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MICROBIOLOGICAL STATUS OF THE MAMMARY GLAND IN LACTATING SHEEPS

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ABSTRACT

The aim of the present study was to determine the microbiological status of the mammary gland in lactating sheeps of the Lacon breed. For this purpose, milk samples from purebred animals, raised in sheep farm located in central region of Bulgaria, were collected. The results show that in 46.6% of the tested milk samples from the farm, were not isolated any pathogenic microorganisms. From the positive samples, S. xylosus was most often found, in most cases in combination with the non-pathogenic streptococcus L. lactis ssp. lactis. Only in one of the samples a combination of two staphylococcal species was found – S. epidermidis and S. xylosus, with the presence of L. lactis ssp. lactis. The number of samples with isolated bacteria with pathogenic potential was 16 (53.3%). Only in seven of them (23.3% of all examined) the amount of detected staphylococci is over 104 CFU/ml. No Gram-negative bacteria were isolated from any of the milk samples. The sensitivity of the isolated bacteria from milk samples to antibiotics from different groups is significant. Resistance has only been established to Colistin, Kanamycin, Amikacin and Gentamicin. The mean values of somatic cells in infected and uninfected halves were 1361058 ± 361307.34 and 171000 ± 36586.04, respectively.

Key words: sheep, mastitis, microorganisms, antibioticogram.

Introduction

Mastitis is one of the most common diseases in dairy farming. Identifying the causative agents and their antibiotic sensitivity is a important step in the treatment and prevention of the disease, as well as to reduce economic losses. Often isolated etiological agents of mastitis in dairy farming are microorganisms of the genus *Staphylococcus* and *Streptococcus*. From a number of studies, it is understood that the main causes of subclinical mastitis are Staphylococcus aureus and representatives of coagulase negative staphylococcus, such as *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus hyicus*, *Staphylococcus simulans*. The genus *Streptococcus* is most often represented by: *Streptococcus agalactiae*; *Streptococcus equinus (bovis)*; *Streptococcus acidominimus*; *Streptococcus equi zooepidemicus*; (Contreras, A et al; 2007; Mavrogianni, Gelasakis et al. 2011; Gelasakis et al. 2015; Queiroga, MC 2017). Other gram-positive bacteria associated with mastitis are: *Clostridium spp.* (Fotou et al., 2011), *Corynebacterium spp.* (Spanu et al., 2011), *Enterococcus spp.* (Marogna et al., 2010), *Listeria monocytogenes* (Brugere-Picox. 2008), *Micrococcus spp.* (Ariziznabarreta et al., 2002), *Mycobacterium spp.* (Nebbia et al., 2006), *Trueperella pyogenes* . (Saratsis et al., 1998;).

Gram-negative bacteria involved in the etiology of mastitis are Citrobacter spp., Escherichia coli, Enterobacter spp., Klebsiella spp., Pasturella multocida, Proteus spp., Pseudomonas aeruginosa (Leitner et al, 2007), Salmonella spp., Serratia spp. (Contreras et al, 2011), Yersinia pseudotuberculosis (Juste et al., 2009), and they represent about 3% of the isolated microorganisms (Bergonier et al., 2003).

The main use of antibiotics in productive livestock is for the treatment of mastitis, the results are not always satisfactory, they depend on the etiological agent and its sensitivity to antimicrobial substances, as well as the adequate functions of the defense mechanisms of the mammary gland

(Thomson K. et al. 2008). Detailed study of microorganisms and their sensitivity to various antibiotics will reduce losses in dairy farming.

The somatic cells in milk are mainly cells of the immune system, such as macrophages, lymphocytes and polymorphonuclear leukocytes. Exfoliated mammary epithelial cells are also classified as somatic cells. (Boutinaud, M., Jammes, H., 2002). In order for the number of somatic cells in milk to serve as a diagnostic method distinguishing healthy from inflamed mammary glands, standards for this indicator should be set. In the European Union, Regulation 853/2004 (EU, 2004) sets hygiene requirements for milk production and sets a limit on the number of somatic cells for cow's milk to 400,000 cells / ml, but no such legal limit has yet been set for the number of somatic cells. in milk from other animal species used to produce milk.

Materials and methods

1. Studied animals

All studied animals were bred for milk production and are of the Lacon breed. The farming system on the farm is intensive, in which the animals are kept entirely in a stable, without free grazing. The farm uses synchronization of the reproductive process and natural insemination with rams bred on site. The milking of the animals is machine in a milking parlor two or three times, depending on the lactation period. The test animals were not treated systemically or locally with antimicrobials for at least 30 days prior to sample collection.

2. Sample collection

We obtained milk samples aseptically from all halves, after examination with CMT (Kruuse, Denmark). Prior to sampling, the papillae and mammary gland were cleaned of mechanical contaminants, followed by dipping the tip with 70° alcohol. From each half, after removal of the first jets of milk, we took double samples in sterile 10 ml test tubes for microbiological examination and in 50 ml milk containers for somatic cell count and physicochemical analysis. The milk samples were transported to the laboratories in a cooler at a temperature of 4 ° C, and the examination of the same was carried out up to 8 hours after their collection.

3. Microbiological analysis of the samples

The microbiological study for isolation and identification of the microbial causative agents of mastitis in sheep in dairy samples was performed according to the accepted methodology for isolation and differentiation of mastitis causative agents.

To isolate microorganisms, cultures were made from milk samples on elective and selective nutrient media – Colorex Chromogenic Orientation Candida agar (HiMeida Laboratories Pvt. Ltd. Mumbai India), Chapman agar, Endo, EMB, Mueller – Hinton and Columbia blood agar. The results were reported after incubation under aerobic conditions at 37°C for 48 – 72 hours.

Taxonomic identification of all isolates was performed by conventional methods according to the 9th edition of the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). For this purpose, microscopic studies of stained by the classical methods of Gram, Pfeiffer, Klet and Moeller, consideration of the cultural and hemolytic properties on solid and liquid media and biochemical characteristics with the help of Polymicrotest was made. As well as additional samples for oxidase, catalase, etc. with reagents from Antisel (Sharlau Chemie S. A., Spain).

Quantitative determination of microorganisms was performed by counting the developed colonies, determining their arithmetic mean and calculating the amount of colony forming units (CFU) in 1 ml of starting material.

4. Physicochemical and cytological analysis for differentiation of subclinical mastitis

Cell counts were indirectly determined by the rapid mastitis tests CMT-Test (Kruuse, Denmark). The results from the CMT-Test were interpreted based on the Smith and Sherman (2009) scale.

The physicochemical and cytological analysis was performed in the "National Reference Laboratory for Milk and Dairy Products at the RVS" – Sofia. Somatic cell levels were determined by the fluoro-opto-electron counting method according to standard EN ISO 13366-2 / IDF 148-2: 2006 using Fossomatic (Foss, Denmark). For the purpose of this study, the mammary half is considered to be affected by subclinical mastitis when there are no clinical signs or altered milk, but the laboratory examination reveals the presence of pathogenic microorganisms and somatic cell count ≥ 500,000 cells / ml.

5. Antimicrobial agents and determination of isolates sensitivity

Determination of antimicrobial sensitivity of all clinical isolates was performed by the classical agar-gel diffusion method of Bauer et al. (1966). Standard disks for antibioticograms (Bul-Bio – Sofia) were used, as well as prepared by us, after inoculation of bacterial suspensions in exponential growth phase with a concentration of 2.106 cells / ml, determined by the Mac Farland optical standard, on blood agar (Bul-Bio – Sofia) or Mueller – Hinton agar (Antisel – Sharlau Chemie S. A., Spain). Cultivation was performed at 37°C for 24 hours. The results were interpreted according to the three-step system of Bauer et al. (1966) after measuring the diameters of the inhibitor zones in millimeters. Determination of minimum inhibitory concentrations (MIC). The determination of MIC was performed using MICROLATEST MIC G +, designed for Gram-positive bacteria. The studies were performed according to the test instructions by instilling 0.1 ml of a suspension with a concentration of 0.5 McFarland in each well of the 96-well plates loaded with different doubling concentrations of 12 antibiotics from different groups. The results were reported after incubation at 37°C for 24 h. Non-growth wells with the lowest concentration of test antibiotic were determined for MIC.

6. Statistical analysis

We performed statistical analysis on the one-way analysis of variance (ANOVA), followed by the Dunnett post-hoc test. The classical Student-Fisher method and the T-test of SPSS 19.0 were also used.

Results

A rapid mastitis test revealed that 18 (60%) of the samples tested negative. A result (+) was detected in 9 (30%) of the samples obtained, and a result of (++) in 3 (10%). Figure 1 shows the CMT results of all samples.

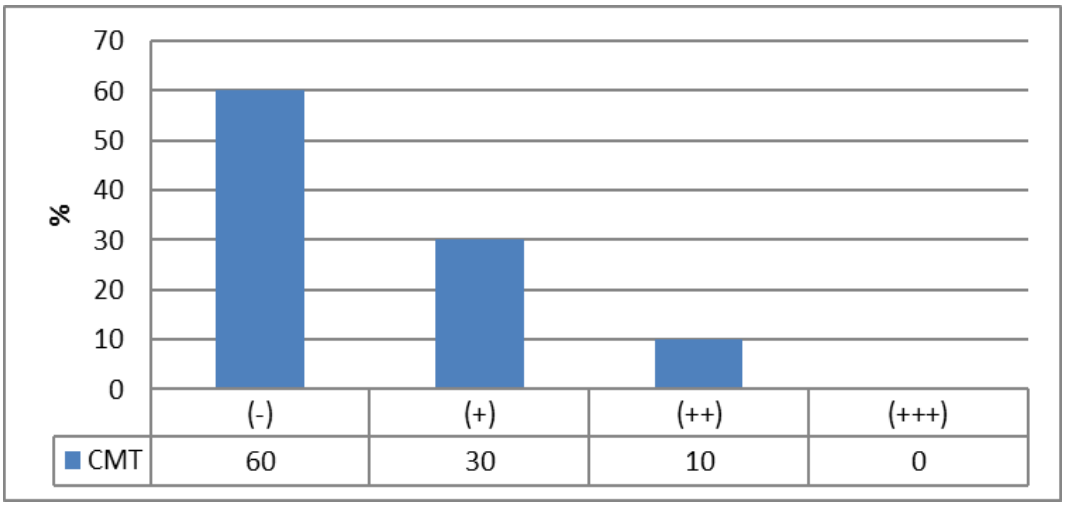

Figure 1: Results of the study conducted with CMT

To accurately identify the halves affected by subclinical mastitis, all collected samples were subjected to a complete physicochemical and cytological analysis in a reference laboratory.

The average values of the individual parameters are presented in Table 1.

Table 1: Physico-chemical and cytological analysis of milk samples

Parameter		Infected	Non-infected
Fat %	**min**	4,42	4,38
	max	7,42	6,18
	±SE	5,42±0,20	5,21±0,14
Protein %	**min**	5,42	5,17
	max	7,16	6,83
	±SE	6,14±0,13	6,27±0,12
TS %	**min**	15,55	15,03
	max	18,87	17,97
	±SE	16,75±0,22	16,81±0,21
SNF %	**min**	10,77	10,54
	max	12,3	12,51
	±SE	11,25±0,10	11,59±0,16
FPD ^{0}C	**min**	-0,56	-0,58
	max	-0,53	-0,54
	±SE	,548 ± 0,002	0,552±0,003
SCC cells/ml	**min**	430000	46000
	max	6266000	441000
	±SE	1361058±361307,34*	171000±36586,04*

The physicochemical analysis shows that there are no significant differences in the levels of fat, protein, total solids, solids non-fat and freezing point depression of milk samples obtained from infected and uninfected animals. The most significant differences are observed in the number of somatic cells in milk from affected and unaffected milk halves.

Figure 2 shows the type and percentage of isolated pathogenic microorganisms from the tested samples.

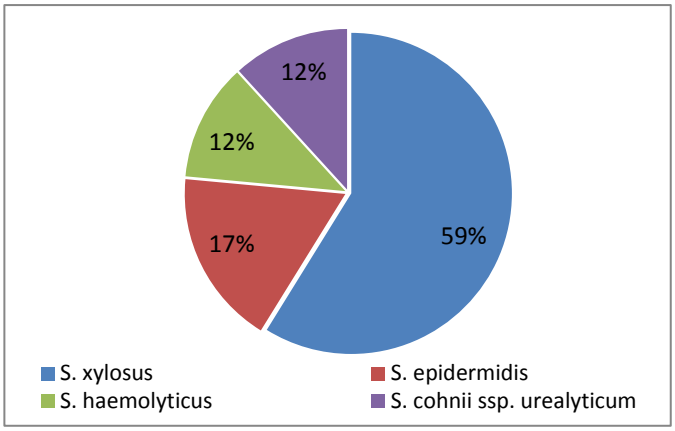

Figure 2: Type and percentage of isolated pathogenic microorganisms from the tested samples.

The results show that in 46.6% of the tested milk samples no microorganisms were isolated. In other samples, *S. xylosus* is most often isoleted, in most cases in combination with the non-pathogenic streptococcus *Lactococcus lactis ssp. lactis*. In only three of the samples were detected staphylococci not in combination with non-pathogenic lactococci. *S. haemolyticus* was found in only two of the samples, in another two – *S. cohnii ssp. urealyticum* and in three more – *S. epidermidis*. Only in one of the samples a combination of two staphylococcal species was found – *S. epidermidis* and *S. xylosus*, together with *L. lactis ssp. lactis*. From two of the milk samples with bacterial microflora were isolated only non-pathogenic bacteria – *Lactococcus lactis ssp. lactis* of one and *Micrococcus sp*. together with *Lactococcus lactis ssp. lactis* – on the other.

The number of samples with isolated bacteria with pathogenic potential was 16 (53.3%). Only in seven of them (7 – 23.3% of all studied) the amount of detected staphylococci is over 10^4 CFU/ml.

The summary results of the determination of the sensitivity of isolated bacteria of all identified species to antimicrobial agents in vitro are presented in Table 2.

Table 2: Susceptibility of isolated bacteria to antimicrobial agents in vitro

Antibiotic agent	Disc concetration µg/disc	S. xylosus	S. epidermidis	S. haemolyticus	S. cohnii ssp urealyticum
Cloramphenicol	30 µg	S	S	S	S
Doxycycline	30 µg	S	S	S	S
Clindamycin	10 µg	S	-	S	S
Penicillin	10 u	S	S	I	S
Oxacillin	1 µg	S	S	S	S
Ampicillin	10 µg	S	S	S	S
Amoxycillin	10 µg	S	S	I	S
Cefuroxime	30 µg	S	S	S	I
Ceftriaxone	30 µg	S	S	S	I
Colistin	10 µg	R	R	R	-
Novobiocin	30 µg	S	S	S	S
Gentamicin	10 µg	S	S	I	R
Kanamycin	5 µg	R	R	R	R
Amikacin	30 µg	S	S	R	S
Ciprofloxacin	5 µg	S	S	S	S
Enrofloxacin	5 µg	S	S	S	S
Sulfamethoxazole+Trimetoprim	23,75/1,25 µg	S	S	S	S

S – sensitive; I – intermediate; R – resistant

As the data in the table show, the sensitivity of the isolated bacteria to antibiotics from different groups is significant. Resistance has only been established to Colistin, Kanamycin, Amikacin and Gentamicin. S. haemolyticus shows intermediate sensitivity to Amoxycillin and Penicillin and S. cohnii ssp urealyticum to Cefuroxime and Ceftriaxone.

The results obtained in determining the MIC of antibiotics from different groups to the most commonly isolated microorganisms (S. xylosus) are presented in Table 3.

Table 3: Minimum inhibitory concentrations of antibiotics from different groups to S. xylosus

Antibiotic agent	MIC	Sensitivity
Penicillin	0.09±0.03	S
Ampicillin	0.34±0.38	S
Erythromycin	0.19±0.06	S
Clindamycin	0.44±0.36	S
Linezolid	1.22±1.61	S
Chloramphenicol	3.06±2.94	S
Tetracycline	1.06±0.62	S
Sulfamethoxazole+Trimethoprim	0.03±0.00	S
Gentamicin	1.25±1.59	S
Vancomycin	1.03±0.66	S
Teicoplanin	0.53±0.31	S
Nitrofurantoin	3.75±2.68	S

S – sensitive; I – intermediate; R – resistant

As can be seen from the presented data, they correspond to the results obtained by the disk agar-gel diffusion method. Isolated bacteria show sensitivity to all tested antibiotics. It is highest to the synergistic combination Sulfamethoxazole + Trimethoprim, as well as to Renicillin G, Erythromycin and Clindamycin.

Discussion

Our study reveals that the positive results of the CMT (40%) are significantly close to the positive results of the performed microbiological examination, where microorganisms with pathogenic potential are detected in 53.6% of the samples. The difference of 13.6% is probably due to the fact that milk from this samples is obtained from mammary glands in the initial stage of mastitis development.

Comparing the results of microbiological studies of the milk samples from our study with the studies of Gelasakis et al; (2015) and Queiroga (2017) we found significant similarity in isolated species of microorganisms. These are mainly species of the genus Staphylococcus, as well as enterococci. Coagulase-negative staphylococci predominate in the studied farm. These results show the exchange of bacteria between the animals on the farms and hence the similarity in their microflora. Data from our previous studies show a similarity of the microflora found in subclinical mastitis in goats (Hristov et al., 2016). However, Gram-negative bacteria were also isolated from them, in contrast to dairy samples from sheep. Also, the percentage of milk samples from the studied goats without isolated microorganisms (17.5%) is significantly lower than that of the sheep we tested (40 – 53%).

In this study, we isolated considered as safe for humans and animals Lactococcus lactis ssp. lactis. This specie is involved in the production of dairy foods for humans. It is possible that it locally

plays the role of a probiotic – an antagonist of the pathogenic microflora, preventing the proliferation of staphylococci and the development of disease. Rodrigues et al. (2016), however, isolated it from cows with mastitis and considered it as a potential etiological agent of the disease.

The number of somatic cells in milk from infected and uninfected halves shows significant differences, with the number of somatic cells increasing significantly in the presence of infectious agents. The results of somatic cell counts confirm the theory of Tvarožková et al., (2020) that the increase in somatic cells in sheep's milk is caused by the presence of pathogenic microorganisms in the mammary gland. The statement that in the presence of subclinical mastitis the number of somatic cells increases above 500,000 cells / ml is also confirmed.

The results of our antibioticograms compared to the results obtained by Katheryne et al. (2016) show some differences. Their study shows that the most common microorganisms show resistance to penicillins (17% of samples), while our results show the highest resistance to Kanamycin and Colistin, and relatively high sensitivity to penicillin antibiotics. Sylejmani et al, (2015) found significant resistance to penicillin antibiotics and the highest sensitivity of the CNS to trimethoprim, and the last result coincides with ours. The study also confirms the results of Ahmad et al .; (2013), who concluded that S. haemolyticus shows the greatest resistance to different types of antibiotics.

Conclusion

As potential causes of subclinical mastitis in sheeps have been isolated CNS (S. epidermidis, S. xylosus and S. haemolyticus) and enterococci. Gram-negative bacteria have not been identified.

In most of the tested samples, the results of the microbiological tests are in accordance with those of the CMT.

Isolated bacteria show in vitro sensitivity to antimicrobials from different groups. Resistance has only been established to Colistin, Kanamycin, Amikacin and Gentamicin.

With the development of subclinical mastitis the number of somatic cells in the milk increases over 500,000

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