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AETIOLOGY OF MASTITES AND ENTEROTOXIN PRODUCTION BY *STAPHYLOCOCCUS sp.* ISOLATED FROM MILK OF TWO SHEEP HERDS

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ABSTRACT

The study was performed in two herds of sheep during 2004–2006 and is focused on two parts: 1. Three years observation of the occurrence the bacterial agents of mastitis in two herds of sheep with different milking technology and proportion of individual pathogens in infection levels. The observation indicates: in the sheep herd with the machine milking the level of infection of the sheep mammary glands caused by *Staphylococcus sp.* ranged within 12.0 %–29.8 %, the level of infections caused by environmental bacteria was within the range of: *Streptococcus uberis* – 1.2 %–3.2 %; *E. coli* – 0.0 %–7.8 %, *Enterococcus sp.* – 0.2 %–1.9 %; – in the herd of sheep with the hand milking the level of infection of the sheep mammary gland by *Staphylococcus sp.* was within the range of 12.9–21.7 %, the level of infections caused by environmental bacteria was within the range of: *Streptococcus uberis* – 1.5 %–3.5 %, *Streptococcus dysgalactiae* – 0.8 %–1.5 %, *Streptococcus agalactiae* – 0.7 %–1.0 %, *E. coli* – 0.0 %–0.3 %, *Bacillus sp.* – 0.0 %–0.3%; 2. Identification, classification and determination of enterotoxin production in 698 *Staphylococcus sp*. The observation indicates: within the identified and classified 698 *Staphylococcus sp.* bacteria following strains are occurred: *Staphylococcus aureus. spp. aureus* (158), *Staphylococcus intermedius* (13), *Staphylococcus hyicus* (11), *Staphylococcus epidermidis* (133), *Staphylococcus caprae* (121), *Staphylococcus haemolyticus* (20), *Staphylococcus gallinarum* (27), *Staphylococcus hominis* (23), S*taphylococcus chromogenes* (31), *Staphylococcus schleiferi spp. coagulans* (19), *Staphylococcus simulans* (44), and *Staphylococcus warneri* (98); – out of 698 tested *Staphylococus sp.* 81 produced enterotoxins. The staphylococcal enterotoxin (SE) of the type C was produced most frequently (47), then type A (8), type D (7), and type E (6). Combination, i.e. simultaneous formation of more SE types produced 2 species of staphylococci – SEA+SED (*Staphylococcus aureus* – 8 and *Staphylococcus simulans* – 4) and SEC+SEE (*Staphylococcus simulans* – 1). *Staphylococcus aureus* (52), *Staphylococcus caprae* (17), *Staphylococcus simulans* (6), *Staphylococcus warneri* (4), *Staphylococcus epidermidis* (2) participated in the production of enterotoxins most frequently.

Key words: mastitis, sheep, bacterial agents of mastitis, staphylococcal enterotoxins, *Staphylococcus sp., Streptococcus sp*.

INTRODUCTION

An increase in the lactating performance burdens the mammary gland to a greater extent which means a greater probability for its inflammatory disease, above all mastitis (Špánik et al., 2004). Mastitis is an important part of sheep morbidity. In Slovakia there are no any complete data on the incidence of mastitis in sheep. According to our experience the incidence of mastitis caused by bacterial agents ranges from 15.6 to 32.2 % depending on the technology of breeding and overall hygiene. The agents are predominantly *Staphylococcus sp.* bacteria, *Streptococcus sp*., and coliform bacteria (Vasil' et al., 2005).

Staphylococcus aureus is the most frequent agent of clinical mastitis in sheep (Vasil' et al., 2007). Recently, coagulase-negative staphylococci (CoNS) have been found to be significantly participated in the onset of mastitis in sheep. The most frequent species are: *S. epidermidis, S. caprae, S. chromogenes, S. hyicus, S, xylosus*, and *S. intermedius*. Sporadically also other pathogenic species are isolated, e.g.: *S. simulans, S.*

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capitis, S. sciuri, and S. *warneri*. These pathogenic bacteria mainly increase the number of somatic cells (SCC). It is reported that *S. epidermidis* increases the level of SCC more than other species, even if the difference is small. Most species of CoNS in sheep are characterised by low pathogenity. However, some species of CoNS cause udder inflammation of the same intensity as coagulase-negative staphylococci (Burriel, 1997; Pengov, 2001; Hadimli et al., 2005). From the breeding and food processing point of view, *Staphylococcus aureus* is classified among the most serious pathogens causing clinical symptoms of various diseases not only in animals, but also in people (Vasiľ et al., 2007; Špánik et al., 1999). Above all, it is the case of staphylococcal enterotoxicoses caused by thermostable enterotoxins (Vasiľ et al., 2007). Bergdoll (1990) confirmed the capability of producing enterotoxins also in *S. intermedius* and *S. hyicus*. Enterotoxins can also produce some species of coagulase-negative staphylococci, e.g. *S. cohnii, S. epidermidis, S. haemolyticus, S. xylosus* (Bautista et al, 1988). *S. chromogenes, S. warnei, S. sciuri, S. saprophyticus, S. lentus* are also determined as enterotoxigenic (Valle et al., 1990; Becker et al., 2001).

Of the coliform bacteria at mastitis in sheep, bacteria of *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia* are commonly isolated. *Pseudomonas aeruginosa* is a common agent of acute mastitis in lactating sheep (Tripathi, 2000). Next agents of mastitis in sheep are environmental *Streptococcus sp.*, fortunately until recently, their portion in the inflammations of the mammary gland in sheep was quite low (3–6 %, or less; Menzies and Ramanoon, 2001). Recently their portion in sheep mastitis has increased. Under our conditions *Streptococcus dysgalactiae* and *Streptococcus uberis* are isolated most frequently.

The goal of our study was to observe aetiology of mastitis in two herds of sheep with different technology of milking, to define species representation of bacteria *Staphylococcus sp.*, and to testify their capability of producing enterotoxins.

MATERIAL AND METHODS

Observation of mastitis aetiology in sheep was performed during 3 years in 2004–2006 in two herds of sheep with different technology of milking in the East Slovakia.

Herds of sheep

In the first herd observed there were about 320 Improved Vallachian sheep. In winter season sheep were housed in the sheep pen consisted of two traditional buildings with deep bedding, milking parlour, which continues with reinforced and sheltered area serving for sheep housing in summer. Milking runs three-times a day in the double-row milking parlour with lowered manipulating corridor that is equipped with the milking device Alfa Laval Agri 2 x 20 (*Alfa Laval, Sweden*). Collected milk, after storage in a cooling tank, is transported by a dairy cistern to a dairy plant for its processing.

The second herd with about 200 Improved Vallachian sheep was raised under traditional chalet conditions with hand milking. The herd was milked twice a day into stainless containers and milk was stored in collecting stainless milk cans, and then transported by a milk cistern to a dairy plant.

Collection and milk sample examination

Collection and milk sample examination in the first herd of sheep was carried out every year three annually in May, July, and August. Collection and milk sample examination in the second herd of sheep was carried out twice a year – in May and August.

After first milking and evaluation of the first sprays of milk and disinfection of the teat endings using cellulose cotton dipped in 70 % benzenealcohol, the individual milk samples (i.e. from both halves in the same ratio) were collected under aseptic condition into sterile test tubes for bacteriological examination in the volume of 10 ml according to the principles given by IDF (IDF Bulletin, No. 211, 1987).

Microbiological diagnostics, cultivation and identification of pathogenic bacteria were carried out according to the valid procedures of IDF (IDF Bulletin, No. 132, 1981). Samples were transported to the laboratory in a portable fridge at 4 ^{o}C and inoculated to the cultivating media within 4 hours after collection. Bacteriological examination was mainly aimed at finding the presence of bacteria of the genus *Staphylococcus sp*., *Streptococcus sp*., *Enterococcus sp*.

Isolation of bacteria was performed on the Columbia Blood Agar Bass (Oxoid, Basingstoke, Hants, England) with the addition of 5% defibrinated ram blood, Staphylococcus medium No 110 (Oxoid, Basingstoke, Hants, England), Baird Parker agar (Oxoid, Basingstoke, Hants, England), Edwards agar (Oxoid, Basingstoke, Hants, England), Endo agar (Biomark, Ahmedabad, India).

Taxonomic classification of the *Staphylococcus* sp. bacteria was performed as follows: firstly bacteria were subjected to a coagulase test using the test Microbiology bactident coagulase (Merck, KGaA Darmstadt, Germany). Then *Staphylococcus* sp. bacteria were tested for the anaerobic utilization of glucose. The bacteria, which anaerobically utilized glucose, were then tested for the anaerobic fermentation of mannitol. In the next step, *Staphylococcus* sp. bacteria were examined by the commercial set STAPHYtest 16, (PLIVA-Lachema, Brno, Czech republic) and results were evaluated using

the identifying programme TNW, version 6.0 (PLIVA-Lachema, Brno, Czech republic). Moreover, these bacteria were tested for the aerobic utilization of following saccharides – melebiose, cellobiose, galactose, fruktose xylitol, melezitose, sorbitol (Sigma-Aldrich, Inc., St. Louis, Moredon USA), fucose, raffinose, arabinose, dulcit, turanose, trehalose, salicyn, xylose, rhamnose (Acros Organics, Ceel, Belgium), mannitol, glucose, lactose, saccharose, mannose, maltose (Hinmedia Labor. Pvt Ltd., Mumbai, India).

Examination of Staphylococcus sp. bacteria for production of enterotoxins

Suspect solitary colonies of the observed staphylococcus were inoculated to 5 ml BHI (Oxoid, Basingstoke, Hants, England) with ceramic isolators and incubated for 18–24 hours at 37°C under continuous shaking. Cultivating fluid was cleared up by separation of the cells of propagated strain using 60 minute centrifugation at 3000 x G. Supernatant was analysed for the presence of staphylococcal enterotoxin (SE) by a commercial kit Ridascreen® set A, B, C, D, E (R-Biopharm, Darmstadt, Germany) according to the producer instruction. The kit has a detection limit of 0.2–0.7 ng.ml^{-1} for the staphylococcal enterotoxin of type A (SEA), type B (SEB), type C (SEC), type D (SED), and type E (SEE).

RESULTS AND DISCUSSION

In table 1 there is a survey of the representation of bacterial agents of mastitis in the sheep breeding with the machine milking during observed period. Occurrence of bacteriologically positive ewes in individual years was following: 42.2 % (2004), 18.8 % (2005), and 31.0 % (2006). Every year in the ewes with infected udder, *Staphylococcus sp.* bacteria had a dominant representation among determined bacterial agents of mastitis– in 2004 it was 29.8 %, but in 2005 only 12.0 %, however in 2006 it was already 26.4 %. The ratio of infections caused by *S. aureus* and cogulase–negative staphylococci was 15.0 % to 14.8 % recorded in 2004; in next years it gradually changed in favour of coagulase-negative staphylococci – in 2005 it was 5.1 % to 6.9 %, and in 2006 5.1 % to 21.3 %. Environmental bacteria participated in the ewe udder infections as follows: *Streptococcus uberis* – in 2004 – 3.2 %, in 2005 – 1.2 %, and in 2006 – 1.4 %. *Escherichia coli* bacteria were in individual years represented: 7.8 % (2004), 1.9 % (2005), 0.0 % (2006). *Enterococcus* sp. bacteria were represented as follows: 2004 – 0.8 %, 2005 – 1.9 %, and 2006 – 0.2 %. Of the group of main pathogens of the mammary gland, only in 2006 *Streptococcus agalactiae* (0.6 % , i.e. 7-times) and *S. dysgalactiae* (0.9 % , i.e. 10-times) were isolated.

Table 1: Number of infected lactating ewes after complex examinations of the sheep herd with machine milking in the years 2004, 2005 and 2006

Mastitis agent (bacteria)	Year of examination					
	2004		2005		2006	
	[1]n	%	[1]n	%	[1]n	%
Mastitis agents–*Staphylococcus sp.*						
Staphylococccus aureus	85	15.0	25	5.1	56	5.1
Coagulasa–negative staphylococci	84	14.8	34	6.9	234	21.3
Total	169	29.8	59	12.0	290	26.4
Mastitis agents–environmental						
Streptococcus uberis	18	3.2	6	1.2	15	1.4
Streptococcus dysgalactiae	0	0.0	0	0.0	10	0.9
Streptococcus agalactiae	0	0.0	0	0.0	7	0.6
E. coli	44	7.8	10	2.0	0	0.0
Enterococcus sp.	5	0.8	9	1.9	2	0.2
Proteus vulgaris	3	0.5	8	1.6	16	1.5
Total	70	12.4	33	6.7	50	4.6
Summary						
Number of infected lactating sheep	239	42.2	92	18.8	340	31.0
Number of uninfected lactating sheep	327	57.8	397	81.2	756	69.0
Total number of lactating sheep	566		489		1096	

[1]n – number of examined dairy sheep

Table 2: Number of infected ewes after complex examinations of the sheep herd with hand milking in the years 2004, 2005 and 2006

Mastitis agent (bacteria)	Year of examination					
	2004		2005		2006	
	[1]n	%	[1]n	%	[1]n	%
Mastitis agents–*Staphylococcus sp.*						
Staphylococccus aureus	3	0.9	5	1.9	10	1.6
Coagulasa–negative staphylococci	35	10.2	47	17.6	80	13.2
Total	38	11.1	52	19.5	90	14.8
Mastitis agents–environmental						
Streptococcus uberis	12	3.5	4	1.5	13	2.1
Streptococcus dysgalactiae	2	0.5	4	1.5	5	0.8
Streptococcus agalactiae	3	0.9	2	0.7	6	1.0
E. coli	1	0.3	0	0.0	1	0.2
Bacillus sp.	1	0.3	0	0.0	0	0.0
Total	19	5.5	10	3.7	25	4.1
Summary						
Number of infected lactating sheep	57	16.6	62	23.2	115	18.9
Number of uninfected lactating sheep	287	83.7	205	76.8	492	81.1
Total number of lactating sheep	344		267		607	

[1]n – number of examined dairy sheep

In the herd with traditional hand milking (tab. 2) following number of bacteriologically positive ewes was recorded: 16.6 % in 2004, 23.2 % in 2005, and 18.9 % in 2006. Infections were caused mainly by *Staphylococcus sp.* bacteria, i.e. 11.1 % (in 2004), 19.5 % (in 2005), and 14.8 % (in 2006). Occurrence of *S. aureus* was minimal – 0.9 % (2004), 1.9 % (2005), and 1.6 % (2006). Substantial part of intramammary infections formed streptococci: *S. uberis* – 3.5 % (2004), 1.5 % (2005), and 2.1 % (2006); *S. dysgalactiae* – 0.5 % (2004), 1.5 % (2005), and 0.8 % (2006); *S. agalactiae* – 0.9 % (2004), 0.8 % (2005), and 1.0 % (2006). *Escherichia coli* and *Bacillus sp.* bacteria had minimal representation in findings.

In table 3 there are the results of species specification of 698 *Staphylococcus sp.* bacteria that were taxonomically classified:

a) to three species of coagulase-positive staphylococci, i.e. totally 182 bacteria: *S. aureus ssp.aureus* – 22.6 %; *S. epidermidis* – 1.9 %; or *S. hyicus* – 1.6 %;
b) to nine species of coagulase-negative staphylococci, i.e. totally 516 bacteria, of which four species were significantly represented: *S. epidermidis* – 19.1 %, *S. caprae* – 17.3 %, *S. warneri* – 14.0 %, and *S. simulans* – 6.3 %. Other species occurred from 2.7 % to 4.4 % in ascending order as follows: *S. schleiferi ssp. coagulans*, *S. haemolyticus, S. hominis*, *S. gallinarum,* and *S. chromogenes*.

Table 3: *Staphylococcus sp.* bacteria isolated from sheep milk samples in the years 2004, 2005 and 2006

Staphylococcus species	Number		%
S. aureus. ssp. aureus	158		22,6
S. intermedius	13	182	1,9
S. hyicus	11		1,6
S. epidermidis	133		19,1
S. caprae	121		17,3
S. haemolyticus	20		2,9
S. gallinarum	27		3,9
S. hominis	23	516	3,3
S. chromogenes	31		4,4
S.schleiferi ssp. coagulans	19		2,7
S. simulans	44		6,3
S. warneri	*98*		14,0
Total	**698**		100,0

Table 4: ***Stahylococcus sp.*** **producing enterotoxins**

Staphylococcus sp.	Number	%[a]	%[b]
Staphylococcus aureus. ssp. aureus	52	64,2	7,4
Staphylococcus caprae	17	21,0	2,4
Staphylococcus epidermidis	2	2,5	0,3
Staphylococcus simulans	6	7,4	0,9
Staphylococcus warneri	*4*	4,9	4,9
Total	81	100,0	11,6

[a] – % of the number of enterotoxins producing staphylococci, n = 81; [b] – % of the total number of isolated staphylococci, n = 698

Table 5: **Production of staphylococcal enterotoxins by individual** ***Staphylococcus sp.***

Staphylococcus sp.		Type SE - number							Total
		A	B	C	D	E	A+D	C+E	
S. aureus		5	0	35	0	0	8	4	52
S. caprae		0	0	12	0	5	0	0	17
S. epidermidis		2	0	0	0	0	0	0	2
S. simulans		0	0	0	3	1	1	0	6
S. warneri		1	0	0	4	0	0	0	*4*
Total	n	8	0	47	7	6	9	4	81
	%	9.9	0.0	58.0	8.6	7.4	11.1	5.0	100.0

The tests aimed at finding of enterotoxin production confirmed that of the total number of 698 tested bacteria 81 (11.6 %) formed some of the five types (A, B, C, D a E) of enterotoxins. From the results in table 4 it follows that of 81 *Staphylococcus sp.* bacteria producing enterotoxins 52 (64.2 %) *Staphylococcus aureus* bacteria produced some of the types of enterotoxins (i.e. 7.4 % of the whole set of 698 tested *Staphylococcus sp.* bacteria). Within the total number of 81 *Staphylococcus sp.* bacteria producing enterotoxins coagulase-negative staphylococci were represented as follows: *S. caprae* – 21.0 %, *S. simulans* – 7.4 %, *S. warneri* – 4.9 %, and *S. epidermidis* – 2.5 %.

In table 5 there is a survey on production of the types of staphylococcal enterotoxins (SE) by individual bacteria of *Staphylococcus sp.* The individual types of SE were identified as follows: SEA – 8 bacteria (9.9 % of the staphylococci producing enterotoxins); SEB – was not determined (0.0 %); SEC – 47 bacteria (58.0 %), SED – 7 bacteria (8.6 %), SEE – 6 bacteria (7.4 %), or more SE simultaneuously, i.e. SEA+SED – 9 strains (11.1 %) and SEC+SEE – 4 strains, which represent 5.0 % of the total number of *Staphylococcus sp*. producing enterotoxins.

Deinhofer and Pernthaner (1993) reported that more species of bacteria participate in the aetiology of mastitis in sheep and goat, and most frequently bacteria of the Micrococcaceae family, when of the 497 samples of sheep and goat milk they isolated 447 bacteria belonging to this family. Though according to Menzies and Ramanoon (2001) *Staphylococus aureus* and *Streptococcus* sp. bacteria are the most frequent pathogens causing sheep mastites, also other species of bacteria are often isolated at sheep mastitis. Saratsis et al. (1998) reported that *Actinomyces pyogenes, Clostridium perfringens*, *Escherichia coli* and *Pasteurella haemolytica* are the common etiological agents at sheep mastitis, but other species are not an exception, and their share to the clinically apparent forms of sheep mastitis is up to 35%. Coliform bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* are common agents of acute, or peracute mastitis, that is difficult to control therapeutically and they result in dying of the affected animal (Tripathi, 2000; Menzies and Ramanoon, 2001).

Vernozy-Rozand et al. (1996), of 187 coagulase–negative strains, isolated from sheep milk and cheese found, that 11 (5.3 %) produced enterotoxins and predominantly type E. Scherrer et al. (2004), at testing the set of 293 strains of *S. aureus* using the PCR method found in 191 (65.2 %) the presence of the gene of these types of enterotoxins: SEC, SEG, SEA, SEJ, SEI, and SEB.

Valle et al. (1990) tested 342 *Staphylococcus* sp. bacteria for their ability to produce enterotoxins, which were isolated from various parts of the body of small ruminants. Staphylococcal enterotoxins (SE) were produced by 74.3 % of 70 coagulase–positíve bacteria and 22 % of coagulase–negative bacteria. Most enterotoxigenic bacteria were isolated from the teat skin and milk. These bacteria most frequently produced staphylococcal enterotoxin of type C, namely either alone (67.9 %) or in combination with other type of SE. *Staphylococcus* sp. bacteria isolated only from the milk produced enterotoxins as follows: of 20 *S. aureus* bacteria 17 produced enterotoxins (SEA – l, SEC – 13, SEA + SEC – 2, SEC + SEE – 1), one bacteria of *S. hyicus* produced SEC, of 5 *S. chromogenes* bacteria 2 produced SEC, and of 4 *S. epidermidis* bacteria one produced SEC. Of the coagulase–negative *Staphylococcus* sp. isolated from milk, enterotoxins were not produced by *Staphylococcus chromogenes* (3 strains), *S. warneri* (5 strains), *S. capre* (5 strains), *S. epidermidis* (3 strains), *S. haemolyticus* (2 strains), and *S. xylosus* (1 strain). The presence of staphylococcal enterotoxins (SEA, SEB, and SEC) was found in 17 milk samples of 133 examined ones.

From our results it follows that within 81 *Staphylococcus sp.* bacteria the production of SEC was recorded most frequently, then SEA, further SED, and SEE, or at combination of two types of enterotoxins simultaneously, combination of SEA+ SED was more frequent than that of SEC+SEE.

CONCLUSION

Based upon the data of three years occurrence of bacterial agents of mastitis in two herds of sheep it follows:

– *Staphylococcus sp.* bacteria had the main representation in the aetiology of sheep mastitis. Their occurrence in the sheep breeding with machine milking was 29.8 % (2004), 12.0 % (2005), and 26.4 % (2006), and in the breeding with hand milking 11.1 % (2004), 19.5 % (2005), and 14.8 % (2006);

– Proportion of the occurrence of *Staphylococcus aureus* bacteria and coagulase–negative staphylococci in the breeding with machine milking gradually changed in favour of coagulase–negative staphylococci (15,0 % : 14,8 %; 5,1 % : 6,9 %, and 5,1% : 21,3% in 2004, 2005, and 2006, respectively).

– During ~~the~~ observed period in the breeding with machine milking, environmental bacteria ranged as follows: *Streptococcus uberis* – 1,2 % – 3,2 %; *E. coli* – 0,0 % – 7,8 %; *Enterococcus* sp. – 0,2 % – 1,9 %, and in the breeding with hand milking: *Streptococcus uberis* – 1.5 % – 3.5 %, *Streptococcus dysgalactiae* – 0.8 % – 1.5 %, *Streptococcus agalactiae* – 0.7 % – 1.0 %, *E. coli* – 0.0 % – 0.3 %, *Bacillus* sp. – 0.0 % – 0.3 %;

– Of the number of 698 *Staphylococcus sp.* bacteria examined for production of enterotoxins 81 (11.6 %) produced some of 5 types (A, B, C, D a E) of enterotoxins. Production of staphylococcal enterotoxin of type C was recorded most frequently (in 47 *Staphylococcus sp.* bacteria). Production of the enterotoxin of type B was not recorded.

More enterotoxins simultaneously produced 2 species of staphylococci: A+D (2) and C+E (1). Coincident production of more types of SE was determined in 2 species of staphylococci – SEA+SED (*Staphylococcus aureus* – 8 and *Staphylococcus simulans* – 4) and SEC+SEE (*Staphylococcus simulans* – 1*)*. In the production of SE, *Staphylococcus aureus* (52), *Staphylococcus caprae* (17), *Staphylococcus simulans* (6), *Staphylococcus warneri* (4), *Staphylococcus epidermidis* (2) participated most frequently.

In conclusion it can be stated, that *Staphylococcus* sp. bacteria participate to a great extent in inflammations of the mammary gland of ewes and a substantial part of species can produce enterotoxins. This fact is significant in concern to health safety of sheep milk as raw material of sheep cheese and other products, and it is necessary to pay attention to it.

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