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RESISTANCE OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM CATTLE WITH SUBCLINICAL MASTITIS

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Summary

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The study was performed on 470 lactating cattle originating from 5 farms in South Bulgaria.

Eighty-eight cases of occult mammary inflammation were found. From them, 74 *Staphylococcus spp*. strains were determined. Fourteen strains were identified as *Staphylococcus aureus*, and the rest were coagulase-negative staphylococci.

After the performance of the methicillin/oxacillin test, 19 staphylococcal isolates (25.7%) were determined as methicillin-resistant (MRS). The sensitivity of methicillin-resistant and methicillin-sensitive strains to 12 of the most commonly used chemotherapeutical drugs was determined. The profiles of cumulative curves were analysed. The results showed that the percentages of resistance of methicillin-resistant *S. aureus* (MRSA) strains to β-lactams, aminoglycosides, tetracyclines, macrolides and lincomycin were higher than those of MRS strains.

Key words: cumulative curves, drug resistance, methicillin resistance, MRSA, subclinical mastitis

INTRODUCTION

Subclinical mastitis is among the most substantial problems of dairy cattle breeding. It is etiologically related to various microbial species - streptococci, staphylococci, corineform bacteria, enterobacteria, *Mycoplasma spp*. etc. (Cullor et al.,1990; Rebhun et al.,1995). According to some authors, subclinical mastitis (occult or apparent) is among the commonest (Wats,1990; Nickerson and Owens, 1993).

It is established that coagulase-positive staphylococci and most commonly *S. aureus* as well as coagulase-negative strains could be responsible for the inflammation of the mammary gland (Eberhart et al., 1981; Timms and Scultz, 1987; Hogan et al., 1987; Owens and Waris, 1988; Harmon and Langlois, 1989; Honkanen-Buzalski, 1990; Henkanen-Buzalski et al., 1994).

It is known that some staphylococci are common for animals and humans, so the former could serve as reservoir for infection of the latter. The problem becomes particularly actual after the series of reports about animals being carriers of methicillin/oxacillin resistant staphylococci – the so-called methicillin-resistant staphylococci (MRS) (Devriese and Hommez, 1975; Ceffai et al., 1994; Anzai et al., 1996; Kawano et al., 1996; Hartmann et al., 1997). Some of the cases refer exactly to strains isolated from the mammary gland of cattle with clinical mastitis (Devriese and Hommez, 1975).

A distinctive feature of methicillin/ oxacillin resistance is that it is not mediated by β-lactamases that destroy the antibiotic, but is realized via the production of a complementary penicillin-binding protein named PBP2a that has a very weak affinity towards β-lactams. Therefore, MRS are not inhibited by β-lactams in their therapeutical concentrations. There is a cross resistance to all penicillins and cephalosporins (Chabmbers, 1988).

MRS are further characterized by a high resistance to an important part of clinically applied antibacterial drugs and are considered as one of the commonest causes of nosocomial infections in humans and therefore, one of the most serious problems of hospital health care (Chabmbers, 1988).

A methicillin/oxacillin resistance is found out in both *S. aureus* (MRSA) and coagulase-negative species (Honkanen-Buzalski et al., 1994; Anzai et al., 1996; Kawano et al., 1996; Chabmbers, 1988).

The question of MRS incidence among animals in the Republic of Bulgaria is hardly studied. Bojkova and Stefanov (1999) are the only investigators that reported evidence for MRS in 31.3% of studied meat and dairy foodstuff samples.

The aim of the present investigation was to study the behaviour of *Staphylococcus* isolates from cattle with subclinical mastitis to antibiotics, most frequently used in veterinary practice and to investigate the possible occurrence of methicillin-resistant strains and their features in the light of considered aspect.

MATERIALS AND METHODS

Four hundred and seventy lactating cattle originating from 5 farms in South Bulgaria were studied via a quick diagnostic tetst (Mastitis test NK, Bioveta). One hundred and twenty-two milk secretion specimens were obtained from all animals that responded positively to the test and were examined for the presence of staphylococci. The specimens were inoculated on blood agar with 5% defibrinated ovine blood and on mannitol-salt agar (BBL). Gram-positive isolates were studied for catalase production (with 3% hydrogen peroxide) and coagulase production (with rabbit plasma from the National Centre of Infectious and Parasitic Diseases) according to routine bacteriological methods.

Additional identification tests were carried out for detection of haemolysis, pigmentation of colonies, presence of oxidase, DNA-ase production and sensitivity to novobiocin.

The behaviour to antimicrobial drugs was determined by the disk diffusion method on Muller-Hinton agar. The results were interpreted with the three scale system of Bauer-Kirby according to Performance Standards for Antimicrobial Disk Susceptibility Tests – Sixth Edition, 1997, NCCLS, M2 – A6, vol.17, No.1.

The strains were divided to sensitive (S) and resistant (R), the latter including the intermediate isolates as well (R+I). The disks contained the following concentrations of antibiotic (μg/disc): penicillin (6); amoxycillin (25); oxacillin (1); gentamicin (10); amikacin (30); spectinomycin (100); tetracycline (30); lincomycin (15); eryhtromycin (15); nalidixic acid (30); enrofloxacin (5) and pefloxacin (5).

The responses to methicillin/oxacillin were tested by measuring the growth on oxacillin-salt screening agar with 6 μg/ml oxacillin and 4% NaCl, as well as by interpretation of the zone of inhibition around a disc containing 1 μg oxacillin.

The presence of methicillin/oxacillin resistance was further studied by an differentiated analysis of behaviours of methicillin-sensitive and methicillin-resistant isolates in a comparative aspect.

The cumulative curves of some antibiotics were analysed by plotting the cumulative percentages of the respective inhibition zones and the diameter of zones.

RESULTS AND DISCUSSION

The screening for subclinical mastitis revealed 88 cases of occult mammary inflammation (18.7%) from the total of 470 studied animals. Seventy four strains, determined as *Staphylococcus spp*. were isolated. Twenty four were identified as *Staphylococcus aureus* and the other 50 belonged to the group of coagulase-negative staphylococci (CNS).

The oxacillin test detected 19 isolates (25.7%) as methicillin-resistant and 55 – as methicillin/oxacillin sensitive. All 19 methicillin-resistant isolates were identified as *Staphylococcus aureus*. The other 5 isolates from this species were methicillin-sensitive.

The results for the behaviour of all 74 *Staphylococcus* isolates to tested 12 antibacterial drugs are presented on Fig. 1. More than one half of isolates showed a relatively high sensitivity to aminoglycosides gentamicin, amikacin and spectinomycin, to β-lactam antibiotics and fluoroquinolones.

The percentages of isolates sensitive to erythromycin and lincomycin were lower that could be explained with their frequent application in mastitis therapy as well as with the long-term utilization of tylosin in cattle breeding and with the cross resistance among macrolides, lincosamides and streptogramin (the so-called MLS

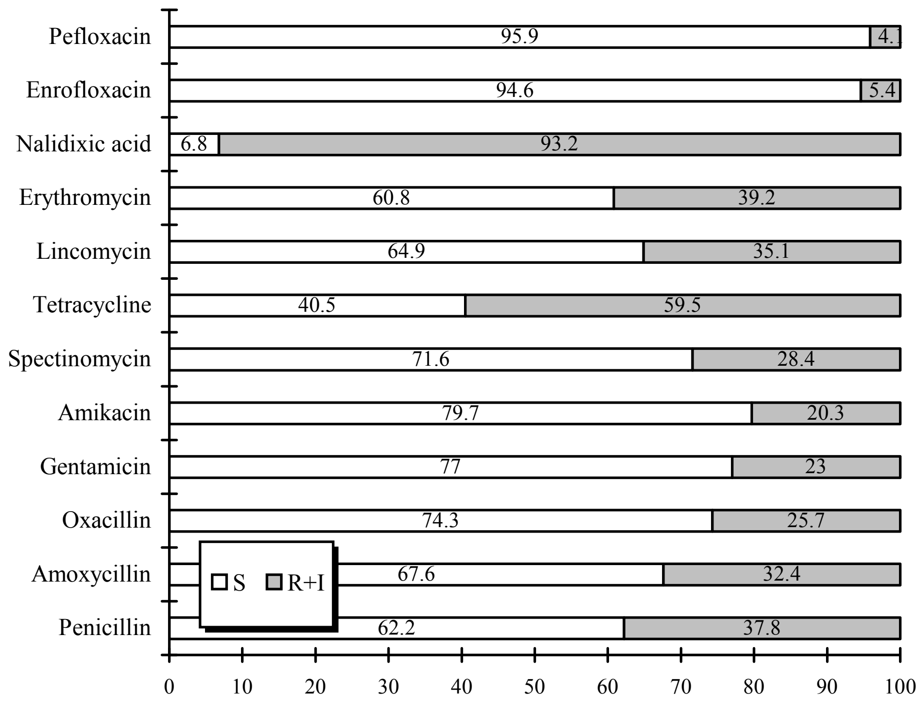

Fig. 1. Percentage of resistant (R) + intermediate (I) and sensitive (S) *Staphylococcus aureus* strains (n=74) isolated from cattle wth subclinical mastitis.

Table 1. Sensitivity of 55 methicillin-sensitive *Staphylococcus aureus* strains isolated from cattle with subclinical mastitis to 12 antibacterial drugs

Antibacterial drug	Sensitive (S)			Resistant (R) + intermediate (I)		
	n	%	Confidence limits	n	%	Confidence limits
Penicillin	45	81.8	71.6÷92.0	10	18.2	8.0÷28.3
Amoxycillin	49	89.1	80.8÷97.3	6	10.9	4.0÷20.6
Oxacillin	55	100.0	98.2÷100.0	0	0	0÷1.8
Gentamicin	48	87.3	78.5÷96.1	7	12.7	3.9÷21.5
Amikacin	49	89.1	80.8÷97.3	6	10.9	4.0÷20.6
Spectinomycin	46	83.6	73.8÷93.4	9	16.4	6.6÷26.2
Tetracycline	24	43.6	43.3÷69.5	31	56.4	43.0÷69.4
Lincomycin	40	72.7	60.9÷84.5	15	27.3	16.3÷40.0
Erythromycin	39	70.9	58.8÷82.9	16	29.1	17.1÷41.1
Nalidixic acid	4	7.3	0.4÷14.2	51	92.7	85.3÷99.6
Enrofloxacin	53	96.4	89.8÷99.8	2	3.6	0.3÷10.2
Pefloxacin	53	96.4	89.8÷99.8	2	3.6	0.3÷10.2

Table 2. Sensitivity of 19 methicillin-resistant *Staphylococcus aureus* strains isolated from cattle with subclinical mastitis to 12 antibacterial drugs

Antibacterial drug	Sensitive (S)			Resistant (R) + intermediate (I)		
	n	%	Confidence limits	n	%	Confidence limits
Penicillin	1	5.26	0.1÷19.9	18	94.7	80.1÷100
Amoxycillin	1	5.26	0.1÷19.9	18	94.7	80.1÷100
Oxacillin	0	0	0÷5.1	19	100.0	94.8÷100
Gentamicin	9	47.4	24.9÷69.8	10	52.6	30.1÷75.0
Amikacin	10	52.6	30.1÷75.0	9	47.4	25.6÷69.8
Spectinomycin	7	36.8	15.1÷58.5	12	63.2	40.5÷83.2
Tetracycline	6	31.6	10.7÷52.5	13	68.4	47.5÷89.3
Lincomycin	8	42.1	19.9÷64.3	11	57.9	35.7÷80.1
Erythromycin	7	36.8	15.1÷58.5	12	63.2	41.5÷84.8
Nalidixic acid	1	5.3	-	18	94.7	-
Enrofloxacin	17	89.5	71.7÷99.0	2	10.5	1.0÷28.2
Pefloxacin	18	94.7	80.1÷100	1	5.26	0.1÷19.9

group).

The highest percentage of resistant isolates (59.5%) were detected against tetracycline. This fact could be explained by the broad range of mastitis drugs from the tetracycline group from one side and by the incorrect use of tetracyclines due to assumption for the broad spectrum of activity of those antibiotics that is in the basis of empirical therapy, on the other.

The results of the differentiated analysis of behaviour of methicillin-resistant isolates of staphylococci (MSS) are presented on Table 1. It is seen that the percentages of MSS isolates resistant against β-lactam antibiotics was relatively low – 18.2% for penicillin and 10.9% for amoxycillin. Most probably, this resistance was a consequence from the production of β-lactamases or from the active efflux of antibiotic substances, still more that there was a multiresistance in part of isolates.

The percentages of strains resistant to aminoglycoside antibiotics and fluoroquinolones enrofloxacin and pefloxacin were low. This could be explained by the fact that they are not used in mastitis therapy and that there is not a possibility for a horizontal transfer of resistance because of the absence of plasmid determination.

The resistance to tetracycline in MSS isolates was high (56.4%).

The results for the behaviour of MRSA strains were different (Table 2). They showed an almost total resistance to the other 2 tested β-lactam antibiotics (penicillin and amoxycillin). The configuration of cumulative curves are accordingly shifted to the right, almost entirely in resistance sectors (Fig. 2 and 3).

The resistance of MRSA to aminoglycosides was also manifested with high percentages – 52.6% and 47.4% for gentamicin and amikacin respectively, although the latter has practically no application in mastitis therapy. The differences vs MSS strains were significant. For gentamicin, they are further illustrated by

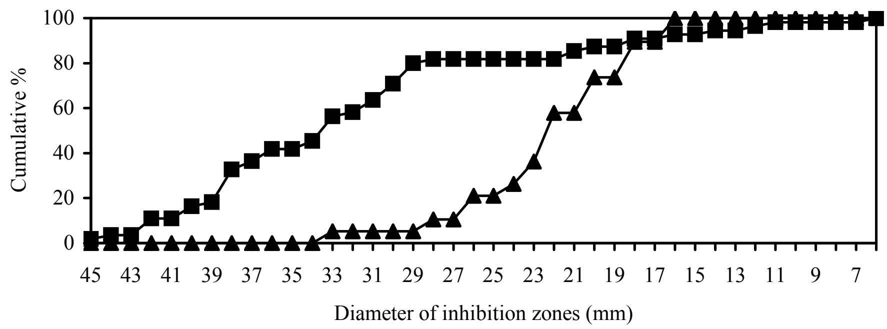

Fig. 2. Cumulative curves of resistance of methicillin-sensitive (– –) and methicillin-resistant (– –) *Staphylococcus aureus* isolates to penicillin.

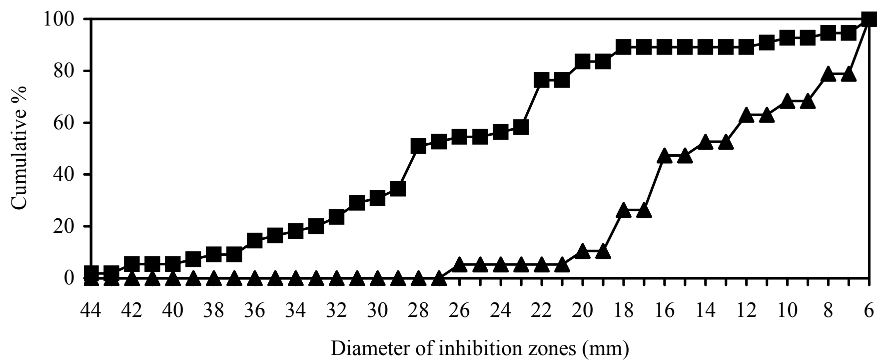

Fig. 3. Cumulative curves of resistance of methicillin-sensitive (– –) and methicillin-resistant (– –) *Staphylococcus aureus* isolates to amoxycillin.

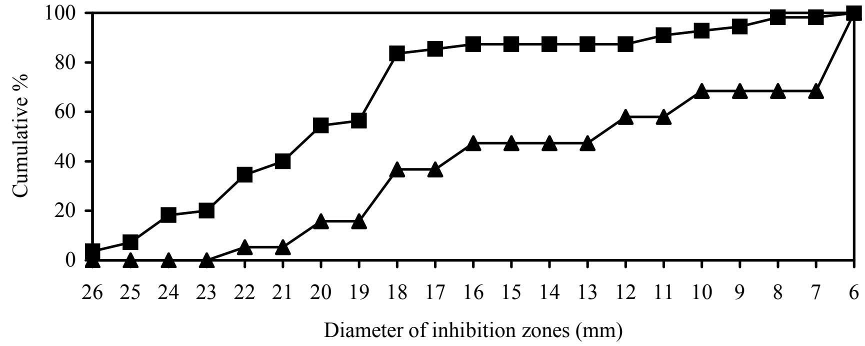

Fig. 4. Cumulative curves of resistance of methicillin-sensitive (– –) and methicillin-resistant (– –) *Staphylococcus aureus* isolates to gentamicin.

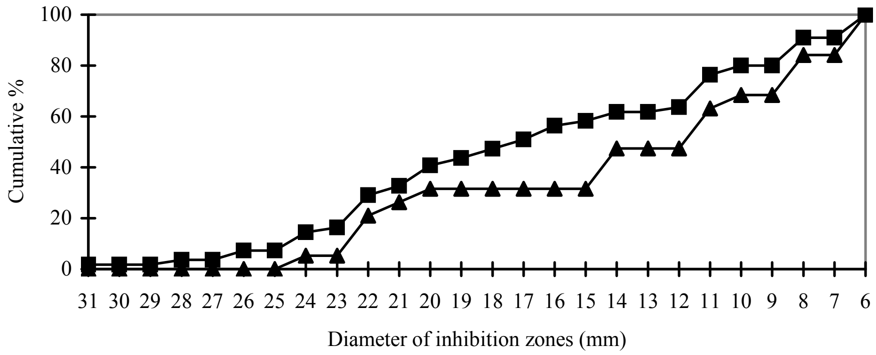

Fig. 5. Cumulative curves of resistance of methicillin-sensitive (– –) and methicillin-resistant (– –) *Staphylococcus aureus* isolates to tetracycline.

cumulative curves (Fig. 4).

Statistically significant differences vs MSS isolates were observed by respect to spectinomycin resistance - 16.4% in MSS and 63.2% in MRSA.

A partial overlapping of confidence limits (i.e. lack of statistical significance) was shown for the resistance of both groups to tetracycline and lincomycin although the percentages of resistant MRSA strains were higher.

The cumulative curves of tetracyclines were very close one to another (Fig. 5). This is a convincing evidence that the resistance to those antibiotics was unvariably high and was possibly mediated by several mechanisms – synthesis of ribosomal protective proteins, active efflux or via permeability barrier effects with all possibilities for a horizontal transfer by plasmids and transposons.

The configurations of cumulative curves of lincomycin for both groups of staphylococci were not as similar (Fig. 6) that was an indirect indication for the increasesing resistance of MRSA. The data obtained for erythromycin were comparable to those of lincomycin, but the differences between both groups were significant.

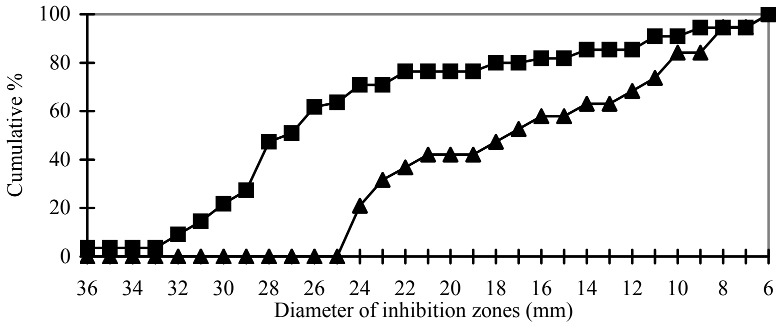

Fig. 6. Cumulative curves of resistance of methicillin-sensitive (– –) and methicillin-resistant (– –) *Staphylococcus aureus* isolates to lincomycin**.**

The lowest levels of resistance were observed against enrofloxacin and pefloxacin, without statistically significant differences between MSS and MRSA. This could be anticipated because they were not used in mastitis therapy and their application for other goals in studied farms was limited.

The analysis of data revealed that bovine mastitis in studied farms was related to strains, resistant to one or several antibiotics – to those, indicated for mastitis therapy as well as to those, rarely used or practically not used for this purpose. Such strains persist on udder skin and/or in the teat channels and could, in our cases, be responsible for subcliical mastitis. We suggest that the selection of resistant strains could be due to the performance of empirical therapy for treatment of mastitis in dairy cows.

The isolation of MRSA is alarming that cows with occult inflammations are a potential source of such isolates for humans without knowing the primary source of infection – whether this is the personnel that takes care for cows, especially the milking staff or the infection is due to genetic transfer via other mechanisms.

In all cases, the veterinary specialists in cattle breeding farms have to face the problem and to perform control by veterinary epidemiological services or the veterinary sanitary services.

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