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# DETERMINATION OF CAUSATIVE AGENTS OF SUBCLINICAL MASTITIS IN SHEEP IN BULGARIA

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## ABSTRACT

Subclinical mastitis is a disease causing significant losses in dairy sheep farming, due to reduction in the quantity and quality of the milk. The main reason for its occurrence are microorganisms of various genera. The aim of the present study was to identify the actual causative agents of subclinical mastitis in lactating sheep in Bulgaria. For this purpose, 156 milk samples were obtained and processed. In 76 (48.76%) of which we found the presence of microorganisms and an increase in the number of somatic cells. The results showed that a broad spectrum of bacterial agent**s** from different genera, (*Staphylococcus, Streptococcus, Bacillus, Enterococcus, Aerococcus, Dermaococcus, Macrococcus, Micrococcus)* and fungi from the genus *Candida,* participate within the etiology of the disease. The most frequently isolated bacteria with pathogenic potential were representatives of coagulase–negative staphylococci (CNS). We characterized 10 different species from them. The results showed the most prevalent were *Staphylococcus xylosus* and *Staphylococcus epidermidis*.

**Key words:** subclinical, mastitis, microorganisms, sheep, Bulgaria.

## Introduction

Mastitis is an inflammation of the mammary gland, characterized by pathological changes within the parenchyma, duct system and interstitial connective tissue, which change the composition and qualities of the milk (De Olive *et al.*, 2013). Subclinical mastitis is characterized by the presence of intramammary infection without clinical signs. This form is accompanied by an increase in the somatic cell count (SCC) in the milk and the presence of pathogenic microorganisms (White and Hinckley, 1999). Subclinical mastitis is a disease causing significant economic losses related to reduced milk production, reduced milk quality and animal treatment costs (Vasileiou *et al.*, 2018).

The main etiological agent of mastitis are various pathogenic microorganisms. A number of them are associated with clinical and subclinical mastitis. In dairy sheep, the most common causes of subclinical mastitis are coagulase negative staphylococci (CNS) and *Staphylococcus aureus* (Bergonier *et al.*, 2003; Vasileiou *et al.*, 2019; Knuth *et al.* 2022). CNS are pathogens of lower virulence that are more commonly associated with subclinical mastitis (Bonnefont *et al.*, 2011), but they can also cause a clinical form of the disease (Fthenakis and Jones, 1990). In Bulgaria, Koleva (1998) found that the most common causative agents of mastitis in sheep are microorganisms from the genera *Staphylococcus* (72.08%) and less commonly from the genera *Streptococcus, Corynebacterium, Escherichia, Pseudomonas*. More recent studies describe the involvement of microorganisms from the genera *Bacillus, Micrococcus, Mannheimia, Trueperella, Aerococcus* and *Lactococcus* (Vasileiou *et al.*, 2019; Knuth *et al.*, 2021).

Knowledge of the etiological agent and its characteristics is a key point for the treatment and prevention of mastitis in sheep.

## Materials and methods

### *Sampling*

The sampling was carried out in 5 sheep farms located in 4 administrative regions of Bulgaria. Seventy–eight sheep were included in the study and each milk half was tested (156 milk samples). The studied animals were from the breeds Assaf, Tsigai, Lacaune and Synthetic population of Bulgarian dairy sheep. The age of the animals varied in the range of 2 to 6 years. All sheep were clinically healthy at the time of the sampling. The sampling date, location, breed, age and clinical status of all sheep were documented. The sampling was done during the lactation period between weeks 8 and 10. We obtained the milk samples aseptically from all milk halves. Each sample was taken after machanical cleaning of the papillae and mammary gland, followed by douching of tip of each teat with 70˚ alcohol. The milk samples were collect in sterile 50 ml containers and stored and transported at 4°C.

### *Mastitis tests*

The condition of the udder halves of each animal was examined for subclinical mastitis directly at the farm, using the rapid mastitis tests CMT–Test (Kruuse, Denmark). Direct determination of the somatic cell counts was done by the BDS EN ISO 13366–2/IDF 148– 2:2006 standard by using Fossomatic (Foss, Denmark) at the National Reference Laboratory for Milk and Dairy Products of the Regional Food Safety Directorate, Sofia.

### *Isolation of microorganisms from milk samples*

The microorganisms isolation were done by culturing on selective nutrient media – Colorex Chromogenic Orientation agar, Candida agar (HiMeida Laboratories Pvt. Ltd. Mumbai India), Mueller – Hinton agar and Columbia blood agar (BB–NCIPD ltd – Sofia Bulgaria).

### *Taxonomic identification*

Taxonomic identification of all clinical isolates was performed by conventional methods according to 9th edition of Bergey's Manual (Guerrero, 2001). The identification of the isolated microorganisms was carried out by microscopic examination of preparations stained by different methods, determination of cultural and hemolytic properties on solid and in liquid media and biochemical features using Polymicrotest (BB – NCIPD). Tests for biochemical identification of staphylococci and enterococci (HiMeida Laboratories Pvt. Ltd. Mumbai India) and additional samples for oxidase and catalase were used. The identification of the species and subspecies of the microorganisms was carried out by means of biochemical tests – STAPHYtest 24, STREPTOtest 24, ENTEROtest 24 и CANDIDAtest (MIKROLATEST, Erba Mannheim).

## Results

The results showed the presence of microorganisms and an increase in the number of somatic cells above 500,000 cells/ml in 48.72% (76/156) of milk samples, while in 51.28% (80/156) of the samples no such were found. In 68.42% (52/76) of positive samples 1 microorganism was isolated (monocultures), while in 31.58% (24/76) more than 1 species was found. The milk samples with two determined microorganisms, showed a combination between the non–pathogenic *Lactococcus lactis ssp. lactis* and representatives of the genus *Staphylococcus*. Thus, the number of isolates with pathogenic potential was 86. Most of the pathogenic isolates were representatives of the genus *Staphylococcus* – 63.95% (55/86), of which *Staphylococcus aureus ssp. aureus* – 13.95% (12/86) and representatives of goagulase–negative staphylococci (CNS) –50% (43/86).The most frequently isolated representative of the CNS was *Staphylococcus xylosus* – 17.44% (15/86) of the isolates, followed by *Staphylococcus epidermidis* – 12.79% (11/86) and *Staphylococcus chromogenes* – 5.81% (5/86), the remaining representatives occupied relatively small partitions.

From all detected pathogenic microorganisms, those of the genus *Bacillus* represented 10.47% (9/86) of all pathogens. The remaining isolated bacteria were from the genera *Enterococcus* – 5,81% (5/86), *Aerococcus* – 4,65% (4/86), *Dermatococcus* – 4,65% (4/86), *Streptococcus* – 4,65% (4/86), *Macrococcus* – 1,16% (1/86) and *Micrococcus* – 1,16% (1/86). Two of the milk samples we found representatives of the genus *Candida*, and in one of them the gram–negative *Edwardsiella tarda* in combination with *Staphylococcus chromogenes*. Figure 1 shows the percentage ratio of isolates with pathogenic potential.

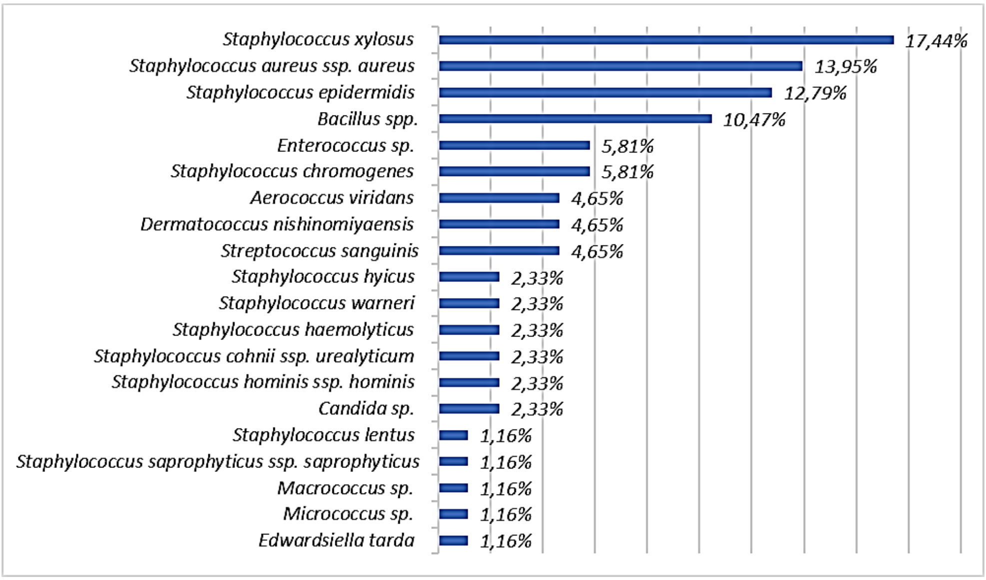

**Figure 1: Percentage ratio of isolates with pathogenic potential**

## Discussion

*Staphylococcus spp*. are gram positive, catalase–positive, facultative anaerobic cocci. They are opportunistic pathogens and inhabit mammals and birds, colonizing on the skin, mucosal surfaces, upper gastrointestinal and respiratory tract as well as the urogenital tract (Shoen *et al.* 2019). Our analysis revealed that representatives of this genus are the main causative agents of subclinical mastitis in sheep. The majority of them are representatives from the CNS group, followed by *Staphylococcus aureus*. These results proved the involvement of the CNS in the etiology of subclinical mastitis, are supported also by other authors (Bergonier *et al.*, 2003; Mork *et al.*, 2005; Contreras *et al.*, 2007, Kern *et al.*, 2013; Queiroga, 2017; Vasileiou *et al.*, 2019, Abed et. al., 2022). Our results are almost identical to those of Koleva (1998), who found that staphylococci represented 72.08% of the causative agents of mastitis in sheep. She concluded that *S. epidermidis* is a major representative of the CNS responsible for the development of the disease, which is in agreement with our results. Similarities between the two studies were also found in the percentage ratio of *S. aureus* and Gram–negative bacteria. In our country, Hristov (2014) also found a predominance of coagulase–negative staphylococci, compared to other bacteria, in his study conducted on goats. The high proportion of staphylococci recorded in our study means that this pathogens continues to be extensively responsible for mastitis problems in Bulgarian dairy herds.

Coagulase–negative staphylococci have long been regarded as apathogenic but their important role as pathogens and their increasing incidence have been recognized and studied in recent years. Although specific virulence factors are not as clearly established, it seems clear that factors such as bacterial polysaccharide components are involved in attachment and/or persistence of bacteria on foreign materials. (Huebner *et al.* 1999) It is known that CNS are opportunistic bacteria that can adhere to metal devices and are able to produce a protective biofilm (El–Jakee *et al.*, 2013). The ability to produce biofilm enables CNS to persist on milking equipment as well as on the milker's hands, which can serves a major source of staphylococcal spread and their high prevalence in affected animals. The two most frequently isolated by us CNS representatives were *Staphylococcus xylosus* and *Staphylococcus epidermidis*. A number of authors report that these two microorganisms are common representatives of the CNS, and the second as the most frequently detected (Las Heras *et al.*, 1999; Onni *et al.*, 2010; Queiroga, 2017, Abed et. al. 2022). In our study, we found that *Staphylococcus xylosus* represented 17.44% of all pathogenic bacteria, in other studies this percentage varied between 5 and 16.3%. Our analysis showed that *Staphylococcus epidermidis* accounted for 12.79% of the pathogens isolated. From the results of the other mentioned authors, a variation between 25% and 50%, is noticeable. The least frequently isolated were *Staphylococcus lentus* and *Staphylococcus saprophyticus ssp. saprophyticus*. This result also correlates with the results obtained by Queiroga (2017) and Abed et. al. (2022)..

From the microbiological analysis, it is clear that *Staphylococcus aureus* ranks second among pathogenic isolates. These data again coincide with the one reported by Koleva – 19.38%, but there is an inverse correlation with the one found by Hristov in goats – 3.67%. This microorganism is associated with both clinical and subclinical mastitis, and some authors define it as the main cause of clinical mastitis. A number of authors found the involvement of *Staphylococcus aureus* in the development of subclinical mastitis, and they reported its isolation in between 3% and 29% of cases (Arsenault *et al.*, 2008; Fotou *et al.*, 2011; Kern *et al.*, 2013; Queiroga, 2017; Vasileiou *et al.*, 2019 Knuth *et al.* 2022).

In the results presented by us, the presence of *Enterococcus spp*., which are Gram–positive facultative anaerobes, is also noticeable. We believe that bacteria of this genus are causative agent of subclinical mastitis, due to the available reaction from the body, expressed in an increase in the total number of somatic cells. This statement is also confirmed by other authors (Arsenault *et al.*, 2008; Kern *et al.*, 2013; Dorea *et al.* 2015).

Like us, other authors concluded that *Bacillus spp.* are bacteria responsible for the development of intramammary infections (Batavani *et al.*, 2003; Arsenault *et al.*, 2008). These authors found a similar percentage ratio of *Bacillus spp.* to ours. Knuth al. (2021) described this genus of microorganisms as the main causative agent isolated from milk samples from infected sheep (16%).

In the results presented by us, the presence of *Aerococcus viridans*, is striking. This bacterium is a widespread pathogen in the environment and clinically is associated with different diseases in animals and humans (Liu et a;. 2015). It has been considered as the etiological agent of ovine mastitis also by Queiroga (2017) who found it in 1.4% of isolates obtained from animals with subclinical mastitis. Saishu et. al (2015) found it in cattle with a clinical form of the disease.

*Dermacoccus nishinomiyaensis* is a Gram–positive aerobic microorganism of the *Dermacoccaceae* family. Its pathogenic influence has been described in human medicine in cases of peritonitis (Tanaka et. al. 2019), bacteremia secondary to catheterization (Joron et. al, 2019) and polymicrobial infection of the skin and urinary tract (Katoulis *et al.* 2015; Seifu *et al.*, 2018). In veterinary medicine, Kurt *et al.* (2021) listed it among the causative agents of clinical mastitis in cattle. We consider *Dermacoccus nishinomiyaensis* as causative agent of subclinical mastitis in sheep, due to the characteristic changes detected in the cytological analysis of the samples in which its presence was established.

In the etiology of mastitis in sheep, in addition to the microorganisms presented so far, a number of others play an important role, albeit with a smaller share. We found those of the genera *Streptococcus* – 4,65%, *Candida* – 2,33% and *Macrococcus* – 1,16%. The participation of these bacteria in the pathogenesis of mastitis is also registered by other authors who isolated them during their research (Queiroga, 2017; Fursova *et al.* 2021; Mousa et. al; 2021).

We also found the presence of the non–pathogenic *Lactococcus lactis ssp. lactis* in most cases in combination with *staphylococci*. It is a Gram–positive microorganism widely used in butter and cheese production technologies (Madigan *et al.* 2005). Most authors consider *Lactococcus lactis ssp. lactis* as non–pathogenic, but has been described as an opportunistic pathogen in humans in several cases (Aguirre *et al.* 1993). Todorov *et al.* (2006) found that this microorganism produces bacteriocin bacHV219 active against *Escherichia coli, Enterococcus faecalis, Lact. casei, Listeria innocua, Proteus vulgaris* and *Pseudomonas aeruginosa*. This statement is in agreement with our results, which show that lactococci occurs in combination with Gram–positive bacteria and in no sample was found in combination with Gram–negative ones or representatives of *Enterococcus*. We do not consider *Lactococcus lactis ssp. lactis* as the causative agent of subclinical mastitis due to the fact that the milk samples from which only it was isolated did not show deviations from the norm of physicochemical and cytological analysis.

The only Gram–negative bacteria we isolated was *Edwardsiella tarda*, a member of the *Enterobacteriaceae family*. It is a small, motile facultative anaerobe. E. tarda is most often isolated from freshwater or saltwater pools, as well as from faecal samples of mammals, fish and reptiles. In human medicine, there are known cases of induced gastroenteritis, endocarditis, peritonitis and meningitis caused by this bacteria (Hirai *et al.* 2015). Regarding its involvement in the pathogenesis of subclinical mastitis, we cannot be definitive due to the fact that a representative of staphylococci was also found in the sample from which it was isolated. Most likely, this bacteria got on the mammary gland through personnel and non–compliance with hygienic conditions.

## Conclusion

The analysis of our results showed that the main etiological agents of subclinical mastitis in sheep in Bulgaria are wide range of gram–positive microorganisms, mainly representatives of the CNS, as well as *Staphylococcus aureus*. The variety of etiological agents of this pathology in sheep requires that each case must be considered individually. The study reveal the importance of microbiological examination in the control of mastitis diseases in dairy farms.

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