

Original Research Article

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Characterization of Microbial Causative Agents of Subclinical Mastitis in Goats in Bulgaria

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ABSTRACT

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Mastitis is the most prevalent mammary gland disease in goats. It has a number of unfavorable health effects in the affected animals and leads to economic losses in the farms. The aim of this study was to isolate and identify microorganisms causing subclinical mastitis (SCM) in goats. Two hundred and twenty-nine milk samples from lactating goats were studied. Microorganisms were isolated from 189 of these samples (82.53%), but not from the remaining 40 ones (17.47%). Isolation and identification of microbial pathogens was carried out using elective and selective growth media for various groups of bacteria. Identification was done using biochemical tests and additional oxidase and catalase tests. The results showed highest prevalence of *Staphylococcus* spp. (52.75%), and particularly, of coagulase-negative staphylococci (CNS). Apart from the samples with a single bacterial species, 42 samples (23.86%) were shown to contain different isolates in associations. The most prevalent associations were those of *Staphylococcus caseolyticus* + *S. adjacens* and *Staphylococcus caseolyticus* + *Proteus penneri* (9.52%).

Introduction

Milk produced from goats with mastitis poses a serious veterinary-sanitary and epidemiological hazard. In addition to causing hygiene and health issues, the mammary gland inflammatory processes also cause economic losses due to reduced milk productivity, early culling of animals and treatment costs. Although the microbial pathogens involved in mastitis have been widely studied, they are still focus of research, as the isolated species of etiological agents change over time. Over 100 species of microorganisms are involved

in mammary gland inflammatory conditions, although it is about 10 species that are most prevalent. A large number of microbiological studies report staphylococci as the most prevalent mastitis pathogens in goats (Bergonier *et al.*, 2003; Contreras, 2003; Ajuwape *et al.*, 2005; Mhase *et al.*, 2007; Moroni *et al.*, 2007; Byeng *et al.*, 2007; Aydin *et al.*, 200; Islam *et al.*, 2012; Silva *et al.*, 2011; Marogna *et al.*, 2012).

The aim of this study was to isolate and identify microorganisms in milk samples

from goats with subclinical mastitis from farms in different parts of Bulgaria in order to determine the species composition of the etiological agents.

Materials and Methods

Milk samples

Microbiological analysis of 220 milk samples was done to isolate and identify mastitis microbial pathogens in goats. The samples were collected from four farms located in different administrative regions of Bulgaria. In three of these farms, the animals had been given regular immunization against contagious agalactia, but not in the fourth one.

Mastitis tests

The condition of the udder halves of each goat was examined for subclinical mastitis on site, at the farm, using the rapid mastitis tests CMT-Test (Kruuse, Denmark) and Porta SCC (Porta Check, USA). 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 procedure

To isolate and identify the mastitis microbial pathogens, the milk samples were inoculated on elective and selective media for different groups of bacteria and for fungi. Incubation was done at 37°C and 28°C for 24–72 h in aerobic conditions. The following growth media were used: Blood Agar, Folate–azide Medium for isolation of enterococci (Bul Bio, NCIPD Ltd, Sofia), Mueller–Hinton Agar and Broth, Eosin Methylene Blue Agar for Gram-negative aerobic and facultative anaerobic bacteria, Cetrimide Agar for

isolation of *Pseudomonas* species; Chapman Stone agar for staphylococci and Sabouraud Agar for fungi (Antisel - Sharlau Chemie S. A., Spain).

Microorganism identification

Identification of the bacterial isolates was done based on microscopic examination of Gram- and Pfeiffer-stained samples, colony characteristics and biochemical tests using Polymicrotest (Bul Bio, NCIPD Ltd, Sofia), as well as additional oxidase and catalase tests using reagents from Antisel - Sharlau Chemie S. A., Spain. The isolation and identification procedures were done according to Bergey's Manual (Holt *et al.*, 1994).

Results and Discussion

Microorganisms were identified to be present in $n=189$ (82.53%) of the 229 tested udder halves, but not in $n=40$ (17.49 %) of the samples. Of the samples containing microorganisms, 77.78% gave a single-species isolate (monoculture), and 22.22%, multiple species isolates.

The relative prevalence (in percent) of all the microorganisms isolated from the studied samples is shown in Figure 1.

Some of the samples from the farm with no immunization against contagious agalactia were microscopically observed to contain small cells with mycoplasma morphology, most likely *Mycoplasma agalactiae*. Such cells were observed both in the monoculture and the mixed-culture pathogen isolates.

The prevalence of microorganisms isolated from the samples with mixed infection is presented in Table 1.

The results showed that the prevalent combinations of microorganisms in the

cases with mixed infections were Gram-positive staphylococci with Gram-negative bacteria.

The main direct etiological factors of all types of mammary gland inflammation are different microbial species. Their ability to penetrate and cause inflammation largely depends on the predisposing factors, such as milking and rearing hygiene, individual resistance of the animals, age, lactation period etc.

There are over 30 different species and subspecies of coagulase-negative staphylococci (CNS) (Aarestrup *et al.*, 1999). However, the role of each of them, especially of the non-pathogenic or less pathogenic ones, in the etiology of clinical or subclinical mammary gland infections in goats is still not fully understood. Our results showed that the most prevalent microbial agents of intramammary infections in goats belonged to genus *Staphylococcus* (52.75%), particularly to the CNS group. These results are in agreement with previous reports (Contreras *et al.*, 2007; Leitner *et al.*, 2007). Bergonier *et al.*, (2003) also observed 25% to 93% prevalence of CNS in different flocks.

In our study, the most prevalent microbial isolate was *S. caseolyticus*. It is, however, not very likely to be definitely considered a single causative agent of subclinical mastitis in goats, since it is mostly isolated in combination with opportunistic Gram-negative bacterial pathogens. It could also be suggested that some opportunistic pathogens, such as intestinal bacteria and *Pseudomonas* spp., which are more penetrative in the mammary ducts due to their motility, create conditions that favor the association of *S. caseolyticus*. It, in turn, degrades milk casein, creating favorable growth conditions for other non-proteolytic

microorganisms, which paves the way for development of inflammatory processes in the mammary gland. Other important causative agents of intramammary infections isolated in our study included CNS members, such as *S. simulans*, *S. haemolyticus*, *S. hyicus*, *S. caprae*, *S. epidermidis* and *S. saprophyticus*. In most cases, they were isolated as single species, which further supports their role in the etiology of mammary gland inflammation. These results are in agreement with the data reported by Deinhofer and Pernthaner (1995) and Moroni *et al.*, (2005), who also identify these microorganisms as main causes of mastitis in goats.

The 3.67% relative prevalence of *S. aureus* in our study is much lower than that reported by others (Ravnaval-Ljutovac *et al.*, 2007; Winter, 2009; Mork *et al.*, 2010). In our study, *S. aureus* was isolated mainly in cases of clinical mastitis and in just a few cases of subclinical infections. Interestingly, *Streptococcus thermophilus* was isolated from some of our samples. It is well-known to play a role in lactic acid fermentation in the production of some dairy products, as well as to have probiotic properties. It is most probably part of the indigenous udder microflora and could be found in the milk in certain conditions without having a pathogenic role. There is little data about the distribution of *Streptococcus adjacens*, which was isolated in our study, and its infectious potential in animals. To the best of our knowledge, this is the first study to report a *Streptococcus adjacens* isolate in Bulgaria. Interestingly, *Streptococcus suis* monoinfection was also identified in a few samples from one of the farms studied by us. This indicates that there is high potential for easy spread of microorganisms among different animal species reared closely together and demonstrates that this streptococcus could also cause mammary

gland infections in goats. Another pathogen that most typically affects cows but less commonly causes subclinical mastitis in goats is *Streptococcus uberis* (Hillerton *et al*, 1993; McDougall, 1998). Although it is generally not considered typical of goats, it was isolated from 4.13% of our samples. These results support the findings of other authors that it can play a role in the etiology of intramammary infections in goats, albeit with a lower prevalence (Ameh and Tari, 2000; Lasagno *et al.*, 2012).

Other bacteria that were identified to have a share in the etiology of mastitis in goats

were *Proteus mirabilis*, *Proteus penner* and *Pseudomonas putida* with 2.76%, *Serratia marcescens* with 2.29% and *Enterobacter* spp. with 0.92% prevalence. These microorganisms were rarely isolated as single agents and, due to their lower pathogenic potential, could not be considered among the main causative agents of mammary gland inflammation in goats. In most cases, their role in the etiology of mastitis was in microbial associations with different staphylococci. These minor species have also been isolated in the study of Bedada and Hiko (2011).

Table.1 Microorganisms isolated from the samples with mixed infection.

| Microorganisms | Number of isolates | Relative shares |
|---|--------------------|-----------------|
| | n | P (%) |
| <i>Staphylococcus simulans</i> + <i>Streptococcus thermophilus</i> | 3 | 7,14 |
| <i>Staphylococcus caseolyticus</i> + <i>Micrococcus (Kocuria) kristinae</i> | 3 | 7,14 |
| <i>Staphylococcus caseolyticus</i> + <i>Streptococcus adjacens</i> | 4 | 9,52 |
| <i>Staphylococcus caseolyticus</i> + <i>Proteus mirabilis</i> | 1 | 2,39 |
| <i>Staphylococcus caseolyticus</i> + <i>Proteus penneri</i> | 4 | 9,52 |
| <i>Staphylococcus caseolyticus</i> + <i>Serratia marcescens</i> | 2 | 4,76 |
| <i>Staphylococcus intermedius</i> + <i>Proteus penneri</i> | 2 | 4,76 |
| <i>Staphylococcus caseolyticus</i> + <i>Pseudomonas putida</i> | 3 | 7,14 |
| <i>Staphylococcus intermedius</i> + <i>Pseudomonas putida</i> | 2 | 4,76 |
| <i>Staphylococcus intermedius</i> + <i>Pseudomonas fluorescens</i> | 1 | 2,39 |
| <i>Staphylococcus haemolyticus</i> + <i>Bacillus brevis</i> | 2 | 4,76 |
| <i>Bacillus brevis</i> + <i>Candida albicans</i> | 2 | 4,76 |
| <i>Corynebacterium sp.</i> + <i>E. Coli</i> | 3 | 7,14 |
| <i>Mannheimia haemolytica</i> + <i>E. coli</i> | 2 | 4,76 |
| <i>Enterobacter spp.</i> + <i>Pasteurella multocida</i> | 2 | 4,76 |
| <i>Streptococcus uberis</i> + <i>Staphylococcus epidermidis</i> | 4 | 9,52 |
| <i>Staphylococcus aureus</i> + <i>E. coli</i> | 1 | 2,39 |
| <i>Staphylococcus aureus</i> + <i>Streptococcus uberis</i> | 1 | 2,39 |

Fig.1 Relative shares of microorganisms causing subclinical mastitis in goats.



One of the clinically important causative agents of mastitis in goats is *E. coli*. It was isolated in 5.50 % of our samples. Its role has been affirmed in nearly all studies on the microbial etiology of mammary gland inflammations in goats (White and Hinckley, 1999; Ajuwape *et al.*, 2005; Gebrewahid *et al.*, 2012). Highly pathogenic strains can cause severe clinical mastitis, often with a lethal outcome. Although *Corynebacterium sp.* was isolated from only 4.13 % of the tested samples, mostly in association with *E. coli*, their role in mammary gland inflammation should not be underestimated. This is in accordance with the results reported by Manser (1986),

McDougal *et al.*, (2002) and Bagnicka *et al.*, (2011), who identify them as part of the microbial agents of mastitis in goats.

The small irregular-shaped cells with a brighter central part observed in some of the samples studied by us could most probably be identified as *Mycoplasma spp.*, although additional methods to confirm this were not included in this study. This suggestion is in line with a large number of reports that identify mycoplasma as causative agents of mastitis (Blood *et al.*, 1990; Ameh *et al.*, 1993; Egwua *et al.*, 2001; Anderson *et al.*, 2002). Presence of mycoplasma in milk has also been demonstrated by Kinde *et al.*,

(1994), who isolated mycoplasma from goat milk from farms with contagious agalactia.

Apart from monocultures, different associations of microorganisms were isolated from 42 (23.86%) of the studied samples. This considerable prevalence among the infected udder halves indicates that mixed infections play an important role in the etiology of subclinical mastitis in goats. Notably, there were mainly different combinations of Gram-positive microorganisms, predominantly staphylococci, with different Gram-negative bacteria. The most prevalent associations were those of *S. caseolyticus* + *S. adjacens* and *S. caseolyticus* + *P. penneri* – 9.52%. *S. caseolyticus* is not considered as a pathogen and is sporadically isolated from healthy slaughtered lambs, as well as from milk (Fuente *et al.*, 1992). However, our results suggest that, in combination with other microorganisms, it could also take part in the etiology of subclinical mastitis in goats.

Another combination of microorganisms that was isolated by us from a considerable number of samples was that of *S. intermedius* with different Gram-negative species. In 4.76 %, it was isolated in combination with *P. penneri*, in the same prevalence, with *Pseudomonas putida*, and in 2.39%, in combination with *Pseudomonas fluorescens*. The role of these microbial associations including *S. intermedius* is probably due to the high pathogenic potential of this staphylococcus and the presence of additional microorganisms.

In conclusion, the polymicrobial etiology of mammary gland infections in goats highlights the importance of identification of the microbial causative agents in each individual case. Effective control of mastitis in goat farms would require differentiation of the causative agents and application of effective treatment schemes.

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