Isolation and Characterization of Bacteria that Cause Subclinical Mastitis in Cows breed Holstein-Friesian in Bitola Region, North Macedonia

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Abstract

The aim of the study was to investigate of bacteria that cause subclinical of mastitis in cows of breed Holstein Friesian. The samples were taken in the in Bitola region, North Macedonia for the period from March 2016 to September 2017. Sixteen strains were isolated and identified based on their growth, colony morphology, gram stain, catalase and oxidase activity, using standard protocols. The study found that most common bacteria that cause mastitis are: *Staphylococcus aureus, Streptococcus uberis, Streptococcus epidermidis, Streptococcus agalactiae, Streptococcus dysagalactiae, Enterobacter aerogenes and Klebsiela pneumoniae.* The obtained findings correspond to the published data in other recent studies.

Key words: cows, mastitis, bacterial causes, Holstein-Friesian breed

Introduction

Disease of the mammary gland

Milk disease has a major importance in the production of milk (Donovan et al., 2005; Souto et al., 2010). From an etiological point of view, diseases of the mammary gland are most often associated with microorganisms and manifest themselves as an inflammatory reaction-mastitis. Milk from mastitis cows contains microorganisms that are harmful to human health (Karima et al., 2006). The intensity of changes in milk varies in mild cases, it can only be observed using special methods, and in severe cases a pathologically altered secretion occurs. With the ultimate goal of preventing the inflammatory response and returning the mammary gland to normal function, treatment is carried out. Most of the imported drugs are excreted through milk, which causes a series of hygienic-health, technological and economic problems. Clinically, mastitis can occur in a clinically visible and subclinical form (Almaw et al., 2008; Getahun et al., 2008; Hashemi et al., 2011). Milk with clinically

visible mastitis is practically not used in people's diet due to altered appearance, color and taste. On the proposal of the International Dairy Federation for mastitis, a definition of mastitis, based on the number of somatic cells and the finding of pathogenic microorganisms in milk has been accepted. The number of somatic cells and the presence of pathogenic microorganisms is determined in milk taken from a single quarter of the udder immediately before milking (International Dairy Federation, 1999). Mastitis is a condition where in the milk tested the number of somatic cells/ml is greater than 400.000 ml (Gharagozloo et al., 2003; Sharma et al., 2011).

In dairy farming in developed countries, the size that shows the rate of mastitis and the possibility of control represent is a national interest and indicates an important indicator of success in animal husbandry (International Dairy Federation, 1999; Sharma et al., 2011).

Mastitis appearance

The mammary gland in wild animals provides food only to the offspring. The appearance and duration of mastitis, in these animals, is correlated with the disease of other organs (Gentilin et al., 2002).

Factors

Three biosystems participate in the formation of mastitis, the cow with its defense mechanisms and predisposition for the occurrence of the disease, the environment with numerous possible transmission of infection and inhibitory factors and the mastitis revival with its virulence and biological nature. All the factors have the same meaning in the technology of the holding milking cattle. Poor care, unbalanced fodder, irregular nutrition and improper watering, as well as technically defective milking machines, act as a stress and have a significant impact on the frequency in appearance and duration of mastitis. Animals exposed to stress, of any nature, react more intensively to the pathological processes and produce poor quality milk. At the heavy duty cows due to the creation of large amounts of milk, are particularly susceptible to inflammatory reactions (Almaw et al., 2008).

Product of microorganisms in mammary gland

Microorganisms usually only inhabit the outlet or the outermost mammary grand. They do not move through *ductus papillaris*. Most often, the possibility of infection leading to causing mastitis is related to physical transportation, as well as during milking or after milking. The penetration of the microorganisms in the mammary gland is limited on several levels. The first defensive line is with characteristic morphology and function. *Ductus papillaris* is covered with a multilayer cutaneous mucous membrane, which rejects the microorganisms from the surface layer. The structure of the channel of mastitis susceptible and mastitis resistant animals varies. The keratin cover contains free fatty acids that act as antimicrobial. The protective function of the extract is such that *Staphylococcus aureus* can stay within 18 weeks without the possibility of infection. Thus the spontaneous disappearance of certain microorganisms and the antibacterial protection mechanism occur (Keefe, 1997; Lucia et al., 2017).

Bacterial cause of mastitis

Bacteria can be found to have subclinical mastitis in cows as: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysagalactiae*, *Enterobacter aerogenes* and *Klebsiela pneumoniae* (Lucia et al., 2017).

Staphylococcus aureus

The most important reservoir of this pathogen is the infected mammary gland, which is usually infected by the udder skin. Recent literature shows that 0.2%–17.6% of the udder is infected with this pathogen. The microorganism is excreted with milk from the diseased quarter of the udder in a number up to 10^4 ml (Lucia et al., 2017; Almeida et al., 1996).

Streptococcus agalactiae

It is an obligate parasite of the mammary gland in cows, depending on the production, its presence is relative. In milk and the surrounding area around the mammary gland, outside the udder, it can stay up to three weeks. Duc to sen-

sitivity of Streptococcus agalactiae to penicillin, the frequency of mastitis caused by this microorganism ranges from 0,1% to 10,4%. With milk 10^2–10^5 ml *Streptococcus agalactiae*/ml (Lucia et al., 2017).

Streptococcus dysgalactiae

A typical cause of mastitis is most often found on the mucous membranes of other organs such as the uterus, vagina, tonsils. Depending on the stage of infection, it ranges from 0.1% to 2.9% (Lucia et al., 2017).

Streptococcus uberis

It is widespread microorganism, found on the skin and mucous membranes as well in the feces and in the soil. Infection ranges from 0.2% to 4.4% (Keefe, 1997).

Enterobacter aerogenes, Klebsiella spp, *and Pseudomonas aeruginosa*

They predispose mastitis and are prevalent in an unhygienic environment. The source of infection is contaminated with water, soil and feces. These microorganisms lead to infection and severe clinical mastitis (Harjanti et al., 2018).

Actinomyces pyogenes

Infection is most commonly associated with other microorganisms (*Pseudomonas indolicus*, microaerophilic microcircuits and *Fuscobacterium necrophorum*). They are particularly related to some geographical and meteorological conditions, and also occur in mammary gland in donkeys who do not give milk. This type of mastitis can be correlated with insects as vectors for transmission. The literature describes it as cholelithiasis or summer mastitis (Keefe, 1997).

Staphylococcus epidermidis and Corynebacterium bovis

These microorganisms are often isolated from the secretion of the mammary gland, regardless of their low pathogenicity. With the primary cause of mastitis, they can act pathogenic but can often lead to excretion of an increased number of cells because they inhabit the milk channels (Gentilin et al., 2002; Taponen et al., 2006).

Serratia marcescens, Bacillus cereus, Clostridium perfringes, Mycoplasma spp, Yeasts

They are sporadic agents of mastitis. The sources of infection are the soil, water, food. It rarely causes a pathological process (Harjanti et al., 2018)

Material and Methods

Sample

For the purpose ot this study, a total of 16 samples were taken over a period of one year and half (March 2016 to September 2017). The cows have a subclinical mastitis. They are from different farms in the area of Bitola region, North Macedonia. The breed is the same Holstein-Friesian and their age ranges from 3.5 to 7.5 years and milk taken from a of the udder. (Table 1).

Table 1. Samples taken from small dairy farms in the Bitola region – North Macedonia for the period from March 2016 to September 2017 (year, age and milk taken from a of the udder)

Sample	Year	Age (Year)	milk taken from a of the udder
1	2016	5.5	double quarters
2	2016	4.7	single quarter
3	2016	4	single quarter
4	2016	6.8	double quarters
5	2016	5.5	single quarter
6	2016	4.9	double quarters
7	2016	7.2	single quarter
8	2016	5.2	single quarter
9	2017	5.4	double quarters
10	2017	6.3	single quarter
11	2017	3.7	single quarter
12	2017	4.1	single quarter
13	2017	7.5	single quarter
14	2017	3.5	single quarter
15	2017	6.6	single quarter
16	2017	3.8	single quarter

Isolation

Sixteen strains were isolated as pure cultures from 16 samples. They are numbered from M1 to M16 (Table 2).

Characterization and identification

To identify the new isolates in our test we use several biochemical tests which are explained below in the text (Catalase test, Oxidase test, Urease test, Gram stainig test, Citrate test, Indole test, Methyl test and API test and other biochemical tests).

Table 2. Isolated and identified strains

Sample	Strains	Sample	Strains
1	M1	9	M9
2	M2	10	M10
3	M3	11	M11
4	M4	12	M12
5	M5	13	M13
6	M6	14	M14
7	M7	15	M15
8	M8	16	M16

Results and Discussion

The results of the primary identification of the isolated 16 pathogenic strains are summarized and presented in Figure 1. The strains belong to 7 species. Four of them refer to the genus Streptococcus and represent 60% from 7 species. It has been published that the species of these streptococcal bacteria – *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis and Staphylococcus aureus* are the main causers of subclinical mastitis (Lucia et al., 2017).

The figure shows that the most common cause of subclinical mastitis in cows in the Bitola region, North Macedonia during the study period is the bacterium *Staphylococcus aureus*. It accounts for almost 1/3 of cases (27%). The second major pathogen is *Streptococcus uberis*. Bacteria of the genus Streptococcus are represented by 4 strains belonging to different species: *Streptococcus uberis* represented by 24%, followed by *Streptococcus epidermides* (16%), *Streptococcus agalactiae* (11%) and *Streptococcus dysagalactiae* (9%). *Enterobacter aerogenes with* (7%) and *Klebsiela pneumoniae* (6%) are relatively poorly represented. The last two pathogens are spread in contaminated water, soil and stool, which become the source of infection.

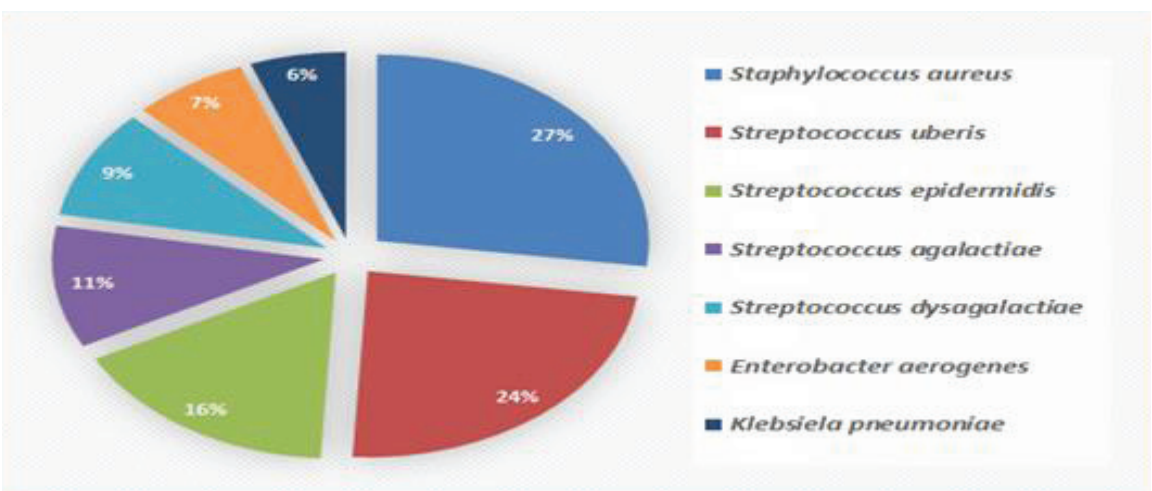

Fig. 1. Microorganisms isolated from clinical cases of cows of breed Holstein Friesian in Bitola region, North Macedonia

Such an unhygienic environment can cause severe clinical mastitis. In this respect, our results are relatively reassuring.

Almaw et al. (2008) established an overall mean cow-level mastitis prevalence of 21.1%, where subclinical (16.3%) is more prevalent than clinical (4.8%) mastitis. Mastitis is found to be prevalent in all of the study areas, with a mean of 49.3%. The common isolates from the clinical mastitic quarters were *Streptococcus agalactiae* (30%, n=3) and *Streptococcus dysgalactiae* (30%, n=3), while from sub-clinical cases were *Staphylococcus aureus* (42.6%, n=83), *Streptococcus epidermidis* (22.1%, n=43), *St. agalactiae* (12.8%, n=25) and *St. uberis* (10.3%, n=20). *Staphylococcus intermedius* and *Streptococcus dysgalactiae* were the species, which showed high level of susceptibility for most of the antimicrobials tested, while the remaining had varying levels of resistance for almost all the antimicrobials used (Getahun et al., 2008).

Milk samples from both clinical and subclinical quarters were collected for bacteriological culture. 4714 (76.28%) quarters were healthy, 1335 (21.6%) quarters were positive by results of CMT (as indicated to subclinical mastitis), 44 (0.71%) quarters showed clinical mastitis signs and 87 (1.41%) quarters were blind. The clinical and subclinical mastitis prevalence at cow level was 2.2 and 42.5%, respectively (Hashemi et al., 2011). Based on the milk samples from 33 buffaloes (Bubalus bubalis), which were positive for subclinical mastitis in Enrekang, one milk sample (3.03%) was positive for *Streptococcus agalactiae*, while four samples (12.12%) were positive for *Staphylococcus aureus*, whereas 28 milk samples from the dairy cows were negative for both *Streptococcus agalactiae* and *Staphylococcus aureus* bacteria (Lucia et al., 2017).

The findings we obtained correspond to the published data of the above authors from the literature.

Conclusion

From the received results it can be concluded that the most common cause subclinical mastitis in cows in the Bitola region, North Macedonia during the study period is the bacterium *Staphylococcus aureus* with 1/3 of cases (27%).

The second major pathogen is *Streptococcus uberis*. Bacteria of the genus *Streptococcus* are represented by 4 strains belonging to different species:

Streptococcus uberis (24%),
Streptococcus epidermides (16%),
Streptococcus agalactiae (11%),
Streptococcus dysagalactiae (9%).

Enterobacter aerogenes (7%) *and Klebsiela pneumoniae* (6%) are relatively poorly represented.

The last two pathogens are spread in contaminated water, soil and stool, which become the source of infection. Such an unhygienic environment can cause severe clinical mastitis. In this respect, our results are relatively reassuring.

Recommendations

• Create a clean, stress-free environment for cows. For optimal milk production, oxytocin is best stimulated under a stress-free environment for cows. Starting with a clean stall and parlor will decrease the presence of mastitis-causing bacteria.

• Remove all solids and clean udder. Cleaning the udder before attaching the milking machine is a very important step in preventing bacteria from getting into the *ductus papillaris* during the milking process.

• Examine the udder. Look for chapped, cracked and bleeding papillae. Examine and score the teat ends. Do not use chapped, cracked or bleeding teats or teat ends. These teats are susceptible to new IMIs and milked carefully.

• Use proven, effective pre-milking teat dips. Teat dips reduce the number of bacteria on teats and thereby help lower the number of new IMIs.

• Use paper towels or reusable cloth towels to clean and dry teats.

• Use milking equipment properly. Persons who use the milking equipment should be trained on how to properly attach, adjust and remove (if required) the milking unit.

• Monitor the milking process. Using milking machines properly is an important part of a milking parlor or routine milking stall, plus an important component of a mastitis management program.

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