



ANTIBIOTIC RESISTANCE IN BACTERIA *STAPHYLOCOCCUS* SPP. ISOLATED FROM SAMPLES OF RAW SHEEP'S MILK

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ABSTRACT

From samples of raw sheep's milk were determined results of bacteriological examination from two herds in region of Eastern Slovakia in three years lasting study. The occurrence of *Staphylococcus spp.* 41.6% (124) was determined from 298 samples. The seven species of staphylococci were on a regular basis isolated: *S. epidermidis* (34), *S. chromogenes* (26), *S. aureus* (16). Alternately have been recorded *S. warneri* (16), *S. schleiferi* (15), *S. haemolyticus* (9) and *S. xylosus* (8). All isolated pathogens were tested by in vitro test on Mueller-Hinton agar by disc methods on resistance to 10 types of antibiotics. Highest value of resistance was determined to Penicilin 21.0%, Neomycin 10.5% and Novobiocin 9.7%. Lower resistance was in to Oxacilin 7.2% and Amoxicilin 6.5%. Minimal resistance was founded to Cefoxitin 0.8%, Linkomycin 2.4%, Erytromycin, and Streptomycin 3.2%. Was founded total resistance (21.0%) to all antibiotics in *S. epidermidis* (34) during the three years, *S. chromogenes* (26) showed resistance to 8 types of antibiotics (12.9%), *S. aureus* (16) to 6 antibiotics (10.5%) and *S. warneri* (16) to 4 antibiotics (5.6%). It was confirmed that sheep's milk remains a major source of staphylococci. Bacteria in comparison with isolates from cows' raw milk, showed lower values of resistance, but were resistant to more than two antibiotics. Recorded occurrence of resistance in staphylococci may be connected with a minimum use of antibiotics in the treatment of mastitis and other diseases in sheep herds. Reported resistance to the tested antibiotics became the basis for the recommendation to use preparations to treat mastitis in sheep principally by the detection of resistance to antibiotics contained.

Keywords: sheep; raw milk; antibiotic; resistance; *Staphylococcus spp.*

INTRODUCTION

The bacteria *Staphylococcus* are important pathogens in human and animals medicine. Specific properties above 50 species of *Staphylococcus spp.* caused differences in pathogenesis of many inflammatory diseases as evidenced by the extensive studies of *S. aureus*, which produces the greatest amount of substances called virulence factors (Spargser et al., 2003).

Staphylococcus spp. is the main causative agent of ovine mastitis, with higher prevalence in cases of clinical and subclinical manifestations (Bergonier et al., 2003; Fagundes et al., 2010).

In the environmental mastitis *Staphylococcus spp.* and *Escherichia coli* are the main pathogens responsible for the inflammation (Baskaran et al., 2009) and, together with coagulase negative staphylococci (CoNS) strains, are the most frequent pathogens, particularly such as *S. epidermidis*, *S. simulans*, *S. hyicus*, *S. warneri*, *S. sciuri* and *S. xylosus* in ovine mastitis (Hariharan et al., 2004).

In sheep, intramammary antibiotic therapy using a combination of penicillin with many drugs has been found to be effective in reducing the load of mastitis pathogens after lambing (Chaffer et al., 2003). Mastitis can be cured by treatment with antibiotics after identification of the causative agents. Antibiotic sensitivity tests can be performed to ensure adequate treatment (Bergonier and Berthelot, 2003).

Owing to the important role of antimicrobials in mastitis control programs, the determination of antimicrobial susceptibilities of mastitis pathogens is necessary for therapy and monitoring the spread of resistant strains among populations (Gentilini et al., 2002; Bengtsson et al., 2009). Also, antimicrobial resistant bacteria of animal origin can be transferred to humans via food chain and can pose public health problems (Skovgaard, 2007).

Antibiotic resistance is one of the important problems encountered in the treatment and control of mastitis. Mastitis caused by resistant bacteria is difficult to cure and has severe consequences. Thus, determination of the antibiotic susceptibilities of pathogens causing mastitis is of crucial importance for the treatment and control of mastitis in dairy ewes (Tel et al., 2012).

Increasing the consumption of antibiotics in veterinary and human medicine is in the last period, accompanied by the phenomenon of an increase in bacterial resistance. The use of antimicrobials in animal nutrition, in the production of plants, feed and food prices can have a negative impact on public health through the increase in resistant bacteria or bacteria producing resistant genes that pass into the organism of people directly or indirectly (Bireš et al., 2009).

Hleba et al. (2010) was focused on monitoring the resistance of bacteria in the work of the family *Enterobacteriaceae*, which are considered a reservoir of genes resistant to antibiotics in animal husbandry. About

bacteria *Staphylococcus*, which are frequently isolated from milk of ruminants, it is generally stated that they can download the following "mediated" resistance genes from another environment (Pyörälä and Taponen, 2009).

Infections caused by resistant species of microorganisms cause costly treatment of animals as well as humans. These infections lengthen a pathological condition and, if not treated with antibiotics are administrative (according to current observed sensitivity of bacteria) can lead to increased mortality (Witte, 2006).

It is therefore the identification of these reservoirs and a mechanism to transfer the key to reduction and the reduction of resistant bacteria in the commercial sphere of the food chain man (Nováková et al., 2009).

Vasiľ et al. (2009) noticed on increasing occurrence of subclinical and latent mastitis caused by CoNS resistant to antibiotics. For this reason, control of antibiotic resistance bacterial pathogens of mastitis should be the starting point for ensure the effectiveness of control methods applied (Virdis et al., 2010; Bennedsgaard et al., 2006).

Recently, it has been recognised that antimicrobial susceptibility of CoNS, which represent 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).

The aim of our study was to determine the occurrence of *Staphylococcus* spp. in samples of raw sheep's milk and their resistance to 10 antibiotics by disc method.

MATERIAL AND METHODOLOGY

Characteristic of experiment and samples collection

During three years, in the frequency of two times a month were bacteriological examined samples of raw and sheep's milk, with a focus on the isolation of the bacteria *Staphylococcus* spp. Samples were collected from the tanks as the total pool after milking from different two sheep holdings on Eastern Slovakia.

The experiment was carried out in two herds - herd A of 450 sheep of Lacaune breed and herd B 400 sheep breed of Improved valaska. During summer season (from April to September) sheep were machine milked two times per day in milk parlous: herd A - 2x14 Miele Melktechnik, (Hochreiter Landtechnik, Germany) and Herd B - 2x20, Alfa-Laval, Tumba, Sweden).

Level on both farms with tradition of machine milking sheep can be characterized as a standard. Sheep have secured adequate food during all year, and grazing on adjacent pastures. It is ensured selection of sheep according health of the mammary gland; decommissioning ewes with chronic form of mastitis from herds of sheep; milking max. 180 seconds and with individual post-milking; observance of hygiene program during milking with an emphasis on teat disinfection after milking; Treatment of clinical mastitis cases on the base of antibiogram results.

Microbiology analyses

Bacteriology analyses were centred on the isolation of *Staphylococcus* spp. Cultivation and identification of bacteria was performed on the 5 % blood agar, Medium No. 110, Baird Parker agar. Colouring by Gram, catalase

activity, coagulation of the rabbit's plasma, haemolysis, and the pigments production were carried out, too. Isolated bacteria were examined by the commercial set Staphytest24 (Erba-Lachema, Brno, Czech Republic), and results were evaluated using the identifying programme TNW, version 7.0 (Erba-Lachema, Brno, Czech Republic).

Resistance to antibiotics

We tested all isolated pathogens by *in vitro* test on Mueller-Hinton agar by zone disc methods (EUCAST, 2015) after 24h incubation at 37 °C on resistance to 10 antibiotics: Ampicilin 10 µg, Amoxicilin 25 µg, Oxacilin 5 µg, Erythromycin 10 µg, Linkomycin 15 µg, Neomycin 10 µg, Novobiocin 5 µg, Penicilin 10 µg, Streptomycin 10 µg, Cefoxitin 30 µg (OXOID Ltd. Basingstoke, Hants, UK). Resistance or sensitivity of the bacteria tested was interpreted by reference zones in accordance with the instructions EUCAST (2015). In tests were used according to recommendations following control strains: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* CCM 4418, *Staphylococcus haemolyticus* CCM 2737. Occurrence of resistance to the tested antibiotics for each species of staphylococci has been registered continuously numerically.

RESULTS AND DISCUSSION

Several authors in their studies from France and Spain recorded, that the species of *Staphylococcus* spp. belongs to general aetiological agents of intramammary infections in small ruminants (*S. aureus* in clinical and CoNS in subclinical cases). From the CoNS is more frequently *S. epidermidis* and *S. chromogenes* what are also determined in our study (Bergonier et al., 2003; Berthelot et al., 2006).

The frequency of occurrence of staphylococci in raw sheep's milk collected twice a month in two different herds of sheep in Eastern Slovakia during the three milking seasons are described in Table 1.

From all isolated species of staphylococci were regular isolated *S. epidermidis* (34), *S. chromogenes* (26), and *S. aureus* (16). Alternately were isolated *S. warneri* (16), *S. schleiferi* (15), *S. xylosum* (8), and *S. haemolyticus* (7).

In general, microbial contamination of raw milk occurs from three main sources: from within the udder, from the exterior of the udder, and from the surface of milk handling and storage equipment (McKinnon et al., 1990).

From the total three years valuation the occurrence of resistance to 10 types of antibiotics, resulted to occurrence of resistance predominantly in two species of staphylococci during year (*S. intermedius* and *S. haemolyticus*). Maximal annual finds had species *S. epidermidis* (16), *S. warneri* (12) and *S. schleiferi* (11).

Continuous valuation of the occurrence of resistance in staphylococci are characterised by minimum values. An objective assessment in the context of individual species and year on year was complicated due to the small number of groups.

In the total group of *Staphylococcus* spp. (n = 124) were determined the resistance to all ten types of antibiotics. Highest value of resistance was determined to Penicilin 21.0%, Neomycin 10.5% and Novobiocin 9.7%. Lower resistance was in to Oxacilin 7.2% and Amoxicilin 6.5%.

Table 1 Resistance to 10 antibiotics in bacteria *Staphylococcus* spp. (n = 124) isolated from 298 samples of raw sheep's milk.

<i>Staphylococcus</i> spp.	year	n	Resistance (%)									
			1	2	3	4	5	6	7	8	9	10
<i>S. aureus</i> (n = 16)	I.	8		1	1					1	2	
	II.	5							1	1	1	2
	III.	3			1				1		1	
<i>S. epidermidis</i> (n = 34)	I.	16	1		3				2	1	5	1
	II.	8	1	1			1	1		3	2	1
	III.	10			1				1		1	
<i>S. chromogenes</i> (n = 26)	I.	10	1	2	1				3	1		
	II.	9		1			2	1		1	2	
	III.	7						1			1	
<i>S. warneri</i> (n = 16)	I.	4								1	1	
	II.											
	III.	12			1				2		2	
<i>S. schleiferi</i> (n = 15)	I.	11		1	1					1	2	1
	II.											
	III.	4							1		1	
<i>S. haemolyticus</i> (n = 9)	I.	7		1					2	1	1	
	II.	2								1	1	
	III.											
<i>S. xylosus</i> (n = 8)	I.											
	II.	3	1				1				1	
	III.	5		1							1	
Resistance (\sum n)		124	4	8	9	4	3	13	12	25	4	1
Resistance (%)*		100	3,2	6,5	7,2	3,2	2,4	10,5	9,7	21,0	3,2	0,8

Note: (1) Ampicilin 10 µg; (2) Amoxicilin 25 µg; (3) Oxacilin 5 µg; (4) Erytromycin 10 µg; (5) Linkomycin 15 µg; (6) Neomycin 10 µg; (7) Novobiocin 5 µg; (8) Penicilin 10 µg; (9) Streptomycin 10 µg; (10) Cefoxitin 30 µg; * – resistance of all 124 tested bacterium on antibiotics.

Minimal resistance was founded to Cefoxitin 0.8%, Linkomycin 2.4%, Erytromycin, and Streptomycin 3.2%.

The resistance in most numbered species (groups with $n \geq 15$) are described in Table 2. Was founded total resistance (21.0%) to all antibiotics in *S. epidermidis* (34) during the three years. *S. chromogenes* (26) showed resistance to 8 types of antibiotics (12.9%), *S. aureus* (16) to 6 antibiotics (10.5%) and *S. warneri* (16) to 4 antibiotics (5.6%).

Our results are consistent with the work Kirkan et al. (2005), where 300 cases of mastitis were isolated 60 bacteria of CoNS (20.0%), which showed resistance to penicillin (90.0%) and oxacillin, (75.0%). For comparison Virdis et al. (2010) report increased resistance of CoNS on ampicillin and kanamycin and of *S. aureus* on the oxytetracycline.

The importance of CoNS in the aetiology of the subclinical mastitis in sheep is the undisputed. However, the frequency of their occurrence is species-specific and is different depending on the technological level of the holding and the geographical conditions of the site (Gentilini et al., 2002). While our work we describe six types of CoNS, Gelasakis et al. (2015) under the transparent processing of the twelve works published over the last ten years in this context, it has registered a total of twenty species have been isolated in the mastitis of sheep. Despite the finding that the CoNS are pathogens of lower virulence, the severity of their occurrence lies in the progressive trend of increasing the share of the prevalence of clinical forms of mastitis. In particular, these are the bacteria *S. epidermidis*, *S. chromogenes*, *S. simulans* and *S. xylosus*, in which was recorded the occurrence of antibiotic resistance (Onni et al., 2010). And for that reason we

Table 2 Resistance in species of the genus *Staphylococcus spp.* (only in groups with $n \geq 15$), which were isolated from raw sheep's milk.

Antibiotics	µg	<i>S. aureus</i> (n = 16)	<i>S. epidermidis</i> (n = 34)	<i>S. chromogenes</i> (n = 26)	<i>S. warneri</i> (n = 16)
Penicilin	10 U	4	8	2	3
Ampicilin	10	–	2	1	–
Amoxicilin	25	1	1	3	–
Oxacilin	5	2	4	1	1
Erythromycin	10	–	1	2	–
Lincomycin	15	–	1	2	–
Streptomycin	10	2	1	–	–
Neomycin	10	2	3	2	2
Novobicin	5	2	4	3	1
Cefoxitin	30	–	1	–	–
Resistance (Σn)		13	26	16	7
*Resistance (%)		10.5	21.0	12.9	5.6

Note: * - resistance from all 124 tested bacteria *Staphylococcus spp.*

prefer to take the view that in the coming period will need to be monitored the antibiotic resistance in CoNS bacteria, in order to ensure the effective clinical therapies of mastitis and, secondly, that it will be important for the early detection of resistance in this important group of bacteria isolated from ewe's milk.

Information about the occurrence of sporadic resistance to antibiotics, are usually a reflection of the range of intramammary drugs the use of veterinary practice in the country. For the acceptance of data in the published studies, is important information about the frequency of resistance isolates of CoNS to tested drugs, as well as the period during which the bacteria have been isolated. **Ebrahimi et al. (2007)** from nine antibiotics recorded, increased resistance of the CoNS to amikacin, and to a lesser extent resistance to ampicillin, penicillin, tetracyclin, and oxytetracyclin. Good sensitivity was detected to chloramphenicol, ciprofloxacin, streptomycin and gentamicin, it is important for potential use in the treatment of clinical mastitis.

The objectivity of the results about resistance to antibiotics of the bacteria can be achieved by using a phenotypical method, in parallel with molecular identification of markers of resistance. This is a PCR detection of number of specific genes responsible for individual properties, which determine the resistance (**Franco et al., 2012**). Record the occurrence of two or more genes may be associated with the multiresistance to antibiotics in bacteria of *Staphylococcus spp.* Increasing incidence of multi-drug resistant strains of CoNS, compared with CoPS in general supports the hypothesis, that the CoNS can play an important role as a source of resistance genes for *S. aureus* (**Taponen & Pyörälä, 2009**).

The results obtained a three-year monitoring of resistance in staphylococci isolated from samples of raw sheep's milk

must be considered as continuous in the light of the ongoing two-year continuation of the experiment yet. It was confirmed that sheep's milk remains a major source of staphylococci; however, the current level of treatment effectively eliminates the potential threat to the health of consumers of dairy. In comparison with numerous types of staphylococci isolates from cow's raw milk, tested strains had lower values of resistance, but were resistant to more than two antibiotics.

CONCLUSION

Using bacteriological examination of 298 samples of raw sheep milk were obtained data on frequency of bacteria *Staphylococcus spp.*, 41.6% ($n = 124$). Recorded the incidence of resistance in staphylococci, which were isolated from raw sheep's milk was linked to minimal use of antibiotics in the treatment of mastitis and other diseases in sheep.

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