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Antimicrobial sensitivity of pathogens causing subclinical mastitis in goats in Bulgaria

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ABSTRACT

The aim of this study was to analyze the antibacterial sensitivity of microbial isolates causing subclinical mastitis in goats. The classical Bauer disk-diffusion assay was used to determine the sensitivity of the coagulase-negative staphylococcal (CNS) isolates and isolates belonging to other genera. In all the tested isolates, the highest rates of resistance were observed to be to Oxacillin (81.25%) and Novobiocin (75%), followed by Penicillin (68.75%), Tetracycline (37.5%) and Amoxycillin (31.25%). There was low prevalence of resistance to Cefotaxime (12.5%), Thiamphenicol (6.25%), Cefuroxim (6.25%), Cefuroxim (6.25%), Amikacin (6.25%), Sulfamethoxazole + Ttrimethoprim (6.25%) and Gentamicin (6.25%). *Staph. intermedius* showed resistance to the highest number of antimicrobial agents, four ones (28.57%), whereas *Staph. haemolyticus* was the least resistant isolate, which was sensitive to nearly all of the tested antimicrobials and showed intermediate sensitivity to only three ones (21.43%). Among the microorganisms other than CNS, *B.brevis* and *Å.coli* showed resistance to the highest number of antimicrobial agents (42.86%).

Key words: Antibacterial sensitivity, Goats, Subclinical mastitis, Microorganisms.

INTRODUCTION

Indiscriminate use of antibiotics for treatment of various conditions in goats has led to emergence of resistant strains of some microorganisms. This leads to unsatisfactory results and economic losses in the treatment of individual clinical cases as well as in programs targeting the treatment of subclinical mastitis (SCM) in whole farms. A number of studies report an increasing number of intramammary infections (IMI) caused by coagulase-negative staphylococci (CNS) in dairy goats (Ryan and Greenwood, 1990, Contreras *et al.* 1995, 1997, 1999., White and Hinckley, 1999). The assessment of the antimicrobial resistance of *Staphylococcus spp*. isolated from goats with SCM is of key importance in the clinical practice. It is also important to identify the antimicrobial agents appropriate for use and for control of the spread of resistant strains in farms. The aim of this study was to obtain up-to-date information about the antimicrobial sensitivity of the isolated CNS and other pathogens causing subclinical mastitis in goats.

MATERIALS AND METHODS

Animals: The study included animals from goat farms of various size. The mean flock size was 100±26 (mean/SD), with the largest farm having 140 goats and the smallest farm, 75 ones. The animals belonged mainly to the Bulgarian white dairy goat (BWD) breed and local crosses. The age range of the animals in the studied farms varied from 1.5 years to over 7 years. All the farms utilized a semi-intensive system of rearing, in which, after the pasture period, the animals were reared in barns and were fed on roughages and concentrated forages during the winter months. The goats had not undergone systemic or local mammary gland antibiotic treatment during the study or the preceding lactation and dry period.

Sampling: Milk samples were collected aseptically from all udder halves (229 halves of 130 goats), that gave a positive result in the CMT-Test (Kruuse, Denmark) and Porta SCC test (Porta Check, USA). Prior to sampling, the mammary papillae and the mammary gland were cleaned up from mechanical contamination, followed by disinfection with 70% ethanol. The first jets of milk from each half were discarded and then 10-ml double samples were collected in sterile tubes for microbiological analysis and 50-ml ones in milk containers for somatic cell counts and physico-chemical analysis. Milk samples were transported to the laboratories in a cooling bag, at 4°C, and all assays were performed within 16 h. from sample taking.

Microbiological analysis: The microbiological analysis for isolation and identification of microbial pathogens causing mastitis in goats was performed in accordance with the adopted method for isolation and differentiation of mastitis causal agents in milk samples. Following plating in elective and selective nutrient media for different groups of bacteria as well as for fungi, the samples were aerobically incubated at 37°C and 28°C for 24–72 h. The following nutrient media

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

Determination of somatic cell counts and differentiation of subclinical mastitis: Somatic cell counts in the samples were enumerated by a fluoro-opto-electronic counter (Fossomatic, Foss, Denmark) according to the EN ISO 13366-2/IDF 148-2:2006 standard at the National Reference Laboratory for Milk and Dairy Products (BFSA, Bulgaria). For the purpose of the present study, an udder half was considered affected by subclinical mastitis if no clinical signs or abnormal milk were present but laboratory tests revealed presence of pathogenic microorganisms and somatic cell counts of > 500,000 cells/ml.

Antimicrobial agents and antibiotic sensitivity testing: The sensitivity of the isolated bacteria to antimicrobial agents was determined using the classical agar gel diffusion assay of Bauer *et al.* (1966). Standard antibiogram discs were used (Bul Bio, NCIPD Ltd, Bulgaria), as well as ones prepared by us by inoculation of exponentially growing bacterial suspensions at a cell density of 2×10^6 cells/ml on Mueller Hinton agar. Incubation was done at 37°C for 24 hours. The results were scored based on the three-level scoring system of Bauer *et al.* (1966) following measurement of the inhibition zone diameters in millimeters.

RESULTS AND DISCUSSION

The sensitivity of some of the isolates to antimicrobial agents *in vitro* is presented in Tables 1 and 2. The obtained results showed that *Staph. intermedius* was resistant to the largest number of antimicrobial agents, four ones (28.57%), whereas the least resistant isolate was *Staph. haemolyticus*, which was susceptible to nearly all of the tested antimicrobials and only showed intermediate resistance to three of them (21.43%). The rest of the *Staphylococcus spp.* isolates showed similar antibiotic resistance, with none of them being susceptible to Penicillin or Novobiocin, and just *Staph. hyicus* being susceptible to Oxacillin. All assayed isolates showed susceptibility to Thiamphenicol, Amikacin, Gentamicin, Enrofloxacin and Ciprofloxacin.

Of the isolates in Table 2, those susceptible to the largest number of tested antimicrobials were *B.brevis* and *Å.coli* (42.86%). *Pr. mirabilis* and *Ps .putida* were resistant to five (35.71%) antimicrobials; *Str.suis, Serr. marcescens* and *Pr. penneri* showed resistance to four antibiotics (28.57), and *E. casseliflavus* was least resistant, showing resistance to just three (21.43%) antimicrobial agents.

Table 3 presents the activity of the tested antimicrobial agents against the studied pathogens causing mastitis in goats.

The results indicated comparatively high sensitivity to the antimicrobial agents from most of the tested groups. The isolates showed highest resistance to Oxacillin (81.25%) and to Novobiocin (75%). The antibiotic that ranked second was Penicillin (68.75%), followed by Tetracycline (37.5%) and Amoxycillin (31.25%). Low prevalence of resistance

Table 1: Antibiotic sensitivity in vitro of Staphylococcus spp. that cause mastitis in goats.

Antimicrobial agent	Concentration in disc ìg/disc	*Staph. caseolyticus*	*Staph. intermedius*	*Staph. simulans*	*Staph. haemolyticus*	*Staph. aureus*	*Staph. saprophyticus*	*Staph. hyicus*	*Staph. adjacens*
Thiamphenicol	30	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Tetracycline	30	***S***	***R***	***S***	***S***	***S***	***S***	***S***	***S***
Lincomycin	15	***S***	***S***	***S***	***S***	***I***	***S***	***S***	***S***
Đenicillin	10 UI	***R***	***R***	***R***	***I***	***R***	***I***	***R***	***R***
Oxacillin	1	***R***	***R***	***R***	***I***	***I***	***R***	***S***	***R***
Amoxycillin	10	***S***	***S***	***S***	***S***	***R***	***R***	***S***	***S***
Cefotaxime	30	***S***	***S***	***S***	***S***	**I**	***S***	***S***	***S***
Cefuroxim	30	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Novobiocin	30	***R***	***R***	***R***	***I***	***I***	***R***	***I***	***R***
Amikacin	30	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Gentamicin	10	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Enrofloxacin	5	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Ciprofloxacin	5	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Sulfamethoxazole + Trimethoprim	23.75 / 1.25	***S***	***S***	***S***	***S***	***I***	***S***	***S***	***S***

S *– susceptible;* ***I*** *– intermediate;* ***R*** *– resistant*

Table 2: Antibiotic sensitivity *in vitro* of microorganisms that cause mastitis in goats.

Antimicrobial agent disc	Concentration in ìg/disc	*Pr. mirabilis* us	*E. casseliflav*	*Str.suis.*	*Serr. marcescens*	*B. brevis*	*Pr. penneri*	*Ps. putida*	*E coli.*
Thiamphenicol	30	***S***	***S***	***S***	***S***	***I***	***S***	***S***	***R***
Tetracycline	30	***S***	***S***	***R***	***R***	***S***	***R***	***R***	***S***
Lincomycin	15	***S***	***S***	***R***	***S***	***R***	***S***	***S***	***R***
Đenicillin	10 UI	***R***	***S***	***S***	***R***	***R***	***R***	***R***	***I***
Oxacillin	1	***R***	***R***	***R***	***R***	***R***	***R***	***R***	***R***
Amoxycillin	10	***S***	***R***	***R***	***S***	***I***	***I***	***I***	***R***
Cefotaxime	30	***S***	***I***	***S***	***S***	**R**	***I***	***R***	***I***
Cefuroxim	30	***S***	***S***	***S***	***S***	***R***	***I***	***S***	***S***
Novobiocin	30	***R***	***R***	***S***	***R***	***R***	***R***	***R***	***R***
Amikacin	30	***R***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Gentamicin	10	***R***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Enrofloxacin	5	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Ciprofloxacin	5	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***
Sulfamethoxazole + Trimethoprim	23.75 / 1.25	***S***	***S***	***S***	***S***	***S***	***S***	***S***	***S***

S *– susceptible;* ***I*** *– intermediate;* ***R*** *– resistant*

Table 3: Antibacterial activity of antimicrobial agents in vitro against microorganisms causing mastitis in goats

Antimicrobial agent	Number of isolates	Sensitivity		
		S	I	R
Thiamphenicol	n	14	1	1
	%	87.5	6.25	6.25
Tetracycline	n	10	0	6
	%	62.5	0	37.5
Lincomycin	n	12	1	3
	%	75	6.25	18.75
Đenicillin	n	2	3	11
	%	12.5	18.75	68.75
Oxacillin	n	1	2	13
	%	6.25	12.5	81.25
Amoxycillin	n	8	3	5
	%	50	18.75	31.25
Cefotaxime	n	10	4	2
	%	62.5	25	12.5
Cefuroxim	n	14	1	1
	%	87.5	6.25	6.25
Novobiocin	n	1	3	12
	%	6.25	18,75	75
Amikacin	n	15	0	1
	%	92.75	0	6.25
Gentamicin	n	15	0	1
	%	92.75	0	6.25
Enrofloxacin	n	16	0	0
	%	100	0	0
Ciprofloxacin	n	16	0	0
	%	100	0	0
Sulfamethoxazole + Trimethoprim	n	14	1	1
	%	87.5	6.25	6.25

was manifested to Cefotaxime (12.5%), Thiamphenicol (6.25%), Cefuroxim (6.25%), Cefuroxim (6.25%), Amikacin (6.25%), Sulfamethoxazole + Trimethoprim (6.25%) and Gentamicin (6.25%). The most potent effect against the assayed mastitis-causing isolates was that of Enrofloxacin and Ciprofloxacin, to which none of the tested microorganisms proved resistant.

The widespread and in some cases indiscriminate use of antibiotics for treatment of a range of conditions in goats has led to emergence of resistant strains of various microbial species. Antimicrobial resistance, along with the diverse microflora causing mastitis, results in unsatisfactory treatment outcomes of individual cases as well as in SCM treatment programs on whole-farm scale.

The sensitivity of the assayed bacterial isolates to the tested antimicrobial agents *in vitro* was shown to be comparatively high. The observed resistance to the penicillin group of antibiotics and partially to tetracycline is most likely due to their history of widespread use in veterinary practice in Bulgaria. In recent years, high levels of resistance of the studied microorganisms to penicillin antibiotics has been observed in isolates from other farm animals, like buffaloes, dogs, cats, lambs and pigs (Parvanov and Popova, 2000; Yordanov *et al.*, 2004; Popova and Kanchev, 2013). At the same time, the high levels of penicillin-resistance of most of the isolates studied here is in contrast to other reports (Moroni *et al.*, 2004) that have indicated penicillin antibiotics as having excellent activity against intramammary pathogens causing inflammation in goats.

The obtained results also showed no resistance to cephalosporins, which is in line with the extremely rare reports of cephalosporin resistance, which mainly depends

on the generation that the agent belongs to in this group. This is also in accordance with the results of Moroni *et al.*, (2004), who observed good activity of Cephalonium and poor performance of Cefoperazone against CNS. The sensitivity that the studied isolates demonstrated to aminoglycosides, quinolones and sulfonamides is an optimistic sign and indicates good treatment options for use of agents belonging to these groups in the treatment of mastitis in goats. It is noteworthy that our study did not identify any pathogens resistant to Ciprofloxacin, which is a second-generation fluoroquinolone antibiotic. This is due to its broad spectrum activity against a wide range of Gram-positive and Gram-negative microorganisms as well as to its excellent permeability in different tissues.

The Enrofloxacin susceptibility observed in this study (i.e. no enrofloxacin-resistant isolates were identified) is in accordance with the data reported by Popova and Todorov (2008), who showed high levels of Enrofloxacin sensitivity in Bulgaira in 93% of the studied microorganisms, intermediate sensitivity in 3% and resistance in 4% only. This agent could be expected to give successful results in the treatment of mastitis in the flocks studied by us, as it shows the best activity against the bacterial isolates from milk samples as well as high activity against *Pseudomonads*, Streptococci and Mycoplasmas (Scheer, 1987).

The Thiamphenicol sensitivity observed in all the assayed microorganisms except for one, a *S. intermedius* strain, could be due to limited use of Amphenicols in productive animals in recent years. Other authors in the country as Popova and Dimitrov (2000, 2001) also establish high sensitivity bacteria from different groups amphenicols.

Our results are in good accordance with other studies similar to ours that analyze the antibiotic sensitivity of pathogens causing mastitis in goats. For example, Virdis *et al.*, (2010), reported CNS to have high levels of sensitivity to Vancomycin (100%), followed by Cefoperazone and Cephalothin (98.7%), Ceftriaxone, Cloxacillin and Novobiocin (97.3%), Oxytetracycline (94.7%) and lowest sensitivity to Ampicillin (64.0%). These results are partly in agreement with ours; however, the reported high sensitivity to Novobiocin was, interestingly, not confirmed by our results. On the contrary, in our study, most of the isolates proved resistant to Novobiocin, with only *Str. suis* showing susceptibility and *Staph. haemolyticus, Staph. aureus* and *Staph. hyicus*, intermediate response.

According to Mhase *et al.,* (2007) in in vitro antimicrobial sensitivity assays, Ciprofloxacin is most effective (100%), followed by Gentamicin (97.62%), Chloramphenicol (95.58%), Tetracycline (81.97%), Ampicillin/Cloxacillin (79.25%), Penicillin (70.75%) and Streptomycin (65.99%).

Similar observations have been previously reported by Mishra *et al.* (1996). Of all isolates from goats with subclinical mastitis, the ones most prevalent are Staphylococci. Antibiotic sensitivity assays of these staphylococci have shown all isolates to be sensitive to *Ciprofloxacin,* Gentamicin and Chloramphenicol. Other antibiotics that are reported to give good results include *Streptomycin* (90.90%) and the combination of *Ampicillin/ Cloxacillin* (54.55%). Bhujbal *et al.* (1999) reported the susceptibility of mastitis organisms from goats showed resistant to Chloramphenicol (12.2%), Gentamicin (21.3%), Oxytetracycline (57.6%), Ampicillin (78.8%) and Amoxycillin (100%

In studies on the sensitivity of CNS isolates from goats with subclinical mastitis in Bulgaria, Bochev and Russenova, (2005), reported high prevalence of resistance to Penicillin (75%) and Amoxycillin (83.3%), Novobiocin (50%), Erythromycin (41.7%) and Lincomycin (33.3%). Compared to this report, our results showed similar prevalence of Penicillin resistance among CNS isolates. However, in contrast to the data reported by Bochev and Russenova, (2005), the results from our study did not confirm high prevalence of Amoxicillin resistance; rather, the prevalence of amoxicillin resistance observed by us was much lower among all the tested isolates, including CNS in particular. Moreover, in our study, the prevalence of Novobiocin resistance proved to be much higher than the 50% reported by Bochev and Russenova, (2005).

In conclusion, this study revealed polymicrobial etiology of mammary gland infections in goats. This, together with the observed resistance to some antimicrobial agents indicate that the causal agents need to be isolated in each individual case of inflammatory process in order to identify the most appropriate antimicrobial agent. To control the emergence of microbial resistance, any antibiotic treatment should be carried out adequately and appropriately. A key step in the development of programs and strategies to combat mastitis in farms is to clearly differentiate the causal agents and apply effective animal treatment schemes. The results clearly show that for the successful treatment of mastitis in goats caused by Staph spp., it is appropriate to use Enrofloxacin, Ciprofloxacin, Gentamicin, Amikacin, Cefuroxime, Cefotaxime.

CONFLICT OF INTEREST

There is no conflict of interest.

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