MNK Publication

International Journal of Latest Research in Science and Technology **ISSN (Online):2278-5299**
Volume 3, Issue 6: Page No.99-102 (2014)
https://www.mnkjournals.com/journal/ijlrst/index.php

ANTIBIOTIC RESISTANCE AND PRODUCTION OF ENTEROTOXINS OF *STAPHYLOCOCCUS* SPP. ISOLATED FROM SAMPLES OF MILK IN DAIRY SHEEP

[1]František Zigo, [1]Milan Vasiľ, [1]Juraj Elečko, [1] Zuzana Farkašová, [1]Martina Chripková
[1]University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic
Corresponding Author Email: frantisek.zigo@uvlf.sk

Received : 02 December 2014; Accepted : 14 December 2014 ; Published : 31 December 2014

Abstract- ***Milk samples from 450 sheep of Lacaune breed with machine milking were collected two times during one lactation season. After microbiological examinations and in vitro test of resistance to antibiotics, by PCR was determined presence of genes coding production of enterotoxins, and by Elisa methods production individual types of enterotoxins. The results suggested on higher occurrence of subclinical (5.9 %) and subacute (4.8 %) forms of mastitis. The coagulase-negative staphylococci (CNS), and S. aureus were identified in 103 (70 %) from all 147 positive isolates. Besides to higher resistance to Oxacilin, Cloxacilin and Penicillin were determined in S. aureus, S. schleiferi, S. epidermidis and S. chromogenes the production of staphylococcal enterotoxins (SE) of type A, B, C and D.***

Keywords - Dairy sheep; Lacaune breed; Mastitis; Resistance; Staphylococcal enterotoxins

I. 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. Mastitis is an important part of sheep morbidity and the occurrence of intramammary infections (IMI) is in interval from 4.0 to 50.0 %. Staphylococci are the main aetiological agents of small ruminant IMI and *Staphylococcus aureus* with coagulase-negative species is the most frequent isolate from subclinical and clinical cases 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, 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).

Seeing that, many bacteria in recent years are becoming resistant to ß-lactamase antibiotics, macrolides and lincosamin, therefore were developed and put into practice methicilin and oxacilin as anti staphylococcal penicillins. After a certain period, there are strains of *S. aureus* resistant also to these antibiotics (Sawant et al., 2009).

The important factor of virulence of *Staphylococcus* spp. is production of enterotoxins, which showed high health risk for human. 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).

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 machine milking technology and determined the resistance of enterotoxigenic bacteria of *Staphylococcus* spp. isolated from milk samples during one milking season.

II. MATERIALS AND METHODS

Animals and milking

The experiment was carried out on herd of 450 sheep of Lacaune breed, which were housed in sheep stable from bricks with deep bedding during the winter period. Machine milking were performed in two-line milk parlour 2 x 14 Miele Melktechnik, (Hochreiter Landtechnik, Germany) two times per day during milking season (from April to September). The milk has been collected within the mobile tank of milk during milking, and milk after milking was transported for further processing.

Examination of health status of sheep and milk samples

Complex examination of health status of udder in sheep was carried out two times, 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, 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. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspect colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* 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).

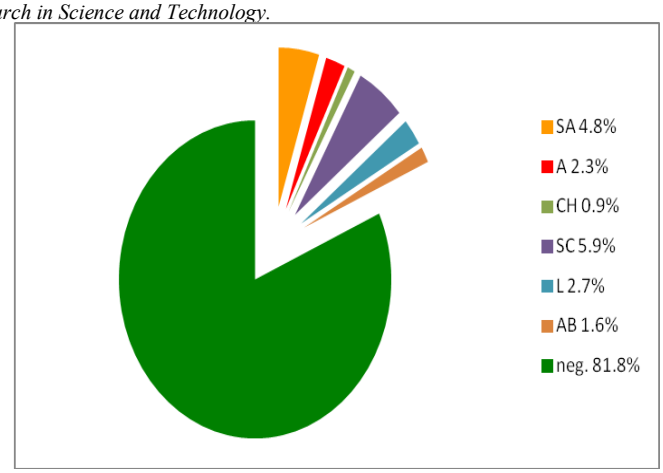
All identified *Staphylococcus* spp. isolated from milk samples were *in vitro* tested on Mueller-Hinton agar (HiMedia, Mumbai, India) by disc method after 24 h incubation at 37°C, on resistance to 13 types of antibiotics: Ampicillin (10 µg); Amoxycilin (25 µg); Cloxacilin (5 µg); Cefaperazone (30 µg); Erytromycin (10 µg); Linkomycin (15 µg); Neomycin (10 µg); Novobiocine (5 µg); Penicillin (10 U); Streptomycin (10 µg); Methicilin (10 µg); Oxacilin (5 µg); Cephalotin (30 µg) (Oxoid Ltd. Basingstoke, Hants, UK). The bacteria were assessed as resistant or sensitive by reference zone according to manual instruction of producer.

III. RESULTS

From total number of sheep (n = 883) in Figure 1. and Table 1, on the base of complete examination were determined 18.2 % occurrence of mastitis (n = 161) during one milking season. The most frequently were found subclinical (5.9 %), subacute (4.8 %) and latent (2.7 %) forms of mastitis in 118 sheep (13.4 %).

Acute forms formed 2.3 % (23 infected sheep). The highest ratio had CNS, which occurrence was determined in 86 cases (9.7 %), at which caused frequently subclinical (4.4 %) subacute (3.2 %) and latent (1.5 %) mastitis. From CNS, *S. chromogenes* (2.4 %), *S. schleiferi* (2.1.9 %) and *S. caprae* (1.1 %) were frequently isolated from positive samples of milk. Bacteria *S. aureus* (1.5 %) and *S. uberis* (1.3 %) were isolated from acute and subacute forms of mastitis.

Figure 1. Occurrence of mastitis[a] in herd of sheep during milking season



[a]SA – subacute mastitis, A – acute mastitis, CH – chronic mastitis, SC – subclinical mastitis, L – latent mastitis, AB – abacterial mastitis, neg. – negative

Table 1. Bacterial agents of ovine mastitis

Bacterial pathogens	∑	%	Mastitis form									
			SA		A		CH		SC		L	
			n	%	n	%	n	%	n	%	n	%
S. chromogenes	21	*2.4*	6	0.7	3	0.3	2	0.2	8	0.9	3	0.3
S. schleiferi	18	*2.1*	4	0.5	-	-	-	-	11	1.2	3	0.3
S. aureus	17	*1.9*	4	0.5	9	1.0	1	0.1	1	0.1	2	0.2
S. epidermidis	15	*1.7*	7	0.8	1	0.1	-	-	5	0.6	2	0.2
S. uberis	14	*1.6*	5	0.6	6	0.7	1	0.1	2	0.2	-	-
E. coli	11	*1.2*	2	0.2	1	0.1	-	-	4	0.5	4	0.5
S. caprae	10	*1.1*	6	0.7	-	-	-	-	2	0.2	2	0.2
E. faecalis	9	*1.0*	2	0.2	-	-	-	-	4	0.5	3	0.3
S. simulans	8	*0.9*	1	0.1	-	-	-	-	5	0.6	2	0.2
S. gallinarum	8	*0.9*	4	0.5	-	-	-	-	3	0.3	1	0.1
S. felis	6	*0.7*	-	-	-	-	1	0.1	5	0.6	-	-
Others[a]	10	*1.1*	2	0.2	1	0.1	3	0.3	2	0.2	2	0.2
positive	147	***16.6***	42	***4.8***	21	***2.3***	8	**0.9**	52	***5.9***	24	***2.7***
negative	736	***83.4***										
Total	883	***100.0***										

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

Resistance of *Staphylococcus* spp. (n = 103) isolated from sheep's milk is described in Table 2. Most strains were resistant to Amoxycilin (9), Ampicillin (8), Oxacilin (8) and Neomycin (8). Besides to one strain of *S. aureus*, showed all tested CNS 100 % sensitivity on Methicilin. In CNS high sensitivity was determined on Methicilin, Cefaperazone, Cephalotin and Novobiocine. In *S. aureus,* highest number of resistant strains (3) to Ampicilin, Amoxycilin and Oxacilin was recorded.

Table 2. Resistance to antibiotics in *Staphylococcus* spp. (n=103) isolated from milk samples of sheep

Staphylococcus spp.	n	Resistance to antibiotics												
		AP	AM	CL	OX	ER	LN	NE	NO	PE	ST	MT	CP	CE
S. chromogenes	21	2	2	2	2	2	2	1	1	2	1		1	
S. schleiferi	18	2	1	2	1			2	1	1	1			
S. aureus	17	3	3	1	3	2	2	2	2	1	1	1		
S. epidermidis	15	1	2	1	2		1	1		2				
S. caprae	10		1	1		1		1						
S. simulans	8										1			
S. gallinarum	8				1									1
S. felis	6							1						
Total	**103**	*8*	*9*	*7*	*8*	*5*	*5*	*8*	*4*	*6*	*4*	*1*	*1*	*1*

AP - Ampicillin (10 µg); AM - Amoxycilin (25 µg); CL - Cloxacilin (5 µg); OX - Oxacilin (5 µg); ER - Erytromycin (10 µg); LN - Linkomycin (15 µg); NE - Neomycin (10 µg); NO - Novobiocine (5 µg); PE - Penicillin (10 U); ST - Streptomycin (10 µg); MT - Methicilin (10 µg); CP - Cephalotin (30 µg), CE - Cefaperazone (30 µg)

The presence of genes coding production of enterotoxins in bacteria *Staphylococcus* spp. (n = 103) described Table 3. Four strains of *S. aureus* showed presence of gene *seb* (1), *sec* (1) and *sed* (2), but by ELISA method was confirmed only production SE types B (1) and D (1) in two strains of staphylococci. In *S. schleferi* was determined presence of three genes *sea* (1) and *seb* (2), but only in two strains were determined production of correspondent SE. In *S. epidermidis* showed presence of one gene *sec* as well as production of SE. The presence of gene *sea,* without production of enterotoxin was determined in one strain of *S. chromogenes*.

Table 3. Staphylococcal enterotoxins and genes coding production of SE in bacteria *Staphylococcus* spp. (n=14) in herd of sheep

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

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

According to Ozenc et al. (2011), the general pathogens, which caused clinical mastitis in dairy sheep herds, are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis*. 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 147 isolated bacterial pathogens, 86 (9.7 %), were represented by CNS, which caused subclinacal (4.4 %), and subacute (3.2 %) forms of mastitis. Bacteria *S. aureus* (1.5 %) and *S. uberis* (1.3 %) were isolated predominantly from acute and subacute mastitis.

Subclinical mastitis is thought to have a prevalence of between 10 % and 30 % in Lowland flocks in Southern England (Conington et al., 2008). From 450 sheep was determined 5.9 % incidence of subclinical mastitis. Most frequently isolated were CNS, predominantly *S. schleiferi* (1.2 %), *S. chromogenes* (0.9 %), *S. epidermidis*, *S. simulans* (0.6 %) and *S. felis* (0.6 %).

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

In addition to high occurrence in cases of subclinical mastitis were in CNS determined antibiotic resistance to Amoxycilin (6), Neomycin (6), Ampicillin (5), and Oxacilin (5).

Similar results of antibiotic resistance of *Staphylococcus* spp. from milk samples recorded Hisira et al. (2013). Bacteria *S. chromogenes*, *S. epidermidis* and *S. xylosus* were characterized by resistance to Ampicilin, Oxacilin, Penicillin, Amoxycilin and Neomycin. Low value of resistance was determined to Methicillin, Cefaperazone and to Tetracyclin.

In our study tested strains *S. aureus* and strains *S. chromogenes* demonstrated higher resistance to Amoxyciline, Ampiciline, and Cloxaciline and highest sensitivity on Methicillin and Cefaperazone. Results were consistent with a previous report from Poland, where strain of *S. aureus*, *S. epidermidis* and *S. chromogenes* were resistant to Ampicillin and Penicillin (Malinowski et al., 2009).

Besides the resistance to Ampicilin and Oxacilin in some strains of *S. aureus* and CNS were determined production of SE. The SE 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).

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 SE of type C, namely either alone (67.9 %) or in combination with other type of SE. From our results it follows that within 103 *Staphylococcus* spp. bacteria the production of SEB was recorded frequently, than SEA, SEC and SED, all the same as a frequently presence of *seb* gene.

The production of enterotoxins SEB, 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. schleiferi, S. epidermidis, S. chromogenes,*) was also certified.

V. CONCLUSION

By complex examination in herd of sheep we determined 7.1 % 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 forms of mastitis can significantly eliminate clinical stage of subacute and acute forms of mastitis.

Besides to higher antibiotic resistance to Amoxycilin, Ampicillin, Oxacilin and Neomycin in strains *S. aureus*, *S. schleiferi, S. epidermidis*, and *S. chromogenes* by PCR were detected the presence of enterotoxins genes *seb* (3), *sea* (2), s*ec* (2) and *sed* (2), as well as production of SE of type B (2), A (1), C (1), and D (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.

VI. ACKNOWLEDGMENT

This work was supported by project APVV-0679-10 and project VEGA-1-0449-13.

REFERENCES

[1] Beatriz, M., Borelli, E.G., Ferreira, I.C., Lacerda, D.A., Santos, L.S., Carmo, R.S., Dias, M., Crisolita, C., Silva, C.A. (2006). Enteroxigenic *Staphylococcus* spp. and other microbial contaminants during production of Canastra cheese, Brazil. *Braz. J. Microbiol*. 37(4):545-550.

[2] Becker, K., Keller, B., Von, E.C., Brück, M., Lubritz, G., Etienne, J., Peters, G. (2001). Enterotoxigenic potential of *Staphylococcus intermedius*. *Appl. Environ. Microbiol*. 67:5551-5557.

[3] Becker, K., Roth, R., Peters, G. (1998). Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for ammplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic syndrome toxin-1 gene. *J. Clin. Microbiol.* 36:2548-2553.

[4] Bergonier, D., Crémoux, R., Rupp, R., Lagriffoul, G. Erthelot, X. (2003). Mastitis of dairy small ruminants. *Vet. Res*. 34:689-716.

[5] Berthelot, X., Lagriffoul, G., Concordet, D., Barillet, F., Bergonier, D. (2006). Physiological and pathological thresholds of somatic cell counts in ewe milk. *Small Ruminant Research*. 62:27–31.

[6] Conington, J., Cao, G., Stott, A., Bůnger, L. (2008). Breeding for resistance to mastitis in United Kingdom sheep, a review and economic appraisal. *Veterinary Record*. 162:369-376.

[7] Contreras, D., Sierra, A., Sanchez, J. C., Corrales, J. C., Marco, M.J., Paape, C., Gonzalo, A. (2007). Mastitis in small ruminants. *Small Ruminant Research*, Special Issue: *Goat and Sheep Milk*. 68, (1–2):145–153.

[8] Fthenakis, G.C. (1994). Prevalence and aetiology of subclinical mastitis in ewes of southern Greece. *Small Rumin. Res*. 13:293–300.

[9] Fthenakis, G.C. (1995). California mastitis test and Whiteside test in diagnosis of subclinical mastitis of dairy ewes. *Small Ruminant Res. 16:*271–276.

[10] Hariharan, H., Donachie, W., Macaldowie, C., Keefe, G. (2004). Bacteriology and somatic cell counts in milk samples from ewes on a Scottish farm. *Can. J. Vet. Res*. July 68(3):188–192.

[11] Hisira, V., Farkašova, Z., Elečko, J., Mudroň, P. (2013). The occurrence of subclinical mastitis in dairy farming with deep litter. *Slovac Veterinary Journal*. 38:270-273.

[12] Malinowski, E., Lassa, H., Kłossowska, A., Smulski, S., Markiwicz, H., Kaczmarowski, M. (2006). Etiological agents of dairy cows' mastitis in western part of Poland. *Pol. Jour. Vet. Sci.* 9:191-194.

[13] Matsunaga, T., Kamata, S., Kakiichi, N., Uccida, A. (1993). Characteristics of Staphylococcus aureus isolated from peracute, acute and chronic bovine mastitis. *J. Vet. Med. Sci*. 55:297–300.

[14] Ozenc, E., Seker, E., Baki Acar, D., Birdane, M.K., Darbaz, I., Dogan, N. (2011). The Importance of *Staphylococci* and threshold value of somatic cell count for diagnosis of sub-clinical mastitis in pirlak sheep at mid-lactation. *Reprod. Dom. Anim.* ISSN 0936-6768. 46:970–974.

[15] Sawant, A.A., Gillespie, B.E., Oliver, S.P. (2009). Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Vet. Microbiol*. 134:73-81.

[16] Valle, J., Gomez, L.E., Piriz, S., Goyache, J., Orden, J.A., Vadilo, S. (1990). Enterotoxins production by Staphylococcal isolated from healthy goats. *Applied and Environmental Microbiology*. 56(5):1323-1326.

[17] Vautor, E., Cocckfield, J., Marechal Le, C., Le Loir, Y., Chevalier, M., Robinson, A.D., Thiery, R., Lindsay, J. 2009. Difference in virulence between *Staphylococcus aureus* isolates causing gangrenous mastitis versus subclinical mastitis in a dairy sheep flock. *Vet. Res*. 6:40 – 56.

[18] Zschöck, M., Botzler, D., Blöcher, S., Sommerhauser, J., Hamann, H.P. (2000). Detection of genes for enterotoxins (ent) and toxic shock syndrome toxin-1 (tst) in mammary isolates of Staphylococcus aureus by polymerase-chain-reaction. *Int. Dairy J*. 10:569–574.

[19] Zschöck, M., Kloppert, W., Wolter, H., Hamann, P., Lämmler, CH. (2005). Pattern of enterotoxin genes *seg*, *seh*, *sei* and *sej* positive *Staphylococcus aureus* isolated from bovine mastitis, *Veterinary Microbiology*. 108(3–4):243–249.