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Original Contribution

STREPTOCOCCUS SPP. AS ETIOLOGICAL AGENT OF SUBCLINICAL AND CLINICAL MASTITIS OF DAIRY COWS IN REPUBLIC OF BULGARIA

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ABSTRACT

The aim of this study was to determine the species composition of streptococci causing subclinical, and clinical mastitis in dairy cows, their prevalence and antimicrobial drug sensitivity with respect to appropriate therapy.

The study subject was the involvement and prevalence of bacteria from the genus *Streptococcus* in the etiology of subclinical, and clinical mastitis in dairy cows. The survey was conducted in eight cattle farms from different regions in Bulgaria in 2020 and 2021. After clinical examination and rapid mastitis test, a total of 239 cows were diagnosed with udder inflammation - 34 (14.2%) with clinical signs and 205 (85.8%) with subclinical mastitis. Laboratory analyses were performed on 346 samples. Two hundred and seventy two bacterial strains were isolated, and 151 (55.5%) were determined to belong to *Streptococcus spp.* Lancefield grouping and species identification of haemolytic variants was done. It was found out that in subclinical forms of mastitis, 54.5% of isolates belonged to *Streptococcus* spp., respectively 63.3 % of isolates in clinical forms of mastitis also belonged to *Streptococcus* spp. Out of them, 38.6% were *S. uberis*, 35.6% were identified as *S. agalactiae* and 25.8% – as *S. dysgalactiae*. *In vitro* tests of the sensitivity to antimicrobial drugs from various groups and classes were conducted with all field isolates. The tests demonstrated a relatively well-preserved sensitivity to antimicrobial agents with the exception of tetracycline.

Key words: cows, mastitis, streptococci, prevalence, sensitivity, antibiotics

INTRODUCTION

Udder inflammation is among the most frequent and relevant health issues in dairy cows. Mastitis is associated with enormous economic losses for farmers. The global dairy industry incurs enormous losses from lower milk yield, lower milk quality, often from irreversible damage of udders, costs related to diagnostics, treatment, personnel, resources and time (1-3). The problem is further aggravated by the public health risk from the presence of bacterial pathogens and their toxins in milk, antimicrobial drug residues and last but not least, the selection of bacterial strains resistant to antibiotics and synthetic chemotherapeutics.

The subclinical inflammations of the udder parenchyma are usually more common and precede the clinical manifestation of mastitis. Due to their more prolonged course, the economic impact of occult mammary inflammations is more protracted and thus more relevant (4-7). Therefore, the early diagnosis identification of the specific etiological agent and its sensitivity to antimicrobial drugs in essential for the practice.

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Mastites are usually polyetiological diseases, with and the role of microbial factor – bacteria, fungi and viruses, is most commonly leading. Among bacterial agents, the role of Gram-positive cocci – streptococci and staphylococci is deemed to be of primary importance. Other isolated organisms are *Mycoplasma* spp., *Trueperella* spp., *Nocardia* spp., yeasts and enterobacteria e.g. *Escherichia coli, Klebsiella* spp. etc. The prevalence of pathogenic streptococci within the infected udder is particularly frequent (8-10). These pathogens are responsible for the occurrence of the major part of both clinical and subclinical intramammary infections (11). Haemolytic streptococci as *S. agalactiae* and *S. dysgalactiae*, able to induce mastitis in cows, belong to Lancefield group B and C, respectively, whereas *S. uberis* do not belong to this classification. Thus, apart the haemolytic toxin, streptococci from Lancefield group B produce also a complex of enzymes including peptidase, lipase and lipoteichoic acid. The production of enzymes with various effects is distinctive for Lancefield group C, whose member is S. *dysgalactiae* subsp*. dysgalactiae.* This organism is very often associated with acute mammary gland inflammations in cattle. It possesses the so-called L-antigen, and produces the enzymes like streptokinase, as well as streptolysins O and S (12).

The third streptococcal species that is commonly detected in bovine subacute mastitis, is *S. uberis.* Its pathogenicity factors are not well studied, yet it is acknowledged that it adheres on epithelial cells of mammary ducts causing their destruction and local inflammation.

Regardless of the streptococcal species isolated from cows with clinical and subclinical mastitis, the application of antimicrobial drugs is advised. The intramammary or parenteral use of antibiotics is increasingly associated with the selection of strains that acquired resistance. This necessitates testing the sensitivity of the specific field isolated before the etiotropic therapy. This is not always possible and that is why drugs are empirically applied. That is why periodical isolation and testing of microbial pathogens for their antimicrobial sensitivity to antibiotics and chemotherapeutics should be an integral part of health management at dairy cattle farms.

The aim of this study was to determine the species composition of streptococci causing subclinical and clinical mastitis in dairy cows, their prevalence and antimicrobial drug sensitivity with respect to appropriate therapy.

MATERIAL AND METHODS

Samples

Investigations were carried out in eight dairy cattle farms from different regions in Bulgaria. After clinical examinations and rapid field screening tests, 239 cows were identified with mastitis. A total of 346 milk samples were obtained from them. Samples were collected in sterile containers after the aseptic preparation of teats and discarding the first amounts of milk. Samples were transported at 8^0C to the bacteriological lab of the Department of Veterinary Microbiology, Infectious and Parasitic Diseases at the Faculty of Veterinary Medicine, Trakia University.

Microbiological examinations

After arrival at the lab, samples were centrifuged at 2000 rmp for 10 min, and the pellet of each sample was inoculated on blood agar with 5% defibrinated sheep blood and on MacConkey agar at 37℃ for 24-48 h. Consequent subculturing was performed to isolate pure cultures. The morphological features of presumptive colonies, the presence of haemolysis and its type were evaluated. Microscopic preparations for Gram staining were prepared from pure colonies.

The biochemical identification of bacterial growth was done by tests for the production of catalase and oxidase, hydrolysis of esculin and Christie, Atkins and Munch-Petersen (CAMP) testing. The group affiliation of haemolytic streptococci was determined by latex agglutination kit (Prolex Streptococcal grouping latex kit, Pro-lab diagnostics, Canada). The method is based on extraction of group specific carbohydrate antigens with three extraction reagents, and subsequent identification with blue polystyrene latex particles. The latter are sensitised with purified group specific rabbit immunoglobulins.

Antimicrobial sensitivity tests

The testing of sensitivity of streptococcal isolates to antimicrobial drugs was done by the disk diffusion method of Kirby-Bauer (13) on Mueller-Hinton agar (Hi Media, India) supplemented with 5% defibrinated sheep blood. The following disks were used: amoxicillin 10 µg, amoxicillin/clavulanic acid - 20/10 µg, cephalothin 30 µg, lincomycin 15 µg, gentamicin 10 µg, tetracycline 30 µg, erythromycin 15 µg, rifampicin 5 µg, enrofloxacin 5 µg (Hi Media, India).

After 24-hour incubation, diameters of zones of growth inhibition were measured and results were interpreted by the 3-degree scale of Bauer–Kirby and reported as sensitive (S), intermediate (I) and resistant (R) as per the current EUCAST standard (14).

RESULTS

Baseline data from the field survey on clinical and subclinical mastitis in the 8 target cattle farms are presented in **Table 1**. The results showed that from all examined 1,731 dairy cattle, 34 cows have demonstrated signs of clinical mastitis. At the same farms, 205 animals were positive in the rapid mastitis test. A total of 346 milk samples were collected. After microbiological examination, 272 bacterial strains were isolated. A total of 151 streptococcal isolates were confirmed, which comprised 55.5% of all isolated bacterial agents. The prevalence of streptococcal mastitis at the individual farms varied from 33.3% (farm 2) to 91.1% (farm 3). **Table 2** presents the distribution of streptococcal species isolated from cows with clinical and subclinical udder inflammation. Out of all 242 subclinical isolated, 132 strains (54.5%) were identified as belonging to *Streptococcus* spp. A high prevalence of streptococci (63.3%) was established in cows affected with clinical mastitis (19 out of 30 isolates).

Table 1. *Surveyed farms, microbiologically examined samples and detected Streptococcus spp. isolates*

Farm	Number of cows	Number of samples with clinical mastitis	Number of samples with subclinical mastitis	Total number of examined samples	Total number of isolates	*Streptococcus* spp. (number)	*Streptococcus* spp. (%)
1	280	2	28	30	22	12	54.5
2	114	10	23	33	24	8	33.3
3	350	10	93	103	67	61	91.1
4	500	3	75	78	64	30	46.9
5	60	1	30	31	26	15	57.7
6	96	1	34	35	29	10	34.5
7	61	6	-	6	4	2	50.0
8	270	1	29	30	36	13	36.1
Total	**1731**	**34**	**312**	**346**	**272**	**151**	**55.5**

Table 2. *Distribution of Streptococcus spp. in cases of clnical and subclinical udder inflammations*

Species	**Subclinical mastitis**		**Clinical mastitis**		**Total**	
	Streptococcus spp. number	%	*Streptococcus* spp. number	%	*Streptococcus* spp. number	%
S. agalactiae	47	35.6	14	73.7	61	40.4
S. uberis	51	38.6	1	5.3	52	34.4
S. dysgalactiae	34	25.8	4	21	38	25.2
Total	**132**		**19**		**151**	

Out of all 151 streptococcal isolates, 40.4% were identified as *S. agalactiae*, 34.4% - as *S. uberis* and 25.2% - as *S. dysgalactiae*. Out of the 132 streptococcal isolates involved in subclinical udder inflammation, the proportion of *S. agalactiae* was 35.6%, of *S. uberis* - 38.6% and of *S. dysgalactiae* 25.8%. The species distribution of the observed 19 cows with clinical mastitis was as followed – 73.7% *S. agalactiae*, 5.3% *S. uberis* and 21 % *S. dysgalactiae*.

The behavior of isolated streptococci to antimicrobial drugs are summarised in **Table 3**. It may be seen that the sensitivity of strains varied substantially both among the farms and with regard to different antimicrobial drugs. The highest percent of strains were resistant to tetracycline (73.5%), and the lowest (0.7%) – against the combination amoxicillin and clavulanic acid. High proportions of resistant streptococcal strains were demonstrated against lincomycin (45%), followed by cephalotin (36.4%). Relatively preserved sensitivity was detected to amoxicillin + clavulanic acid (99.3%), erythromycin (78.1%) and amoxicillin (75.5%). The highest share of multiresistant streptococcal isolates from clinical and subclinical mastitis was found out in farms No. 1 and No 3, whereas the lowest – at farm No. 7.

Table 3. *Antimicrobial behavior of isolated Streptococcus spp. strains to 9 antimicrobial drugs (%)*

Farm	Tested strains	Result	Amx	Aug	Cep	E	L	G	T	Rif	Enr
1	12	S	83.3	100	91.7	41.7	41.7	58.3	16.7	75	16.7
		R+I	16.7	0	8.3	58.3	58.3	41.7	83.3	25	83.3
2	8	S	100	87.5	100	87.5	75	75	50	87.5	62.5
		R+I	0	12.5	0	12.5	25	25	50	12.5	37.5
3	61	S	67.2	100	18	93.4	62.3	39.3	16.4	57.4	63.9
		R+I	32.8	0	82	6.6	37.7	60.7	83.6	42.6	36.1
4	30	S	73.3	100	93.3	70	36.7	90	50	70	76.7
		R+I	26.7	0	6.7	30	63.3	10	50	30	23.3
5	15	S	60	100	93.3	60	100	100	0	73.3	100
		R+I	40	0	6.7	40	0	0	100	26.7	0
6	10	S	90	100	90	70	0	100	30	60	100
		R+I	10	0	10	30	100	0	70	40	0
7	2	S	100	100	100	100	100	100	50	100	100
		R+I	0	0	0	0	0	0	50	0	0
8	13	S	100	100	100	76.9	46.2	84.6	38.5	61.5	84.6
		R+I	0	0	0	23.1	53.8	15.4	61.5	38.5	15.4
Total	**151**	**S**	**75.5**	**99.3**	**63.6**	**78.1**	**55**	**67.5**	**26.5**	**65.6**	**70.9**
		R+I	**24.5**	**0.7**	**36.4**	**21.9**	**45**	**32.5**	**73.5**	**34.4**	**29.1**

Legend: **S**-sensitive; **I**-intermediate; **R**-resistant; **Amx**-amoxicillin; **Aug**- amoxicillin+clavulanic acid; **Cep**-cephalotin; **E**-erythromycin; **L**-lincomycin; **G**-gentamicin; **T**-tetracycline; **Rif –** rifampicin; **Enr**-enrofloxacin.

DISCUSSION

The analysis of results from this survey confirmed the important role of pathogenic streptococci in the etiology of clinical and subclinical mastitis in dairy cows, as evidenced by the high percentage of isolated streptococcal strains.

Data demonstrated that *S. uberis* was the commonest streptococcal agent of bovine subclinical mastitis. This is partly in agreement with other reported data (11, 15-17), affirming that *S. uberis* is responsible for a considerable proportion of both subclinical and clinical infections in mammary gland. According to our results however, the most frequently isolated

clinical mastitis agent from the genus *Streptococcus* was *S. agalactiae*. It should be noted that in a similar survey, other researchers have also found out a high prevalence of this species (12.28%) during three consecutive years (18).
The increasing involvement of *S. dysgalactiae* is mastitis etiology is also frequently pointed out. Its significance as mastitis pathogen is often considered to be equal to or even exceeding the prevalence of *S. uberis* (19, 20). In our survey, the participation of *S. dysgalactiae* is also significant, but its occurrence exceeded that of *S. uberis* only in cows affected with clinical mastitis.

The results from the performed *in vitro* testing of the behavior of streptococcal isolates from the udder of cows with subclinical and clinical mastitis to a set of antimicrobial drugs demonstrated a relatively preserved sensitivity to antibiotics and chemotherapeutics that are most frequently applied in the clinical practice. Substantial variations were also observed, as well as the presence of resistance at the farm level. This supports the consideration of numerous researchers that the increasing resistance and selection of multi-resistant strains is most cases is a function of imprudent antibiotic policy at dairy cattle farms (21, 22).

CONCLUSION

Our study allows concluding that the *Streptococcus spp.* members are among the commonest microbial pathogens incriminated as agents of bovine mastitis. They are responsible both for overt and occult mammary gland diseases. In this sense, they cause serious economic losses to dairy cattle farmers. Having in mind the economic impact of this disease, the survey of most commonly involved microbial agents, resistance indices, and therapeutic effects of antimicrobial drugs used in the clinical practice, becomes essential. This is a prerequisite for quality monitoring of the commonest circulating microbial pathogens, the dynamics of antimicrobial resistance, and therefore, more efficient control.

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