Bulgarian Journal of Veterinary Medicine (2005), 8, No 2, 109–118

RESISTANCE OF *STAPHYLOCOCCUS SPP*. STRAINS ISOLATED FROM GOATS WITH SUBCLINICAL MASTITIS

I. BOCHEV & N. RUSSENOVA

Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Summary

Bochev, I. & N. Russenova, 2005. Resistance of *Staphylococcus spp*. strains isolated from goats with subclinical mastitis. *Bulg. J. Vet. Med.*, **8**, No 2, 109–118.

One hundred and sixty cases of subclinical mastitis were detected in a study on 478 goats from 6 herds in Southeastern Bulgaria. From positive milk samples, 96 *Staphylococcus spp*. strains were isolated. Nineteen isolates were determined as *Staphylococcus aureus* (19.8%), and the other 77 (80.2%) – as coagulase-negative staphylococci. The sensitivity of all strains to 13 chemotherapeutics was tested through the disk diffusion method of Bauer-Kirby. Via the oxacillin agar screening test, 15 strains (15.6%) were determined as methicillin-resistant: out of them 12 isolates or 80% were coagulase-negative (MRCNS) and only 3 isolates (20%) – methicillin-resistant *Staphylococcus aureus* (MRSA). The cumulative curves of resistance were drawn and their profiles were analysed. The patterns of resistance of MRCNS and methicillin-sensitive coagulase-negative staphylococci (MSCNS) were determined. The results showed that the percentage of resistance of MRCNS to β lactams (75% to penicillin, 83.3% to amoxycillin), macrolides (41.7% to erythromycin), lincomycin (33.3%) and novobiocin (50%) was higher compared to the respective percentages in MSCNS.

Key words: coagulase-negative staphylococci (CNS), cumulative curves, goats, methicillin/oxacillin resistance, MRSA, subclinical mastitis

INTRODUCTION

During the last years, the rearing of goats in Bulgaria is becoming more and more important because of its favorable geographic conditions and the economical advantages of goat rearing compared to that of cattle or sheep. At the same time, the importance of mammary gland pathology especially udder inflammation is increasing too. The effective prevention and etiotropic therapy of these diseases require a precise bacteriological diagnostic and testing the sensitivity of microbial agents to various antimicrobial drugs (Burriel, 1997; Galabinova *et al.*, 1999; Malinowski *et al.*, 2002).

The role of staphylococci in the infectious pathology of men and animals is becoming more and more important due to the nosocomial infections with a high lethality, increasing distribution of resistance and multiresistance to various groups of antimicrobial drugs. The problem has initially appeared with methicillin-resistant *Staphylococcus aureus* (MRSA) and then, with methicillin-resistant coagulase-negative staphylococci (MRCNS) (Tyagunenko & Sotirova, 1997).

More recent studies showed an increasing significance of coagulase-negative staphylococci (CNS) as causative agents of subclinical mastites (Ryan & Greenwood, 1990; Idrissi *et al.*, 1994; Poutrel *et al.*, 1997; Maisi & Riipinen,

1988; Ndegwa *et al.*, 2001). MRCNS are isolated from cows and sheep with clinical and subclinical mastitis (Owens & Watts, 1988, Honkanen-Buzalski *et al.*, 1994, Corrente *et al.*, 2003) and from chickens (Kawano *et al.*, 1996). The infections caused by methicillin-resistant staphylococci (MRS), including MRCNS, cannot be effectively treated with beta-lactam antibiotics (Mitov *et al.*, 2000).

In Bulgaria, there are studies about the detection of MRS in meat and dairy foodstuffs (Bojkova & Stefanov, 1999) and in milk from cows with subclinical mastitis (Urumova *et al.*, 2002). Despite the increasing goat population in our country, there are no studies about the presence of MRS in goats.

The aim of the present study was to perform a bacteriological analysis of sublinical mastitis in goats from both etiological and etiotropic point of view. The isolation of methicillin-resistant staphylococcal isolates is an important prerequisite for more extensive studies on an epidemiological background with regard to the sensitivity of isolates to used antibacterial agents.

MATERIALS AND METHODS

Obtaining and analysis of samples

A preliminary analysis for presence of sublinical mastitis was done in 478 goats from 6 herds in Souteastern Bulgaria via CMT (Kruuse, Denmark, cat. No. 170366). Prior to sampling, the udders were inspected and palpated for estimation of pathological alterations. When such changes were not observed and the CMT was postitive, milk samples were aseptically obtained in sterile 10 mL flasks. The samples were stored at 4˚C and were processed within 2 hours. Milk samples were inoculated on blood agar with 5% defibrinated ovine blood, on mannitol-salt agar and McConkey agar (National Centre of Infectious and Parasitic Diseases, Sofia). The incubation was done aerobically at 37 ˚C for 24-48 h. The presence of more than 3 colonies of a similar morphotype was accepted as positive bacteriological finding (Deinhofer & Pernthaner, 1995).

Identification tests

The tests for routine identification of strains were: test for presence of catalase (with 3% hydrogen peroxide), test for presence of oxidase activity, test for free coagulase (with lyophilized rabbit plasma (National Centre of Infectious and Parasitic Diseases, Sofia).

Other identification tests were those for detection of DNA-ase, urease, alkaline phosphatase, sensitivity to novobiocin and bacitracin, O/F test for determination of carbohydrate utilization. The haemolytical properties of isolates and the pigmentation of colonies were also recorded.

Determination of the sensitivity to antimicrobial drugs

All staphylococcal isolates were tested for their sensitivity to 13 antimicrobial drugs by the disk diffusion method on Mueller-Hinton agar. The strains were determined by the three scale system of Bauer-Kirby as sensitive (S), intermediate (I) and resistant (R) according to Performance Standards for Antimicrobial Disk Susceptibility Tests (Anonymous, 1997).

The used antibacterial drugs and their quantities were as followed: penicillin (6 μg); amoxycillin (25 μg); oxacillin (1 μg); amoxycillin+clavulanic acid (30 μg); gentamicin (10 μg); amikacin (30 μg); tetracycline (30 μg); erythromycin (15 μg); lincomycin (15 μg); nalidixic acid (30

μg); pefloxacin (5 μg); enrofloxacin (5 μg) and novobiocin (5 μg). Filter paper antibiotic disks with a diameter of 6 mm were used.

Methicillin/oxacillin resistant isolates were further screened by inoculation on Mueller-Hinton agar with 4% NaCl and 6 μg/mL oxacillin and incubation for 24–48 h at 35 ˚C.

Analysis of bacterial resistance

Cumulative curves plotting. The cumulative percentage for each inhibition zone diameter was obtained by addition of isolate's percentages for each respective larger inhibition zone to those of larger inhibition zones. The cumulative percentages (Y axis) were plotted against the diameters of inhibition zones (X axis) (Microsoft Word 97). The trendlines were calculated using a 6^{th} degree polynomial approximation of experimental data. The regression equations and the correlation coefficients were calculated using the same software.

Determination of inhibition zone diameter range. Similarly to the MIC range, this parameter was introduced as the difference between the largest and the smallest diameter of inhibition zones of the respective isolates.

Determination of ZD_{50} and ZD_{90}[*]. From the point where the theoretical cumulative curve intercepts the 50% inhibition line, a perpendicular to the X-axis was drawn, and by the point of interception, the value corresponding to ZD_{50} was determined. Usually, the point was between two adjacent diameters. According to the definition, the smaller diameter was chosen because ≥50% of studied strains had inhibition zones with this or larger diameter. ZD_{90} was determined identically.

Determination of resistance patterns. The percentages of resistant isolates showing the respective combinations by respect to their resistance to several chemotherapeutic drugs were determined.

Statistical analysis

The proportion of MRCNS and methicillin-sensitive CNS (MSCNS) isolates, sensitive or resistant to any given antibiotic vs the overall number of isolates in the respective group was assessed by the method of alternative analysis (Sepetliev, 1980). The confidence limits were calculated using the angular transformation by the equation $\varphi=2\arcsin.\sqrt{p}$, where p is the value of the proportion of a given parameter in percents. The maximum error of φ (Δ) was calculated as $\Delta=t/\sqrt{n}$ where t was a coefficient depending on the preselected confidence level (here, 0.95 corresponding to t=1.98) and n was the number of MRCNS or MSCNS isolates. Both confidence limits were determined as $\varphi_1=\varphi-\Delta$ and $\varphi_2=\varphi+\Delta$ (Sepetliev, 1980).

RESULTS AND DISCUSSION

Out of all 478 goats subject to the study, 160 cases of subclinical mastitis were identified. Out of them, in 96 cases (60%), the isolates were *Staphylococcus spp.* strains, 19 (19.8%) of them were coagulase-positive staphylococci (CPS) (*Staphylococcus aureus)*, and the remaining 77 (80.2 %) belonged to the group of CNS.

The screening of the 96 strains on oxacillin agar revealed resistance in 15 (15.6%) strains. Out of them, 12 strains were CNS (15.6% of all 77 strains) and 3

[*] ZD_{50} and ZD_{90} = inhibition zones beyond which ≥ 50% and ≥ 90% of strains are located, respectively.

isolates were CPS (15.8% of all 19 strains).

The data about the behaviour of MRCNS to some antibacterial drugs are presented on Table 1. The highest resistance percentages were those to β-lactams (75% to penicillin and 83.3% to amoxicillin), whereas the resistance to lincomycin, erytromycin and novobiocin were 33.3%, 41.7% and 50% respectively. The isolates were not resistant to aminoglycosides and tetracycline. The behaviour of MSCNS to tested antibacterial drugs is presented in Table 2. The resistance to β-lactams was 49.2 % to penicillin and 30.8% to amoxycillin.

In the 3 CPS resistant isolates (MRSA), 2 were resistant to β-lactams and one isolate – resistant to β-lactams, macrolides, lincosamides and novobiocin.

The high percentage of resistance to β-lactams in both groups of staphylococci is probably related to their wide use in the therapy of mastitis. The mechanisms of resistance to these antibiotics include the synthesis of alternative (with a very low affinity towards β lactams) penicillin-binding protein as well as the production of more than 200 types of β-lactamases (Hawkey, 2000). Among staphylococci, a cross-resistance to all penicillins and cephalosporins exists (Chambers, 1998).

MSCNS exhibited a lower percentage of resistance to erythromycin (1.5%), pefloxacin (1.5%) and novobiocin (3.4%) and no resistance to lincomycin and enrofloxacin. Both groups of staphylococci were not resistant to aminoglycosides. In MSCNS, a certain resistance to tetracycline was established (23.1 %) unlike MRCNS, that did not exhibit any resistance. The causes for these behaviours to tetracycline remain unclear.

The higher resistance of MRCNS to macrolides and lincomycin could be explained by their frequent use in veterinary practice and the existing cross-resistance to them.

The low percentages of resistance to fluroquinolones (enrofloxacin and peflo-

Table 1. Sensitivity of methicillin-resistant coagulase-megative staphylococci (MRCNS), isolated from goats with subclinical mastitis, to 13 antibacterial drugs; n=12

Antibacterial drugs	Sensitive (S)		Intermediate + resistant (I+R)	
	%	Confidence limits	%	Confidence limits
Penicillin	25.0	17.5÷33.3	75.0	66.8÷82.4
Amoxycillin	16.7	10.5÷24.1	83.3	76.0÷89.5
Oxacillin	0	0	100.0	99.7÷100.0
Amoxycillin + clavulanic acid	83.3	76.0÷89.5	16.7	10.5÷24.1
Gentamicin	100.0	99.7÷100.0	0	0
Amikacin	100.0	99.7÷100.0	0	0
Tetracyclin	100.0	99.7÷100.0	0	0
Lincomycin	66.7	57.9÷75.0	33.3	25.1÷42.1
Erythromycin	58.3	49.3÷67.1	41.7	32.9÷50.8
Nalidixic acid	8.3	4.0÷14.0	91.7	86.0÷86.2
Enrofloxacin	91.6	85.9÷86.1	8.4	4.1÷14.1
Pefloxacin	83.3	76.0÷89.5	16.7	10.5÷24.1
Novobiocin	50.0	40.0÷59.1	50.0	40.0÷59.1

Table 2. Sensitivity of methicillin-sensitive coagulase-megative staphylococci (MSCNS), isolated from goats with subclinical mastitis, to 13 antibacterial drugs; n=65

Antibacterial drugs	Sensitive (S)		Intermediate + resistant (I+R)	
	%	Confidence limits	%	Confidence limits
Penicillin	50.8	49.3÷52.4	49.2	47.7÷50.8
Amoxycillin	69.2	67.8÷70.6	30.8	29.4÷30.9
Oxacillin	100.0	99.9÷100.0	0	0
Amoxycillin + clavulanic acid	100.0	99.9÷100.0	0	0
Gentamicin	100.0	99.9÷100.0	0	0
Amikacin	100.0	99.9÷100.0	0	0
Tetracyclin	76.9	75.6÷78.2	23.1	21.8÷24.4
Lincomycin	100.0	99.9÷100.0	0	0
Erythromycin	98.5	98.1÷98.9	1.5	1.2÷1.9
Nalidixic acid	10.8	9.9÷11.8	89.2	88.2÷90.2
Enrofloxacin	100.0	99.9÷100.0	0	0
Pefloxacin	98.5	98.1÷98.9	1.5	1.2÷1.9
Novobiocin	96.6	99.4÷99.8	3.4	2.9÷4.0

xacin) in MRCNS and MSCNS are probably due to the fact that they are not used in mastitis therapy and to the lack of plasmide transfer.

Figures 1, 2 and 3 present the experimental cumulative curves of the sensitivity of staphylococci to penicillin, amoxycillin and lincomycin. The cumulative curves of MSCNS and MRCNS to penicillin (Fig. 4), lincomycin (Fig. 5), amoxycillin (Fig. 6), erythromycin (Fig. 7) and novobiocin (Fig. 8) are also depicted. The experimental cumulative curves show the relationship between the inhibition zones (X-axis) and cumulative percentages (Y-axis).

The experimental cumulative curve of 96 isolates to pencillin (Fig. 1) shows that the diameters of inhibition zones ranged between 40 (the highest) and 6 mm (the lowest). The value of ZD_{50} was 8 mm and that of ZD_{90} − 7 mm, as the higher inhibition diameter corresponding to ≥ 90% of

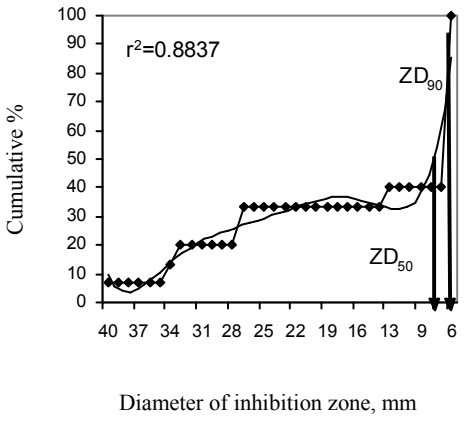

Fig. 1. Cumulative curve of resistance of *Staphylococcus spp.* strains isolated from caprine milk samples to penicillin (−♦−); theoretical curve (−); r^2=coefficient of correlation between both curves, ZD_{50} and ZD_{90} −inhibition zones, beyond which ≥ 50% and ≥90% of strains are located respectively.

isolates was 7 mm. For amoxycillin, the respective values of ZD_{50} and ZD_{90} were 8 and 7 mm (Fig. 2). The inhibition diameters ranged between 33–6 mm. The diameter range for lincomycin (Fig. 3) was 40–6 mm. The ZD_{50} value was 25 mm, and the ZD_{90} value – 7 mm.

Fig. 4. presents the experimental cumulative curves of sensitivity of MRCNS and MSCNS isolates to penicillin. The cummulative curve to penicilin in MSCNS strains was located on the left (towards larger zones) and illustrated a lower resistance. The diameters of inhibition zones of MSCNS strains ranged between 48 and 6 mm, whereas those for MRCNS strains – between 45 and 6 mm. The ZD_{50} and ZD_{90} values for MSCNS determined on the basis of the theoretical curve, were 32 and 14 mm respectively. For the MRCNS strains, ZD_{50} was 7 mm. Fify percents of MRCNS isolates exhibited an inhibition zone of 6 mm.

Fig. 5 presents the experimental cumulative curves of MRCNS and MSCNS sensitivity to lincomycin. The range of inhibition zones for MSCNS strains was from 43 to 22 mm and the curve was located to the left in the region of bigger inhibition zones. The ZD_{50} value was 34 mm, and that of ZD_{90} was 31 mm. The distribution of isolates depending on the maximum theoretical percentage in this group was monomodal. In the group of MRCNS strains, the inhibition zone diameters ranged between 37 and 6 mm, ZD_{50} was 26 mm and the other half of isolated had an inhibition zone of 6 mm.

The cumulative curves of sensitivity to the other β-lactam, amoxycillin are given on Fig. 6. The diameters of inhibition zones were between 45 and 6 mm (for MSCNS isolates) and from 33 to 6 mm (for MRCNS strains). The values of ZD_{50} and ZD_{90} for methicillin-sensitive isolates were 28 and 9 mm, respectively. More than a half of MRCNS isolates,

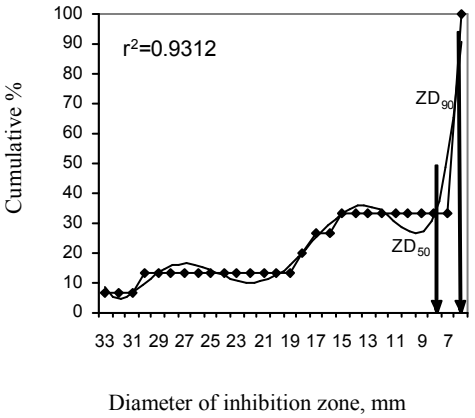

Fig. 2. Cumulative curve of resistance of *Staphylococcus spp.* strains isolated from caprine milk samples to amoxycillin (–♦–); theoretical curve (–); r^2=coefficient of correlation between both curves, ZD_{50} and ZD_{90}– inhibition zones, beyond which ≥ 50% and ≥ 90% of strains are located respectively.

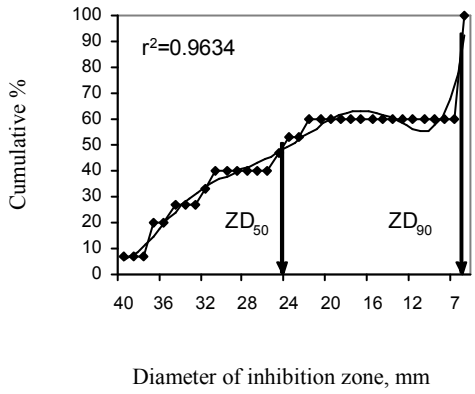

Fig. 3. Cumulative curve of resistance of *Staphylococcus spp.* strains isolated from caprine milk samples to lincomycin (–♦–); theoretical curve (–); r^2=coefficient of correlation between both curves, ZD_{50} and ZD_{90}– inhibition zones, beyond which ≥ 50% and ≥ 90% of strains are located respectively.

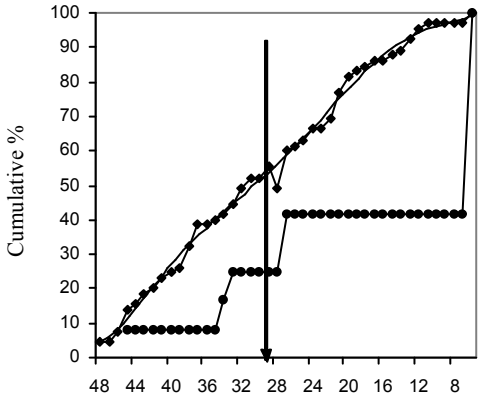

Fig. 4. Cumulative curve of resistance of MSCNS (–♦–) and MRCNS (–•–) strains isolated from goats with subclinical mastitis to penicillin. The arrow indicates the break point between sensitive and resistant isolates

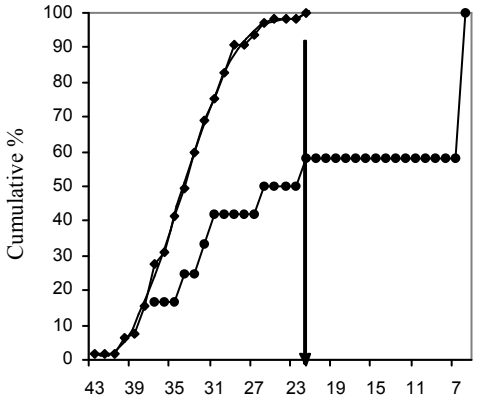

Fig. 5. Cumulative curve of resistance of MSCNS (–♦–) and MRCNS (–•–) strains isolated from goats with subclinical mastitis to lincomycin. The arrow indicates the break point between sensitive and resistant isolates.

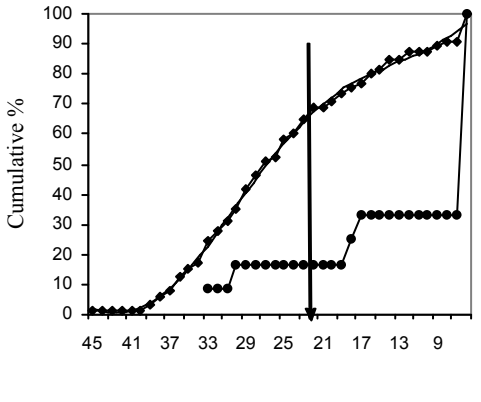

Fig. 6. Cumulative curve of resistance of MSCNS (–♦–) and MRCNS (–•–) strains isolated from goats with subclinical mastitis to amoxycillin. The arrow indicates the break point between sensitive and resistant isolates.

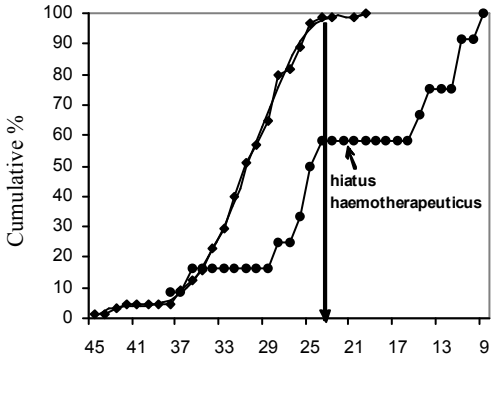

Fig. 7. Cumulative curve of resistance of MSCNS (–♦–) and MRCNS (–•–) strains isolated from goats with subclinical mastitis to erythromycin. The arrow indicates the break point between sensitive and resistant isolates.

similarly to the behaviour to penicillin, gave an inhibition zone of 6 mm.

The experimental cumulative curves of the sensitivity to erythromycin (Fig. 7) showed inhibition zone of 45–20 mm for MSCNS strains and 38–9 mm for MRCNS strains. The experimental cumulative curve of MSCNS was well visible on the left side of the MRCNS curve. More than 90% of isolates in this groups showed an inhibition zone of 23 mm. The values of ZD_{50} and ZD_{90} in the other group of strains (MRCNS) were 25 and 12 mm respectively. A plateau, hiatus haemotherapeuticus, was observed.

The inhibition zones of MSCNS strains to novobiocin (Fig. 8) were 43–13 mm, whereas those of MRCNS isolates were 33–6 mm. More than 90% of MSCNS isolates yielded an inhibition zone of 21 mm. In the second group, ZD_{50} was 27 mm whereas ZD_{90} – 11 mm.

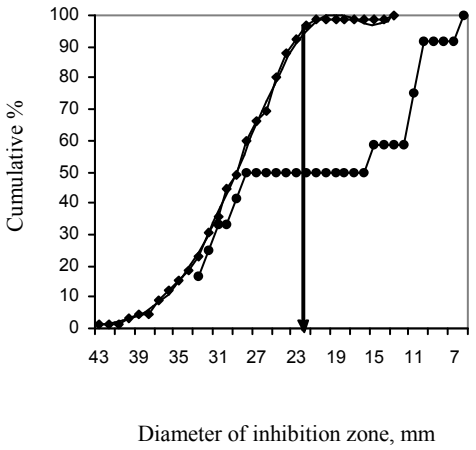

Fig. 8. Cumulative curve of resistance of MSCNS (–♦–) and MRCNS (–•–) strains isolated from goats with subclinical mastitis to novobiocin. The arrow indicates the break point between sensitive and resistant isolates.

The comparison of resistance percentages to penicillin, amoxycillin, erythromycin, lincomycin and novobiocin of MRCNS and MSCNS isolates using the method of alternative analysis revealed a statistically significant difference among them ($P \leq 0.05$).

The commonest resistance patterns were: penicillin/amoxycillin (P-Ax) – 83.3% for MRCNS and 26.2% for MSCNS, penicillin/amoxycillin/novobiocin (P-Ax-Nb) – 50% for MRCNS and 1.5% for MSCNS and penicillin/amoxicillin/lincomycin (P-Ax-L) – 50% for MRCNS.

The results showed a resistance to oxacillin in 15.6 % of isolates. This perceentage of resistance is lower than that in sheep (24%; Corrente *et al.*, 2003) and chickens (25.7%; Kawano *et al.*, 1996). In cows with intramammary infections, the results are variable. Some authors did not evidence MRSA (Gentilini *et al.*, 2000), whereas others reported an incidence of MRSA in 25.7% of cases (Urumova *et al.*, 2002).

Our study showed that in general, the number of MRS isolates and especially that of MRSA was relatively low. The obtained results confirm the increasing role of CNS in the aetiology of various human and animal infections (Burriel & Scott, 1998). It was shown that subclinical mastitis in sheep, provoked by CNS, resulted in reduction in milk production with up to 37% and in lower weight of suckling lambs by up to 30% compared to healthy animals (Watkins *et al.*, 1991). Also, the importance of MRCNS as a pool of resistance for other staphylococci is identified (Gentilini *et al.*, 2002). According to the same authors, cows with intramammary infections caused by MRCNS or multiresistant CNS should be culled.

Our data showed a considerably higher percentage of resistance in MRCNS than in MSCNS, isolated from goats to some chemotherapeutics as β-lactams, macrolides, lincomycin and novobiocin.

CONCLUSIONS

In our study on goats with sublicnial mastitis, CNS strains were found to prevail over CPS isolates (80.2% and 19.8% respectively).

Out of the total number of CNS isolates, 15.6% were methicillin-resistant. The respective percentage in CPS (15.8%) was similar.

In MRCNS, a high percentage of resistance (over 50%) was observed to penicillin, amoxycillin and nalidixic acid whereas in MSCNS – only to nalidixic acid.

The commonest resistance pattern in both groups of staphylococci was P-Ax – 83.3% in MRCNS and 26.2% in MSCNS.

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Paper received 15.04.2004; accepted for publication 26.01.2005

Correspondence:
Dr. Iliyan Bochev,
Section of Microbiology,
Faculty of Veterinary Medicine,
Trakia University,
Student's Campus,
6000 Stara Zagora, Bulgaria