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STUDIES ON THE CAUSES OF BACTERIAL MASTITIES IN TWO DAIRY BOVINE FARMS IN NORTHEASTERN BULGARIA AND THEIR SENSITIVITY TO ANTIMICROBIAL PRODUCTS

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ABSTRACT

The prevalence of clinical and subclinical mastitis in two dairy farms with 500 and 114 cows each was studied. The causative agents have been identified and their antimicrobial susceptibility has been determined.

In farm A, 44 cows (8.8%) with mastitis were observed - 3 with clinical and 41 with subclinical forms. Pathogenic microflora was isolated from 35 cows (79.5%). Were identified 64 strains: 30 (46.9%) *Streptococcus spp*. and 34 (53.1%) *Staphylococcus spp*. All staphylococci and streptococci showed sensitivity to Amoxicillin/Clav. acid. All staphylococci also showed sensitivity to Cephalothin, Gentamicin and Rifampicin.

In farm B, 26 cows (22.8%) with mastitis were diagnosed - 10 with clinical and 16 with subclinical forms. Pathogenic microflora was isolated from 19 cows (73.1%). Were identified 24 strains: 8 (33.3%) *Streptococcus spp.* and 16 (66.7%) *Staphylococcus spp.* All streptococci showed sensitivity to Amoxicillin, Amoxicillin/Clav. acid, Cephalothin, Erythromycin, Gentamicin, Tetracycline, Rifampicin and Enrofloxacin. Susceptibility to Amoxicillin/Clav. acid, Cefoxitin, Cephalothin, Lincomycin, Gentamicin, Tetracycline, Rifampicin and Enrofloxacin was found in all staphylococci.

Key words: cows, mastitis, causative agents, antibiotics, resistance.

Introduction

Inflammatory processes in mammary glands are one of the most significant health and economic problems in dairy farming worldwide (Lopes et al., 2012; Ruegg, 2012; Costa et al., 2013). They cause huge losses, both for producers and for the industry as a whole, due to the reduction in the quantity and deterioration of the milk produced or due to permanent damage to the udder, which requires culling of the animals. In addition, they contribute to the high treatment costs and can have negative consequences for public health. The prevalence of latent (subclinical) mastitis in some dairy farms is particularly unfavorable. According to Ramírez et al. (2014) and Abebe et al. (2016), subclinical mastitis occurs with a higher incidence in animals and if not detected in time can lead to clinical manifestations and further complications. Therefore, early diagnosis of latent mastitis and identification of their etiological agents are key to reducing economic losses and health consequences for animals. A wide range of pathogenic microorganisms can cause both latent and clinically manifested mastitis in cattle. Some species of streptococci such as *S. agalactiae*, *S. uberis, S. dysgalactiae, Staphylococcus aureus* and some coagulase-negative staphylococci, coryneform bacteria, *Trueperella pyogenes*, some bacilli and enterobacteria, of which *E. coli* species are most significant, are most often identified as the main pathogens. Rational etiotropic therapy requires, in addition to determining the etiological agent, also testing its in vitro susceptibility to antimicrobial agents. This is especially important given the growing problem of antimicrobial resistance, which is usually realized through genetically determined mechanisms. Studies on the causes of clinical and especially subclinical mastitis and their susceptibility to antimicrobials provide a better understanding of their epidemiology, the causes and ways of their spread in farms and areas and allow for a motivated approach to strategy and tactical approaches to their control.

The aim of the present study was to start a monitoring study on the species composition of bacterial mastitis in cattle (both subclinical and clinical) and to determine their susceptibility to antimicrobials in connection with the development of a comprehensive concept for their successful control.

Materials and methods

Farms, animals, samples:

The studies were conducted in two dairy cattle farms in Northeastern Bulgaria in the fall of 2020. A total of 70 animals were tested, and 111 milk samples were obtained respectively. Cases of clinical mastitis were determined based on the presence of macroscopic changes in milk and clinical signs of udder inflammation. The California mastitis test was used as a screening method for detecting and diagnosing subclinical mastitis. The classification of the changes was carried out according to a four-cross system. The samples were taken in sterile containers after aseptic treatment of the milk papillae and after separation of the first streams of milk. They were transported to the laboratory under refrigerated conditions. The microbiological tests were conducted in the bacteriological laboratory of the Department of "Veterinary Microbiology, Infectious and Parasitic Diseases" of the Faculty of veterinary medicine at Trakia University.

Bacteriological tests:

After centrifugation at 2000 rpm for 15 minutes, a culture was prepared from each sample on blood agar (with 5% defibrinated sheep blood) and on Mac Conkey agar (Hi Media, India). The cultures were incubated at 37 ºC for 24–48 hours under aerobic conditions. Suspicious colonies were subcultured on the same bacterial culture media to isolate pure cultures for further identification. The morphological characteristics of the colonies such as shape, periphery, size, pigmentation and hemolysis were assessed on a stereoscope. Microscopy of Gram-stained preparations took into account the shape and size of the bacterial cells, their location and Gram staining. Catalase and oxidase tests were performed.

Isolates identified as *Staphylococcus spp*. were tested for plasma coagulase production (with lyophilized rabbit plasma, Hi Media, India). The growth incriminated as *Streptococcus spp.* was further investigated in a set of biochemical tests with a Christie, Atkins and Munch-Petersen reaction (CAMP) and for the hydrolysis of esculin for further differentiation.

Antimicrobial susceptibility testing:

In vitro sensitivity of the isolates to antimicrobials were tested by the Kirby-Bauer (1966) disk diffusion method on Mueller-Hinton agar (Hi Media, India) and interpreted on a three-point scale as sensitive (S), intermediate (I) and resistant (R). For this purpose, a 24-hour culture suspension of each strain analyzed was adjusted to a density corresponding to 0.5 on the McFarland scale and applied to the agar surface with a sterile swab. Antibiotic discs with the appropriate concentrations were used as follows: Amoxycillin 10 mcg, Amoxycillin/Clav. acid – 20/10 μg , Oxacillin 1 μg, Cefoxitin 30μg , Cephalothin 30 μg, Lincomycin 15μg , Gentamicin 10 μg, Tetracycline 30μg , Erythromycin 15μg , Rifampicin 5 μg, Enroflaxacemin 25 μg. After 24 hours of incubation, the zones of growth inhibition were counted by measuring their diameter. The information from the manufacturer (Hi Media, India) and the EUCAST standard were used as criterion evaluation system.

Results

The results of the conducted field research are reflected in Table 1. Of the 614 lactating cows surveyed, 57 had **subclinical mastitis.** Of the 228 milk quarters tested, 90 milk quarters responded to the rapid California test, of which a total of 90 samples were obtained and tested. As a result of the bacteriological examination, a total of 71 bacterial strains were isolated and incriminated as possible etiological agents of inflammatory processes in the mammary gland. For farm A, there were a total of 56 strains and for farm B, there were 15 strains. Data on **clinical mastitis** was available in 13 animals, from which 21 samples were obtained. Of these, 17 strains were isolated – 8 in farm A and 9 in farm B.

Table 1: Results of the examination of milk samples from cows with clinical and subclinical mastitis

Farm	n animals/ quarters	Subclinical mastitis			Clinical mastitis			Total n of strains
		n cows	n sampl.	n isolated strains (%)	n cows	n sampl.	n isolated strains (%)	
A	500/2000	41	70	**56** (87.5%)	3	8	**8** (12.5%)	**64**
B	114/456	16	20	**15** (62.5%)	10	13	**9** (37.5%)	**24**
Total	614/2456	57	90	**71** (80.7%)	13	21	**17** (19.3%)	**88**

In farm A, out of a total of 78 milk samples tested (70 subclinical mastitis samples, 8 clinical mastitis samples), a microbiological finding was detected in 69 of them (no pathogenic microflora was isolated from 9 samples). Based on the morphological characteristics of the prepared microscopic preparations, as well as the characteristics of the colonies, Gram stain, as well as catalase and oxidase tests were identified totally 64 strains: 34 strains (53.1%) were attributed to *Staphylococcus spp* and 30 strains (46.9%) to *Streptococcus spp*. From the test for plasma coagulase production in staphylococci, 14.7% coagulase-positive isolates and 85.3% coagulase-negative staphylococci were found. Streptococcal isolates were subjected to CAMP as well as an esculin hydrolysis assay. Based on these biochemical analyses, the presence of *S. agalactiae* (23.3%), *S. uberis* (50%) and *S. dysgalactiae* (26.7%) was established in streptococci.

A total of 33 milk samples were obtained and tested from farm B. The presence of a bacterial finding was detected in 25 of them (8 samples were without isolated pathogenic microflora). After microscopic and culture examinations, as well as on the basis of the results of biochemical tests, a total of 24 strains were isolated, of which 16 (66.7%) were identified as belonging to *Staphylococcus spp*, and 8 (33.3%) were incriminated as *Streptococcus spp*. Of the staphylococcal isolates, 68.8% were identified as *S. aureus* and 31.3% were coagulase-negative species. Of the streptococci, 50% were identified as *S. agalactiae* and 50% as *S. uberis.*

The results for the species diversity of the pathogenic microflora isolated from subclinical mastitis are shown in Table 2. In the both of the farms the diversity was reduced to the limited type of pathogenic microorganisms mainly representatives of the Gram-positive microbial flora. Thirty nine staphylococcal strains were detected, 28 of which were in farm A and 11 in farm B. Of these, 8 were identified as *S. aureus* - 1 in farm A and 7 in farm B. 31 strains were Coagulase - negative species (CNS), 27 of which were in farm A and 4 in farm B. A total of 32 streptococcal strains were isolated, 28 of which were isolated in farm A and 4 in farm B. In farm A, 5 of them were identified

as *Streptococcus agalactiae*, 15 as *Streptococcus uberis* and 8 as *Streptococcus dysgalactiae*. In farm B, one isolate was identified as *Streptococcus agalactiae* and 3 as *Streptococcus uberis.*

Table 2: Microbial strains from dairy samples of cows with subclinical mastitis.

Microbial strains of cows with subclinical mastitis.	Farm A n (%)	Farm B n (%)	Total n (%)
Staphylococcus spp.	28	11	39
Staphylococcus aureus	1 (1,8%)	7 (46,7%)	8 (11,3%)
CNS*	27 (48,2%)	4 (26,7%)	31 (43,7%)
Streptococcus spp.	28	4	32
Streptococcus agalactiae	5 (8,9%)	1 (6,7%)	6 (8,5%)
Streptococcus uberis	15 (26,8%)	3 (20,0%)	18 (25,4%)
Streptococcus dysgalactiae	8 (14,3%)	-	8 (11,3%)
Total n of isolates	**56**	**15**	**71**

**CNS – coagulase-negative staphylococci*

The results of the test of susceptibility of the isolates to antimicrobial agents are expressed in Table 3. They showed that in farm A, *S. aureus* strains was sensitive to all tested antimicrobials, and for farm B, they were 100% sensitive to amoxicillin + clavulanic acid, oxacillin, cefoxitin, cephalothin, lincomycin, gentamicin, tetracycline and rifampicin and enroflaxacin. A smaller percentage of them (18.2%) were sensitive to amoxicillin.

Table 3: Antimicrobial sensitivity /%

	n strains	Amx	Amx + Clav	Oxa	Cxt	Cft	E	L	G	T	Rfp	Enr	Sulf+TMP
Farm A													
S. aureus	5	5 100%	5 100%	5 100%	5 100%	5 100%	-	5 100%	5 100%	5 100%	5 100%	5 100%	5 100%
CNS	29	26 89.7%	29 100%	15 51.7%	28 96.6%	29 100%	-	9 31%	29 100%	11 37.9%	29 100%	27 93.1%	28 96.%
S. agalactiae	7	6 85.7%	7 100%	-	-	7 100%	6 85.7%	6 85.7%	7 100%	7 100%	7 100%	7 100%	-
S. uberis	15	11 73.3%	15 100%	-	-	14 93.3%	12 80%	3 20%	14 93.3%	5 33.3%	12 80%	12 80%	-
S. dysgalactiae	8	6 75%	8 100%	-	-	8 100%	4 50%	2 25%	7 87.5%	3 37.5%	7 87.5%	6 75%	-
Farm B													
S. aureus	11	2 18.2%	11 100%	11 100%	11 100%	11 100%	-	11 100%	11 100%	11 100%	11 100%	11 100%	9 81.8%
CNS	5	4 80%	5 100%	2 40%	5 100%	5 100%	-	5 100%	5 100%	5 100%	5 100%	5 100%	3 60%
S. agalactiae	4	-	-	-	-	-	-	-	-	-	-	-	-
S. uberis	4	4 100%	4 100%	-	-	4 100%	4 100%	3 75%	4 100%	4 100%	4 100%	4 100%	-

In coagulase-negative staphylococci in farm A, the highest (100%) percentage of sensitivity was to amoxicillin + clavulanic acid, cephalothin, gentamicin and rifampicin, and the lowest to lincomycin (31%). Similarly, in farm B, the percentage of sensitivity was 100% for amoxicillin + clavulanic acid, cefoxitin, cephalothin, lincomycin, gentamicin, tetracycline, rifampicin and enrofloxacin, while only 60% of the strains were sensitive to trimethoprim-potentiated sulfonamide.

For *S. agalactiae* strains isolated from farm A, maximum susceptibility (100%) was again found for amoxicillin + clavulanic acid, cephalothin, gentamicin, tetracyclines, rifampicin and enrofloxacin, and lowest susceptibility was towards amoxicillin, erythromycin and 85% lincomycin. In farm B, these strains showed no growth on Mueller-Hinton agar and were not tested by the disk diffusion method. Farm A strains identified as belonging to *S. uberis* showed a maximum percentage (100%) of sensitivity to the combination of amoxicillin and clavulanic acid, and the lowest susceptibility to lincomycin (20%). The same species isolated from farm B showed 100% sensitivity to all tested antibiotics except lincomycin (75%).

In farm A, *S. dysgalactiae* strains showed the highest percentage of sensitivity to amoxicillin + clavulanic acid and cephalothin (100%) and the lowest to lincomycin (25%). No strains belonging to *S. dysgalactiae* were isolated in farm B.

Discussion

The results of this study show a different prevalence of clinically manifested and subclinical mastitis in the two farms. In farm A, the percentage of affected animals was very low – 8.8%, as 6.8% of them had clinical and 93.2% had subclinical mastitis. In farm B, average ¼ of them (22.8%) had data for mastitis. In 38.5% of animals, it was clinically manifested, and in 61.5% it was manifested in its' subclinical form. In our opinion, the reason for this difference is due to the different level of hygiene and asepsis, with the human factor playing a key role in this direction.

In the two studied farms, causative agents belonging to the same microbial species were identified. According to our study, these were mainly Gram-positive cocci. This correlates with the studies of other authors (Sentitula & Kumar, 2012; Gomes et al., 2016; Mesquita et al., 2019). In both farms, almost half of the isolates were staphylococci, respectively 53.1% and 66.7%, and the rest of the isolates, respectively, 46.9% and 33.3% were represented by 3 types of streptococci. In farm A, where clinical mastitis was 6.8%, the share of *S. aureus* was 14.7% of staphylococci and 7.8% of all isolates on the farm, and in farm B, where clinical mastitis was 38.5%, *S.aureus* strains were 68.8% and 45.8%, respectively. The involvement of coagulase-negative staphylococci in the etiology of clinical and subclinical mastitis should not be overlooked. A large percentage of these bacteria were found in both farms. In farm A, they represented up to 85.3% of staphylococcal and 45.3% of all isolates. In farm B, these percentages were 31.3% and 20.8% respectively. Coagulase-negative staphylococci were isolated from the majority of subclinical mastitis as participants in mixed infection or as the main causes. Similar results have been reported by other authors (Yang et al., 2015; Condas et al., 2017; Sztachańska et al., 2016; Zeryehun & Abera, 2017).

In farm A, where streptococci were detected in almost half of the isolates, the species *S.uberis* dominated, contributing to 50% of streptococci and 23.4% of all isolates. In farm B, the streptococcal isolates were significantly lower, but the S.uberis was isolated again with a high frequency, 50% and 16.7%, respectively. The absence of streptococci belonging to the *S.dysgalactiae* species in this farm was impressive.

The results of the *in vitro* sensitivity testing of the isolated strains to antimicrobial agents by the disk-diffusion method showed a relatively preserved sensitivity to the representatives of the main classes of antibiotics and chemotherapeutics used in clinical practice.

In farm A, staphylococci showed the highest percentage of lincomycin resistance (58.8%), followed by tetracyclines (52.9%) and in farm B the highest resistance was noted to amoxicillin - 62.5%. It was noteworthy that 41.2% of staphylococci in farm A and 18.8% in farm B were resistant to oxacillin. This fact should be taken as an alarming signal for the spread of methicillin/oxacillin resistance, which is of great social and health importance (Otto, 2013; Kalayu, 2020). Streptococcal strains were also found to be sensitive to the main classes of antimastitis agents. Polyresistance to six antibiotics was observed in only one strain.

From our study can be concluded that the successful prevention and treatment of mastitis and especially subclinical mastitis should be comprehensive and may include screening tests with rapid field tests, microbiological testing and determination of antimicrobial susceptibility of isolated pathogens. In this way, prevention and control of the spread of resistance among microorganisms could be achieved.

CONFLICT OF REPORT OF INTEREST

The authors declare that they have no conflict of interest regarding authorship and / or the publication of this article. This study is funded by project № 12/2020 / at FVM, Trakia University, Stara Zagora, Bulgaria.

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