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OCCURRENCE OF MASTITIS AND THE DETECTION OF VIRULENCE FACTORS IN STAPHYLOCOCCI ISOLATED FROM MASTITIC EWES

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ABSTRACT

Milk and milk products are important global dietary products, consumed by more than 6 billion people worldwide. Despite the increasing level of zoohygienic provision of dairy farming, inflammation of the mammary gland - mastitis is still one of the main health problems. The aim of study was examination of the udder health with detection of contagious and environmental pathogens causing mastitis in 940 ewes from four dairy herds localized in east of Slovakia. Particularly, in isolated Staphylococci were determined the presence of selected virulence factors such as formation of hemolysis, gelatinase, biofilm, hydrolyze DNA, and resistance to antibiotics with detection of methicillin resistance gene – *mecA*. The results of the study indicate that, in addition to the major udder pathogens (*S. aureus*, *S. uberis*, and *S. agalactiae*) causing mastitis, non-aureus Staphylococci (NAS) is a major risk to ewes. NAS, such as *S. chromogenes*, *S. warneri*, and *S. xylosus* isolated from infected ewes with clinical and chronic mastitis, had the highest representation of virulence factors in comparison to less virulent strains. In addition, isolates of *S. aureus* and NAS showed 44.2% resistance to one or more antimicrobial classes from mastitic milk samples. The 22 isolates (25.6% of all isolated staphylococci) from ewes in which phenotypic resistance was confirmed to *β*-lactam antimicrobials were tested by PCR for methicillin resistance with the detection of the *mecA* gene. We can conclude that despite the increased resistance to *β*-lactam antimicrobials, the presence of the *mecA* gene was not confirmed in the tested staphylococci.

KEYWORDS: sheep, milking, mastitis, PCR, resistance.

INTRODUCTION

About 150 million families around the world are engaged in milk production however, inflammation of mammary gland - mastitis remains a major problem in dairy animals that affects quality and milk worldwide production.[1]

This disease is associated with pain and adversely affects animal health, welfare, milk quality and the economics of milk production. Direct and indirect losses caused by mastitis lead to economic losses. For direct losses we can include: treatment costs, discarded milk, labor time, fatalities and the associated costs with repeated cases of mastitis. Regarding indirect losses we can include: increased culling, decreased milk production, decreased milk quality, loss of premiums, pre-term drying-off, animal welfare aspects and other associated health problems.[2]

Due to their polyethological origin, infections of the mammary gland are most often caused by a complex of interactions among the host, environment, and infectious agents result in mastitis, one of the most frequent diseases of ewes. In comparison with most other animal diseases, mastitis differs by the fact that that several diverse kinds of bacteria can cause the infection. These pathogens are capable of invading the udder, multiplying there, and producing harmful, inflammation causing compounds.[3]

Up to this date, more than 137 different organisms have been recognized as causative agents of ruminants intramammary infection (IMI). They include bacteria, viruses, mycoplasma, yeasts, and algae, but bacteria have been identified as the principal causative agents of mastitis (95% of all IMI).[4]

In recent years, *S. aureus* and NAS belong to the most common microorganisms causing mastitis in dairy animals and health disorders among consumers of milk and dairy products. According to the World Health Organization, 420,000 lives are lost due to food poisoning; and *Staphylococcus* spp. is characterized as an important agent that can cause foodborne diseases. Poisoning occurs due to the ingestion of preformed enterotoxins in food. Symptoms include vomiting, diarrhea and cramps; and an outbreak could lead to a public health problem.[5,6]

The main complications associated with the treatment of *S. aureus* and NAS infection include the fact that many strains can cause this disease and increasing number of them becomes resistant to increasing range of antimicrobials (ATB) available for veterinary use. Increase in the occurrence of NAS on dairy farms was observed after decrease in the incidence of mastitis caused by the main pathogens; the causative NAS show increased resistance to common ATBs and disinfectants.[7] Compared to *S. aureus*, NAS usually exhibit lower number virulence factors. The essential factor of pathogenicity of NAS is biofilm formation that allows them to survive application of disinfectants and other sanitation procedures. Nascimento et al.[8] reported that the NAS (*S. epidermidis*, *S. saprophyticus*, *S. hominis*, and *S. aerletae*) isolated from mastitic ruminants, were resistant to the ATBs used to treat of cows during lactation and were able to produce some of the staphylococcal enterotoxins.

Especially, multiresistant strains of Staphylococci associated with resistance to more than one ATB are a serious risk to public health. Recent studies also suggest that multi resistant Staphylococci, especially to *β*-lactams ATBs indicates the presence of methicillin – resistant Staphylococci (MRS) that have been identified in raw milk and dairy products, including cheeses.[8 – 10]

In addition to the increased antibiotic resistance of Staphylococci, the authors Vasil et al.[5] confirmed biofilm formation and lysines in mastitic milk samples and considered them as important virulence factors involved in the development of CM. Previous research has linked Staphylococci and their virulence factors to the pathogenesis and clinical manifestations of mastitis. Therefore, the study was aimed at the etiology and determination of contagious and environmental udder pathogens in dairy ewes' herds. Particularly in isolated Staphylococci, the presence of selected virulence factors such as hemolysis, gelatinase, biofilm, hydrolyze DNA, and resistance to antimicrobials with detection of methicillin resistance gene - *mecA* and their effect on the severity of mastitis were determined.

MATERIAL AND METHOD

Monitored farms

The practical part of the study was carried out in four different sheeps' herds located in east of Slovakia with conventional (non-organic) farming. The farms were in herd sizes ranging from 200 to 400 animals and consisted of Improved Valachian, Lacaune, and Tsigai breeds. After their lambs were weaned in early April, the ewes were milked twice a day on each farm. In the same month, the ewes were on pasture during the day and received concentrates

in amounts of 200 g per day during milking. In first two herds, machine milking was performed using a two-line milk parlour 2×14 Miele Melktechnik, (Hochreiter Landtechnik, Germany) and in two other herds the sheep were milked in two-line milk parlour 2× 16 Alfa Laval Agri (Alfa Laval, Sweden). From all the monitored sheep farms, during first month of pasture (April), were investigated 940 animals: 220 ewes from first, 250 ewes from second, 270 ewes from third, and 200 ewes from the fourth herd.

Udder health examination

Complex examination of health status of udder in ewes was carried out at the start of milking season (April). The clinical examination such as swelling and presence of lesions or anatomical malformation was carried out according to Fthenakis [11] and milk from individual halves was evaluated by CMT. CMT scores were 0, +, ++ and +++ for "negative", "weak positive", "positive" or "strong positive", respectively. Emphasis has been placed on aseptic sampling and transport of individual halves milk samples intended for bacteriological examination. Subsequently, from 940 examined ewes, 756 animals had negative CMT score and from 184 animals with CMT score trace or 1-3 were taken of 12 mL mixed halves milk samples for laboratory analyses.

Bacteriological examinations were performed according to commonly accepted rules Holko et al.[12] Milk samples (0.05 ml) were inoculated onto blood agar (Oxoid, UK) and cultivated at 37 °C for 24 hour. Based on the colony morphology and by Gram staining, 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 24 hour and detailed identified biochemically using the STAPHY-test, STREPTO-test, resp. ENTERO-test and identification by software TNW Pro 7.0 (Erba-Lachema, CZ). The species of gram-negative rods were identified used by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) according to Gregova and Kmet.[13]

Health udder and individual forms of mastitis (subclinical, clinical and chronic) based on clinical signs, abnormal udder secretions, CMT scores, bacteriological examination and history of treatment, with positive culture result were classified according to Fthenakis.[11]

Detection of virulence factors in Staphylococci

Confirmed Staphylococci based on MALDI-TOF analysis wasexposed to deoxyribonuclease (DNase test) and to produce extracellular proteolytic enzymes (Gelatin hydrolysis test) according to Hiko.[14] The formation of biofilm was determined by phenotypic method by growth on Congo Red agar (CRA) according to Vasil' et al.[5]

The ability of Staphylococci to produce of hemolysins was also determined. According to Moraveji et al.[14] types of hemolysis were phenotypically characterized based on the lysis zone of each staphylococcal isolate on plates of blood agar base supplemented with 5% sheep blood after 24 and 48 h incubation at 37 °C.

The susceptibility of staphylococci isolated from sheeps' (n=86) infected milkt was tested in vitro against 14 antimicrobial agents. The susceptibility tests of isolates were carried out on Mueller Hinton agar using a standard disk diffusion procedure.[15] In the current study, antibiotic discs containing penicillin (PEN;10 µg), ampicillin (AMP; 10 µg), amoxicillin (AMC; 10 µg), amoxicillin+clavulanic acid (AXC; 20/10 µg), ceftiofur (CEF; µg), oxacillin (OXA; 1 µg), cefoxitin (CFX; 30 µg), ciprofloxacin (CPR; 5 µg), lincomycin (LNC; 15 µg), neomycin (NMC; 10 µg), novobiocin (NVB; 5 µg), rifaximin (RFX; 15 µg), streptomycin (STR; 10 µg), and tetracycline (TET; 30 µg) were used. The zone of inhibition was recorded in millimeters, and results were interpreted as previously described. The determined diameters of the respective inhibition zones were evaluated (susceptible, intermediate, resistant) according to CLSI breakpoints.[16] The choice of antimicrobials reflects the range contained in a number of intramammary products to treat mastitis, which are available in Slovakia.

Phenotypical positive Staphylococci (26 isolates from mastitic milk samples) based on their antimicrobial resistance to *β*-lactams antimicrobials were subjected to PCR to test for methicillin resistance according to Poulsen et al.[17] *S. aureus* CCM 4750 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a reference strain for PCR in this study.

Statistical Analysis

Data were entered into Microsoft Excel 2007® (Microsoft Corp., Redmond, USA) and analyzed using Excel, State 11, and SPSS version 20 (IBM Corp., Armonk, USA). The dependence of the production of virulence factors on the most frequently isolated

staphylococci from clinical, chronic and subclinical mastitis was statistically analyzed using the chi-squared test with the significance level $\alpha = 0.05$; critical value $\chi^2 = 1.824$ for testing Staphylococci isolated from mastitic ewes and testing value - G. Statistical independence between isolates with virulence factors and isolates without virulence factors within each species was confirmed when $G > \chi^2$; the independence was not statistically significant when testing values of $G < \chi^2$.

RESULTS AND DISCUSSION

The incidence of mastitis is extremely variable, of course, highly dependent on the lactation stage and health status of dairy animals.[18] In our study, we monitored the occurrence and etiology of mastitis in four sheeps' dairy farms at the start of milking season (April). Of the milk samples taken from 184 examined ewes based on the anamnesis and positive CMT score was identified as bacterial agents causing a clinical or subclinical mastitis in 155 samples (16.4%) and in 29 samples from examined ewes with positive CMT score were identified as negative or contaminated for presence of udder pathogens (Figure 1). Based on the clinical examination of the MG, assessment of CMT, and laboratory diagnosis of milk samples, the occurrence of CM in the monitored sheeps' dairy farms was 4.5%. The most common form of IMI in monitored ewes was subclinical mastitis with an incidence of 10.2%. The occurrence of chronic mastitis was 1.8% in monitored dairy herds.

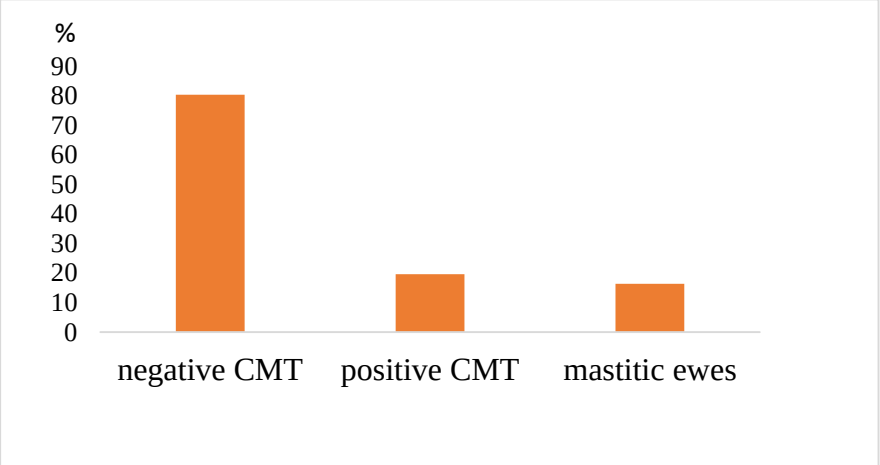

Figure 1: Evaluation of CMT from 940 monitored ewes.

To compare our results, Fthenakis[11] found the occurrence of mastitis in sheep is between 4 - 50%. The incidence of mastitis in our study at the beginning of the pasture season was 16.4% in monitored sheep's herds with the most frequently of subclinical form (11.5%). The

occurrence of CM was 4.9% which is considered an acceptable value. On the contrary, study from British slaughterhouses reported very high prevalence of CM ranging from 13 - 50%. This suggest that CM or chronic mastitis is an major cause of culling of ewes in the UK.[19]

Of the ewes' positive samples, 86 cases (55.4% of the infected samples) contained the most commonly isolated Staphylococci. The NAS represented the most commonly detected bacteria (39.9% of positive findings) causing mainly subclinical mastitis. The bacteria *S. aureus* were the second most abundant pathogen (18.2% of positive findings) causing mainly clinical or chronic mastitis, followed by *E. coli*, streptococci and enterococci (Table 1). Generally, IMI begins when pathogens pass through the teat canal, interact with the mammary tissue cells, multiply and disseminate in the cisterns and throughout the duct system. The manifestation of mastitis depends mainly on the degree of reaction of the udder tissue to injury or infection.[3] The most clinical cases caused by Staphylococci are manifested with increased body temperature, inappetence, redness, swelling and/or painful udder and/or abnormal milk. In the subclinical forms that were most often confirmed in our study, are no apparent clinical signs, but an increase in SCC is observed in milk.

Table 1: Udder pathogens isolated from milk samples of four monitored sheep herds.

Pathogens	Ewes		Clinical[1]		Subclinical		Chronic	
	n	%	n	%	n	%	n	%
NAS	59	*39.9*	9	*6.1*	44	*29.7*	6	*4.1*
S. aureus	27	*18.2*	11	*7.4*	9	*6.1*	6	*4.1*
Escherichia coli	18	*12.2*	7	*4.7*	10	*6.7*	1	*0.7*
Str. uberis	0	0	0	0	0	0	0	0
Str. agalactiae	4	*2.7*	4	*2.7*	0	0	0	0
Streptococcus sp.	9	*6.0*	1	*0.7*	6	*4.1*	2	*1.4*
Enterococcus sp.	24	*16.2*	5	*3.4*	17	*1.5*	2	1.4
Mixed infection	7	*4.7*	5	*3.4*	10	*6.7*	0	0
Total	155	*100*	42	*27.1*	96	*62.0*	17	*11.0*

Note: Clinical IMI[1] - clinical mastits represent mild, moderate or severe form of intramammary infection.

High detected incidence of Staphylococci in our results is consistent with the study Holko et al.[12] who recorded a high incidence of NAS and *S. aureus* isolated from infected milk samples during the examination of 42 dairy farms in the west of Slovakia. The NAS represented 35.9% of positive findings and were the most commonly detected bacteria. The authors confirmed high resistance to aminoglycosides and β-lactam antimicrobials without the presence of methicillin resistance genes, which confirmed our study.

In recent years, increasing studies have reported *S. haemolyticus*, *S. chromogenes*, *S. warneri* and *S. xylosus* as the dominant strains of NAS isolated from mastitis in dairy ruminants.[19 – 21] In addition to subclinical forms of IMI, NAS have been largely isolated from CM[20], which was confirmed in our study. The CM mastitis caused by NAS were associated with increased SCC, ability to form biofilm (Table 2) and resistance to aminoglycosides and *β*-lactam antimicrobials, especially to penicillin, amoxicillin, ampicillin and oxacillin (Figure 2).

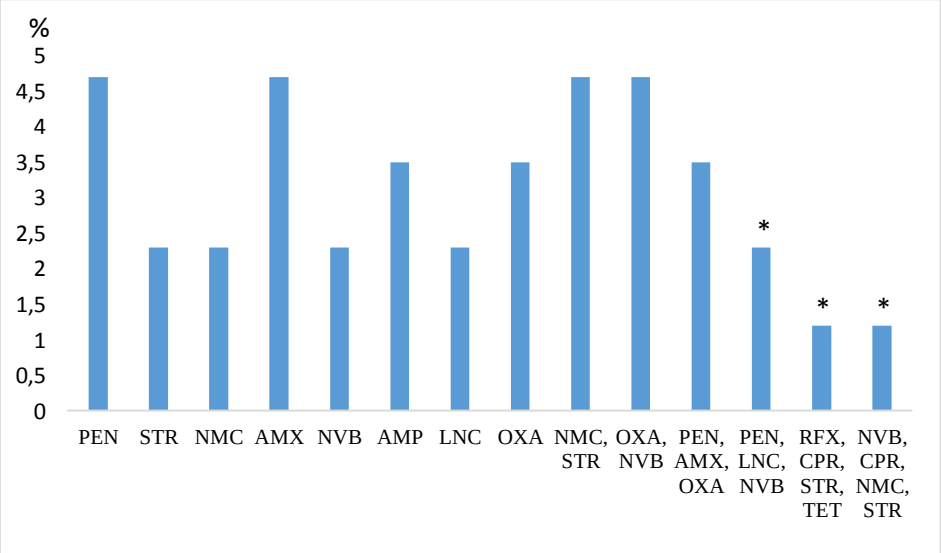

Figure 2: Phenotypic resistance profile in isolates of *Staphylococcus* spp. from mastitic ewes.

Note: *MDR - multi drug resistant isolates to three or more antimicrobial classes; AMX - amoxicillin, AMC - amoxicillin+clavulanat acid, AMP - ampicillin, CEP - cephalexin, CPR - ciprofloxacin, FOX - cefoxitin, LNC - lincomycin, NMC - neomycin, NVB -novobiocin, OXA - oxacillin, PEN - penicillin, RFX - rifaximin, STR - streptomycin, TET - tetracycline.

The increased incidence of staphylococcal infection in dairy ruminants also encourages the highest degree of pathogenicity in the production of more virulence factors, which are of crucial importance in persistent and CM cases.[21,22] Table 2 summarizes, in descending frequency, the isolated strains of Staphylococci and indicates their role in the type of mastitis and the occurrence of selected virulence factors. Isolated *S. aureus* from clinical, chronic or subclinical cases of mastitis has a highest ability to report virulence factors compared to NAS and showed hemolysis in blood plates, production of gelatinase, biofilm, and the ability to hydrolyze DNA. From mastitic ewes were isolated six species of NAS with the following recorded: *S. warneri* (23.7%), *S. chromo*genes (18.6%), *S. xylosus* (18.6%), *S. haemolyticus*

(15.2%), *S. caprae* (13.6%) and *S. epidermidis* (10.2%). From all mastitic samples caused with NAS, 26 (44.1%) cases involved the production of hemolysins, 11 (18.6%) the hydrolysis of DNA and 12 (20.3%) the production of gelatinase as well as 14 (23.7%) involved biofilm production. The significance level of $\alpha = 0.05$ was confirmed in the isolated Stafylococci *S. aureus*, *S. chromogenes* and *S. warneri* from CM and chronic cows' mastitis, which had the most numerous representation of virulence factors (production of hemolysins, gelatinase, the ability to hydrolyze DNA and biofilm) in comparison to less virulent strains. In addition, the presence of *mecA* gene has not been confirmed in tested *S. aureus* and NAS.

Table 2: The role of *S. aureus* and NAS in the form of mastitis from infected ewes and their virulence factors.

Staphylococcus *spp.*/number	IMI[1]/number	Hemolysins[2]	DNAse[3]	Gelatinase	Biofilm	*mecA* gene	Testing value
S. aureus (27)	clinical (11)	4α/2δ/2β	6	9	4	0	3.288*
	chronic (6)	2α/1β	3	6	4	0	
	subclinical (10)	4α/2β	4	8	3	0	
S. warneri (14)	clinical (3)	1α/1β	1	2	2	0	2.305*
	chronic (3)	2β	1	1	1	0	
	subclinical (8)	2α/2β/	3	4	3	0	
S. chromogenes (11)	clinical (2)	1β	0	0	1	0	1.824*
	chronic (2)	1β	1	1	1	0	
	subclinical (7)	3β/1δ	3	2	2	0	
S. xylosus (11)	clinical (1)	1β	1	0	0	0	1.140
	subclinical (10)	4α/2β	2	2	2	0	
S. haemolyticus (9)	clinical (2)	1β	0	0	0	0	0.435
	chronic (1)	0	0	0	1	0	
	subclinical (6)	3β	0	0	0	0	
S. caprae (8)	clinical (1)	1β	0	0	0	0	0.341
	subclinical (6)	0	0	0	0	0	
S. epidermidis (6)	subclinical (7)	0	0	0	1	0	0.215

Note: IMI[1] - number of isolates and their influence on type of mastitis; hemolysins[2] - production of hemolysin type α, β or δ; DNAse[3] - ability of staphylococci to hydrolyze DNA; *Chi-squared test significance level $\alpha = 0.05$; critical value $\chi^2 = 1.808$; Testing value (G) and statistical independence of virulence factors in isolated Staphylococci was confirmed when $G > \chi^2$; the independence was not statistically significant when testing value was $G < \chi^2$.

The WHO classified *S. aureus* as a high-priority pathogen, and it has gained most attention among the resistant Staphylococci.[23] However, methicillin resistance has also been described in several species of the NAS group.[17,18,21] which was not confirmed in our study.

In 86 isolates of Staphylococci from mastitic milk samples, *in vitro* resistance to 14 antimicrobials was tested by the standard disk diffusion method. Generally, low resistance was shown to tetracycline, amoxicillin reinforced with clavulanic acid, rifaximin and cephalexin. Of the tested Staphylococci, 38 isolates (44.2%) showed resistance to one or more antimicrobials. Multi-drug resistance to three or more antimicrobial classes were recorded in 4 isolates (4.7%). Tested Staphylococci showed multi-resistance to a combination of antimicrobial classes, such as aminoglycosides, β-lactams, macrolides and cephalosporins (Figure 2). Although our results showed a higher resistance of the tested staphylococci to *β*-lactam antimicrobials we can state that the presence of *mec A* gene has not been confirmed in monitored ewes.

CONCLUSION

Our study confirm that more than half of the IMIs were caused by Staphylococci, especially NAS, followed by *S. aureus* in monitored cows' and ewes' dairy farms. In addition to *S. aureus*, *S. chromogenes*, *S. warneri* and *S. xylosus* isolated from CM and chronic mastitis indicated a high degree of pathogenicity in the production of more virulence factors in comparison to other strains of NAS. Resistance to aminoglycoside and β-lactam antimicrobials were frequently detected in the tested Staphylococci, possibly because these are the antimicrobials most frequently used in the drying and mastitis treatment of dairy ruminants. Based on the phenotypic manifestation of antimicrobial resistance, detection of the presence of the *mecA* gene was confirmed in MRS (2.9%) in two isolates of *S. aureus* and two isolates of NAS (one isolate each of *S. chromogenes* and *S. warneri*) from mastitic cows. We can state that *S. aureus* still comes on top in the number of chronic or severe mastitis cases, as well as the number of virulence factors but some NAS species could have same aggressive potential based on their production of gelatinase, hemolysis, biofilm, hydrolyzed DNA and multi-drug resistance.

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