphylococci were isolated of which 48.7% (133) *S. aureus* was isolated in 8.1% (23) *S. intermedius*, 5.9 % (16) *S. hyicus,* and 37.0% (101) *S. schleiferi.*

S. aureus was isolated during the reporting period, although most frequently at the beginning of the reference period (50). *S. intermedius* was isolated in the two last years, most frequently in the second year (12), S. *hyicus* was isolated in the first (12) and second (4) years of the experiment. *S. schleiferi* was isolated during the reporting period, most frequently at the end of the experiment (48).

In the reporting period, 158 coagulase-negative staphylococci were isolated, of which *S. epidermidis* was isolated in 55.7% (88), *S. chromogenes* 31.0% (49), *S. caprae* 3.8% (6), *S. xylosus* 4.4% (7), other species were rarely isolated.

Table 2 shows the reported overview of the occurrence of resistance to 14 antibiotics in four tested species of staphylococci (n = 371), which were most frequently isolated during the experiment.

Although intramammary infections caused by CoNS are usually self-limiting, there are reports of clinical mastitis cases that often require antimicrobial treatment **(Taponen, 2006; Pieterse and Todorov, 2010)**.

Occurrence to resistance to 14 antibiotics in *S. aureus* and *S. schleiferi*, which were isolated from sheep´s milk during the three-year period is reported in Table 3. In the evaluation, the tests of sensitivity of the two most numerous CPS (*S. aureus, S. schleiferi)* were numerically expressed as numbers of (S) – sensitive, (IM) – intermediate, and (R) – resistant as well as the values of the resistance in percentage (%).

Staphylococcus aureus (Table 2 and Table 3) showed the highest resistance to erythromycin 12.0%, novobiocin 10.5%, and neomycin 9.0%. We may consider that there is an adverse incidence of intermediate susceptibility of penicillin (16), novobiocin, and erythromycin (14 strains), to oxacillin and cloxacillin (12 strains), neomycin (11 strains), and lincomycin (9 strains). For other antibiotics, the incidence of resistant strains of *S. aureus* was relatively low.

From all 101 tested strains of *Staphylococcus schleiferi,* it was discovered that 5.9% resistant was to novobiocin, and 5.0% to erythromycin. Intermediate sensitivity was detected to erythromycin (9), amoxicillin and novobiocin (8), ampicillin, and lincomycin (7), and penicillin, methicilin, and cefoperazone (5 strains). Penicillin antimicrobials were reported to be effective against CoNS infections **(Becker, Heilmann and Peters, 2014; Bhattacharyya et al., 2016)**. However, studies declare an increasing prevalence of antimicrobial resistance in CoNS from clinical mastitis cases **(Schmidt, Kock, and Ehlers, 2015; Beuron et al., 2014)**, including resistance to penicillin, tetracycline, lincomycin, and streptomycin **(Taponen et al., 2006)**.

Recent evidence from Europe indicates insignificant problems of resistance to antibiotics commonly used for cases of mastitis in sheep. **Vautor et al**. **(2009)** reported only sporadic resistance in *S. aureus* isolated in France. **Onni et al**. **(2011)**, in Italy, likewise found limited resistance in *S. epidermidis*, except to penicillin for which the resistance rate was 38%. Similar results were observed in Turkey, where in coagulase-negative isolates from subclinical mastitis only resistance to β-lactams was noteworthy (43%), whilst there was a much smaller frequency of resistance to tetracycline (11%) and even less to other agents **(Ergün et al., 2012)**. Moreover, research in Turkey corroborated these findings, the rate of resistance to penicillin was 27% and to tetracycline 8% **(Unal and Çinar, 2012)**. **Martins et al. (2017)** published similar results; 17% of isolates were resistant to penicillin and 11% to tetracycline. Finally, evidence from Greece was consistent with the abovementioned, as the frequency of resistant isolates was 35% of staphylococcal isolates tested **(Vasileiou et al., 2019)**.

The incidence of the following characters (S, IS, R) was compared in two groups, the most numerous of staphylococci *S. aureus* and *S. schleiferi* using a statistical method, the Chi-squared test. On the significance level $\alpha = 0.05$ (5%) was recorded in fourteen antibiotic substances test value (G <χ2), the statistical independence of tracked characters was confirmed. The antibiotic substance methicillin, penicillin, and oxacillin when applied, G >χ2, in the test groups of *S. aureus* and *S. schleiferi,* statistical dependence of the observed characters was confirmed, which means that the occurrence of the characters was not random.

Table 1 Bacteria *Staphylococcus* spp. isolated from sheep milk from two herds of sheep during the three-year period in Slovakia.

Bacteria *Staphylococcus* spp.	2017	2018	2019	Total	%
S. aureus	43	40	50	133	30.9
S. intermedius	3	12	8	23	5.3
S. hyicus	12	4	-	16	3.7
S. schleiferi	9	44	48	101	23.4
S. caprae	1	4	1	6	1.4
S. epidermidis	47	21	20	88	20.4
S. chromogenes	29	8	12	49	11.4
S. sciuri	3	-	-	3	0.7
S. simulans	3	-	-	3	0.7
S. warneri	-	2	-	2	0.5
S. xylosus	4	-	3	7	1.6
∑	154	135	142	431	100.0

Table 2 Total overview of the incidence of resistance to 14 tested antibiotics in the two species of CPS and CNS staphylococci (n = 371), which were the most frequently isolated from sheep's milk, during the three years.

Bacteria	R	n	Antibiotics													
			AMP	AML	CFP	FOX	OB	E	MY	MET	N	NV	OX	P	S	T
S.aureus (n = 133)	1.	43	4	3	1	1	1	2	3	1	6	8	4	10	1	1
	2.	40	1	2	-	2	1	8	2	-	5	3	1	3	-	-
	3.	50	1	2	1	2	1	6	2	1	1	3	2	3	-	-
	∑	133	6	7	2	5	3	16	7	2	12	14	6	7	1	1
S.schleiferi (n = 101)	1.	9	-	-	1	-	-	-	-	-	-	1	-	-	-	-
	2.	44	-	1	-	-	2	1	-	-	2	1	-	1	11	-
	3.	48	2	1	-	-	-	4	1	-	2	4	-	1	-	-
	∑	101	2	2	1	-	2	5	1	-	4	6	-	2	1	-
Resist. ∑ KPS		234	8	9	3	5	5	21	8	2	16	20	6	9	2	1
S. epidermidis (n = 88)	1.	47	2	2	1	-	1	2	1	-	2	2	-	2	-	-
	2.	21	1	1	1	-	2	1	2	-	2	3	1	2	1	-
	3.	20	-	-	1	-	-	3	1	-	2	2	-	1	-	-
	∑	88	3	3	3	-	3	6	4	-	6	7	1	5	1	-
S. chromogenes (n = 49)	I.	29	-	-	-	-	-	1	1	-	1	1	-	1	-	-
	II.	8	-	-	2	-	-	-	-	-	1	1	-	-	-	-
	III.	12	1	-	-	1	-	-	-	-	-	1	-	1	-	-
	∑	49	3	-	-	1	-	1	1	-	2	3	-	2	-	-
Resist. ∑ KNS (∑n)		137	6	-	3	1	3	7	5	-	8	10	1	75	4	-

Note: (AMP) Ampicillin 10 μg; (AML) Amoxicillin 25 μg; (CFP) Cefoperazone 30 μg; (FOX) Cefoxitin 30 μg; (OB) Cloxacilin 5 μg; (E) Erythromycin 10 μg; (MY) Lincomycin 15 μg; (MET) Methicilin 10 μg; (N) Neomycin 10 μg; (NV) Novobiocine 5 μg; (OX) Oxacillin 5 μg; (P) Penicillin 10 IU; (S) Streptomycin 10 μg; (T) Tetracycline 10 μg.

Table 3 An overview of the sensitivity and the occurrence of resistance to 14 tested antibiotics in *S. aureus* and *S. schleiferi* representatives of the CPS, isolated from sheep's milk in the years 2017 to 2019.

Antibiotics	*S. aureus* (n = 133)				*S. schleiferi* (n = 101)				TEST *
	S	IS	R	%	S	IS	R	%	G
Ampicillin	119	8	6	4.5	92	7	2	2.0	1.165
Amoxicillin	121	5	7	5.3	91	8	2	2.0	3.397
Cefoperazone	126	5	2	1.5	95	5	1	1.0	0.306
Cefoxitin	125	3	5	3.8	97	4	-	-	4.388
Cloxacilin	118	12	3	2.6	95	4	2	2.0	2.357
Erythromycin	103	14	16	12.0	87	9	5	5.0	3.887
Lincomycin	117	9	7	5.3	93	7	1	1.0	3.169
Methicilin	131	-	2	1.5	96	5	-	-	8.153*
Neomycin	110	11	12	9.0	93	4	4	4.0	4.396
Novobiocine	105	14	14	10.5	87	8	6	5.9	2.189
Oxacillin	115	12	6	4.5	97	4	-	-	7.296*
Penicillin	110	16	7	5.3	94	5	2	2.0	7.757*
Streptomycin	127	5	1	0.8	97	3	1	1.0	5.509
Tetracycline	127	5	1	0.8	199	2	-	-	4.512

Note: Sensitivity (S); Intermediate sensitivity (IS); Resistance (R); % resistance on the base n;* χ^2 test (significance level $\alpha = 0.05$ (5%); critical value $\chi2 = 5.991$; G – testing value).

CONCLUSION

By the bacteriological examination of individual and pool samples of sheep's milk during the three seasons of machine milking was isolated and taxonomically classified 11 species from the total number of 431 bacteria *Staphylococcus* spp. From the coagulation of positive staphylococci (KPS) (n = 273), *S. aureus* was isolated throughout the period considered, but most often in the last year (50). The incidence of *S. intermedius* was highest in the second year (12). *S. hyicus* was isolated in the first and second years of experiment (16) and *S. schleiferi* was most experienced in the last year (48). From the group of coagulase-negative staphylococci (KNS) (n = 158), *S. epidermidis* occurred in 55.7% (88), *S. chromogenes* 31.0% (49), *S. caprae* 3.8% (6), *S. xylosus* 4.4% (7), and other species rarely occurred. *S. aureus* bacteria (n = 133) showed maximum resistance to erythromycin 12.0% and novobiocin 10.5%, and to neomycin 9.0% as well. The incidence of intermediate sensitivity was observed in penicillin (16 strains) of novobiocin, erythromycin (14 strains), oxacillin and cloxacillin (12 strains), neomycin (11 strains), and lincomycin (9 strains). *Staphylococcus schleiferi* bacteria (n = 101) showed the highest resistance to novobiocin (5.9%) and erythromycin (5.0%), however, a high incidence of intermediate susceptibility to erythromycin (9), amoxicillin and novobiocin (8), ampicillin and lincomycin (7), penicillin, methicilin and cefoperazone (5 strains) can be identified as adverse. This work was aimed at testing the most frequent representatives of the genus *Staphylococcus* spp., a relatively adverse development of resistance to the most commonly used antibiotics for the treatment of inflammation of the udder in sheep.

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