

PRODUCTION OF ENTEROTOXINS OF *STAPHYLOCOCCUS* SPP. ISOLATED FROM SAMPLES OF SHEEP MILK

František Zigo, Milan Vasil', Juraj Elečko, Zuzana Farkašová, Martin Lapin

ABSTRACT

In our study was followed occurrence of mastitis in herd of 430 sheep of breed zoslachtена valaska with hand milking technology examined two times during one lactation season. Individual examination consisted from clinical examination of udder and microbiological examination of milk samples. By PCR was determined presence of genes coding production of enterotoxins, and by ELISA methods production individual types of enterotoxins. From individual forms of mastitis were frequently detected subacute (6.7%), subclinical (5.7%) and acute (2.9%). The coagulase-negative staphylococci (CNS) were identified in 102 (65.4%) from all 156 positive isolates. The CNS and *S. aureus* caused subacute (5.1%), subclinical (3.9%) and acute (2.4%) forms of mastitis. The most frequently isolated were *S. epidermidis*, followed by *S. chromogenes* and *S. xylosus* from ewes with subacute and subclinical mastitis. From acute and chronic forms of mastitis were predominantly isolated *S. aureus*, *S. uberis* and *S. epidermidis*. The production of staphylococcal enterotoxins (SE) - SEA, SEB, SEC, SED and the presence of genes *sec* (3), *sea* (2), *seb* (2) and *sed* (2) were determined in *S. aureus*, *S. epidermidis*, *S. schleiferi* and *S. chromogenes*, respectively. The results suggested on the high occurrence (12.4%) of subacute and subclinical forms. Confirmed production of enterotoxins and presence of genes coding their production present a risk for human health and decreased a quality of milk and products from sheep's milk.

Keywords: sheep; hand milking; mastitis; pathogens, staphylococcal enterotoxins

INTRODUCTION

Mastitis has been considered an economically important disease in the production of sheep. The occurrence of mastitis in sheep is in interval from 4.0 to 50.0%. Staphylococci are the main aetiological agents of small ruminant intramammary infections (IMI), and *Staphylococcus aureus* is the most frequent isolate from clinical mastitis cases and coagulase-negative species are the most frequent in subclinical IMI. The annual incidence of clinical IMI in dairy sheep is generally lower than 5%, but in a small percentage of herds the incidence may exceed 30-50% of the animals, causing mortality (gangrenous mastitis) or culling of up to 70% of the herd (Fthenakis, 1994, 1995; Vautor et al., 2009).

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 (Contreras et al., 2007).

The important factor of virulence of *Staphylococcus* spp. besides to antibiotic resistance is production of enterotoxins, which showed high health risk for human. Milk and other dairy products are reported to be frequently associated with SE food poisoning. It was supposed that milk of infected animals constitute the main source of staphylococcal enterotoxigenicity of animal origin (Omoe et al., 2002; Scherrer et al., 2004).

During the many years the production of enterotoxins was connected only with *S. aureus*. Many authors report, that other species of CNS (*S. intermedius*, *S. hyicus*) may

producing of enterotoxins (Becker et al., 2001, Beatriz et al., 2006).

The aim of our study was the observed the occurrence of mastitis in herd of sheep with hand milking and determined of enterotoxigenic bacteria of *Staphylococcus* spp. isolated from milk samples during one milking season.

MATERIAL AND METHODOLOGY

Animals and milking

The experiment was carried out in herd of 430 sheep of breed zoslachtена valaska, which during winter season were stabled in bricked shed with deep bedding. During milking season (from April to September) was hand milking carrying out in cover strung cage with three fixation places two times per day.

Examination of health status of sheep and milk samples

Complex examination of health status of udder in sheep was carried out at the start (April) and at the end of milking (September). The clinical examination was carried out according to Hariharan et al. (2004) and milk from individual halves was evaluated by NK-test (Bioveta a.s., Inovice na Hané, Czech Republic) according to Fthenakis (1994).

Laboratory analyses

From the every individual milk sample were inoculated 0.05 ml, onto blood agar (Oxoid LTD, Hampshire, UK) and cultivated at 37°C for 24h. Based on the colony morphology, bacteria *Staphylococcus* spp. was selected for

the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspect colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriaceae* spp. were isolated on blood agar, cultivated at 37°C for 24h and identified biochemical using the STAPHYtest, STREPTOtest, ENTEROtest (Erba-Lachema, Brno, Czech Republic) and identification by software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic).

Identification of genes coding staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*) was carried out by PCR method according to Becker et al. (1998). DNA was isolated by QiAMP tissue kit (Qiagen, Hilden, Germany). For control were used reference strains for types of SE: SEA, SEB, SEC, SED, SEE (Bergdoll; CNCTC, Brno, Czech Republic). For the detection of genes was used oligonucleotide primers *sea* - *see* (Becker et al., 1998). Validation of production of SE *in vitro* in every strain was confirmed by ELISA method, by set Ridascreen® Set A, B, C, D, E (R-Biopharm AG, Darmstadt, Germany).

RESULTS

From total number of sheep (n = 820) in Figure 1, on the base of complete examination were determined 19.5% occurrence of mastitis (n = 160) during one milking season. The most frequently were found subacute (6.7%), subclinical (5.7%) and acute (2.9%) forms of mastitis in 126 sheep (15.4%).

The highest ratio had CNS, which occurrence was determined in 102 cases (12.4%), at which caused subacute (4.8%) and subclinical (3.8%) mastitis. From CNS, *S. epidermidis* (16.0%), *S. chromogenes* (11.9%), *S. simulans* (7.0%) and *S. schleiferi* (6.4%) predominantly caused subacute and subclinical forms of mastitis. Bacteria *S. aureus* (6.4%) and *S. uberis* (4.5%) were isolated from acute and subacute forms of mastitis (Table 1).

The presence of genes coding production of enterotoxins in bacteria *Staphylococcus* spp. (n = 115) described Table 2. Four strains of *S. epidermidis* showed presence of gene *sea* (1), *seb* (2) and *sed* (1), but by ELISA method was

confirmed only production SE types A (1) and B (1) in two strains of staphylococci. In *S. chromogenes* and *S. schleiferi* was determined presence of one gene *sec*, and gene *sed* in *S. schleiferi* was conjugated with production correspondent type of SE. Three strains of *S. aureus* was characterized by presence of genes *sea* (1) and *sec* (2), but the production of SEC was determined only in one strain.

DISCUSSION

How common mastitis is in sheep is extremely variable. Studies of cull ewes at slaughter in Britain show a very high prevalence ranging from 13 to 50%, indicating that clinical mastitis is likely an important cause of culling of ewes in the UK (Conington et al., 2008).

In our study we confirmed 19.5% occurrence of mastitis, predominantly subacute (6.7%), subclinical (5.7%) and acute (2.9%) forms caused by CNS, *S. aureus* and *S. uberis*. According to Ozenc et al. (2011), the general pathogens, which caused clinical mastitis in dairy sheep herds, are *Staphylococcus aureus* and *Streptococcus agalactiae*. Subclinical forms are refers to CNS, which often grows to subacute and acute forms. In compare with *S. aureus* have CNS lower frequency of virulence factors, however their representation in clinical forms of mastitis in sheep is becoming increasingly a problem in the holdings as confirmed in our study. From 156 isolated bacterial pathogens, 102 (12.4%) were represented by CNS, which caused subacute (4.8%) and subclinical (3.8%) forms of mastitis. Bacteria *S. aureus* (1.6%) and *S. uberis* (1.5%) were isolated predominantly from acute and subacute mastitis.

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 cases and CNS in subclinical). From the CNS is more frequently *S. epidermidis* what is also determined in our study. No less important bacterial pathogens are *Corynebacterium*

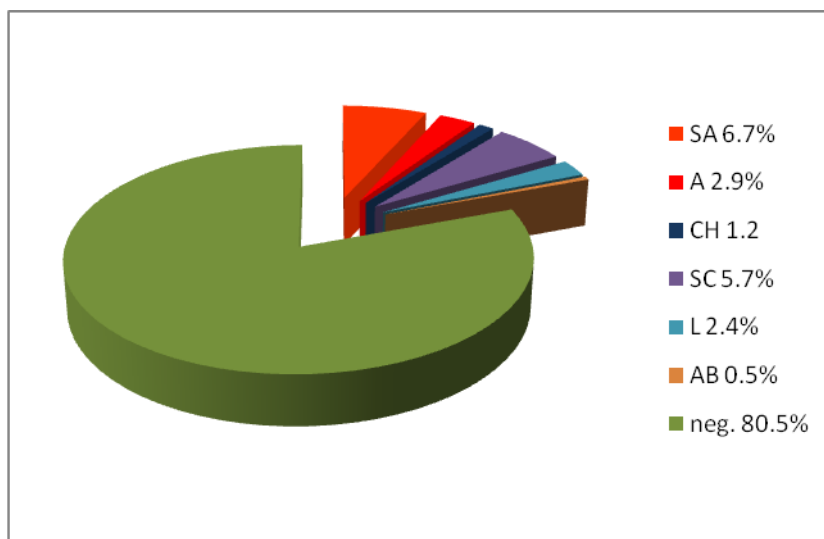


Figure 1 Occurrence of mastitis in herd of sheep during milking season

SA - subacute mastitis, A - acute mastitis, CH - chronic mastitis, SC - subclinical mastitis, L - latent mastitis, AB - abacterial mastitis, neg. - negative

spp., *Enterococcus* spp. and *Micrococcus* spp. (Bergonier et al., 2003; Berthelot et al., 2006; Ozenc et al., 2011).

Subclinical and subacute mastitis are thought to have a prevalence of between 10% and 30% in Lowland flocks in Southern England (Conington et al., 2008). From 430 sheep was determined 5.7% incidence of subclinical mastitis. Most frequently isolated were CNS, predominantly *S. epidermidis*, *S. chromogenes*, *S. simulans* and *S. schleiferi*.

Hariharan et al. (2004) showed 6.7% positive samples of milk from 492 examined sheep in which were isolated CNS. The most frequently isolated were *S. equorum*, *S. xylosus* and *S. simulans* from ewes with subclinical

mastitis during lactation.

Burriel (1997) reported that *S. simulans*, *S. xylosus*, *S. hyicus* were the predominant species of CNS in the milk from meat ewes, *S. epidermidis* and *S. schleiferi* was the predominant species in the milk of dairy ewes, what was also confirmed in our study. In addition to the high incidence of subclinical and subacute mastitis, which were caused by CNS and *S. aureus*, in some strains were determined production of SE.

The staphylococcal enterotoxins are recognised as being agents of intoxication such as staphylococcal food poisoning syndrome in man and they may be involved in other types of infections (Zschöck et al., 2005).

Table 1 Bacterial agents of ovine mastitis

Bacterial pathogens	Σ	%	Mastitis form									
			SA		A		CH		SC		L	
			n	%	n	%	n	%	n	%	n	%
<i>S. aureus</i>	13	1.6	3	1.9	7	4.5	2	1.3	1	0.6	-	-
<i>S. epidermidis</i>	37	4.5	14	9.0	7	4.5	2	1.3	11	7.1	3	1.9
<i>S. chromogenes</i>	22	2.7	8	5.1	3	1.9	2	1.3	7	4.5	2	1.3
<i>S. schleiferi</i>	17	2.1	6	3.8	2	1.3	1	0.6	4	2.6	4	2.6
<i>S. simulans</i>	14	1.7	5	3.2	1	0.6	-	-	6	3.8	2	1.3
<i>S. caprae</i>	7	0.9	4	2.6	-	-	-	-	1	0.6	2	1.3
<i>S. xylosus</i>	5	0.6	2	1.3	-	-	1	0.6	2	1.3	-	-
<i>Bacillus</i> spp.	9	1.1	3	1.9	1	0.6	-	-	4	2.6	1	0.6
<i>S. sanguinis</i>	3	0.4	2	1.3	-	-	-	-	1	0.6	-	-
<i>S. uberis</i>	12	1.5	4	2.6	3	1.9	-	-	4	2.6	1	0.6
<i>E. coli</i>	6	0.7	1	0.6	-	-	-	-	2	1.3	3	1.9
Others*	11	1.3	3	1.9	-	-	2	1.3	4	2.6	2	1.3
positive	156	19.0	55	6.7	24	2.9	10	1.3	47	5.7	20	2.4
negative	664	81.0										
Total	820	100.0										

Others* - *Arcanobacterium* spp., *Proteus* spp., *Corynebacterium* spp., *Enterococcus* spp., Σ - number of sheep, SA - subacute mastitis, A - acute mastitis, CH - chronic mastitis, SC - subclinical mastitis, L - latent mastitis

Table 2 Staphylococcal enterotoxins and genes coding production of SE in bacteria *Staphylococcus* spp. (n=15) in herd of sheep

<i>Staphylococcus</i> spp.	production of SE				presence of genes			
	SEA	SEB	SEC	SED	sea	seb	sec	sed
<i>S. aureus</i>	1		1		1		2	
<i>S. epidermidis</i>	1	1			1	2		1
<i>S. chromogenes</i>			1				1	
<i>S. schleiferi</i>				1				1
Total	2	1	2	1	2	2	3	2

Valle et al. (1990) tested 342 *Staphylococcus* spp. bacteria for their ability to produce enterotoxins, which were isolated from various parts of the body of small ruminants. Staphylococcal enterotoxins were produced by 74.3% of 70 coagulase-positive 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. From our results it follows that within 115 *Staphylococcus* spp. bacteria the production of SEC and SEA was recorded frequently, than SEB and SED, all the same as a frequently presence of *sec* gene.

The production of enterotoxins SEA, SEC and SED by *S. aureus* field strains isolated from mastitis animals has been investigated in several studies (Matsunaga et al., 1993; Zschöck et al., 2000). In our study presence of genes coding SE in species of CNS (*S. epidermidis*, *S. chromogenes*, *S. schleiferi*) was also certified.

CONCLUSION

By complex examination in herd of sheep we determined 12.4% occurrence of subacute and acute forms of mastitis caused predominantly by CNS, *S. aureus* and *S. uberis*. Very important is the early diagnosis of mastitis in sheep during the milking season. At the start of the treatment of subclinical mastitis showing the subclinical forms of mastitis can significantly eliminate clinical stage of subacute and acute forms of mastitis. In bacteria *S. aureus*, *S. epidermidis*, *S. schleiferi* and *S. chromogenes* were by PCR detected the presence of enterotoxins genes *sec* (3), *sea* (2), *seb* (2) and *sed* (2), as well as production of SE - SEA (2), SEC (2), SEB (1) and SED (1) by ELISA method. Because of the importance of these toxins for public health and food safety, an efficient screening for the prevalence of enterotoxigenic strains in mastitis is required.

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Contact address:

MVDr. František Zigo, University of Veterinary Medicine and Pharmacy in Košice, Department of Animal

Husbandry, Komenského 73, 041 81 Košice, Slovakia, Email: frantisek.zigo@uvlf.sk

Doc. MVDr. Milan Vasil', University of Veterinary Medicine and Pharmacy in Košice, Department of Animal Husbandry, Komenského 73, 041 81 Košice, Slovakia, E-mail: milan.vasil@uvlf.sk

MVDr. Juraj Elečko, CSc., University of Veterinary Medicine and Pharmacy in Košice, Department of Animal Husbandry, Komenského 73, 041 81 Košice, Slovakia, E-mail: juraj.elecko@uvlf.sk

MVDr. Zuzana Farkašová, PhD., University of Veterinary Medicine and Pharmacy in Košice, Department of Animal Husbandry, Komenského 73, 041 81 Košice, Slovakia, E-mail: zuzana.farkasova@uvlf.sk

MVDr. Martin Lapin, University of Veterinary Medicine and Pharmacy in Košice, Department of Animal Husbandry, Komenského 73, 041 81 Košice, Slovakia, E-mail: martin.lapin@student.uvlf.sk