Acta Veterinaria (Beograd), Vol. 52. No. 5-6, 337-344, 2002.

UDK 619:618.19-002:636.2

ANTIBIOTIC SUSCEPTIBILITY AND β-LACTAMASE PREVALENCE FOR STAPHYLOCOCCI ISOLATED FROM BOVINE MASTITIC MILK SAMPLES

TURUTOGLU H, MUDUL S and PEHLIVANOGLU F

Department of Microbiology, Faculty of Veterinary Medicine, Akdeniz University, Burdur - Turkey

(Received 14. june 2002)

In this study, it was aimed to investigate the mastitis pathogens and to determine the β-lactamase activities of staphylococci isolated from bovine mastitic milk samples and the sensitivities of these isolates to various antibiotics.

For this purpose, 1180 dairy cows On 131 farms were examined for mastitis and 496 mastitic milk samples were taken from 249 cows. Staphylococcus aureus was present in 33.16 %, coagulase negative staphylococci in 31.10 %, Escherichia coli in 11.57 %, Streptococcus uberis in 6.43 %, Streptococcus dysgalactiae in 5.39 %, Streptococcus agalactiae and Streptococcus spp. in 3.86 %, Candida spp. in 1.29 %, Pseudomonas aeruginosa in 1.03 %, Bacillus spp. in 0.77 %, Citrobacter freundii and Proteus vulgaris in 0.51 %, Actinomyces pyogenes and Enterobacter aerogenes in 0.26 % of the samples.

The resistance rates of 250 staphylococci to amoxi-cillin +clavulanic acid, danofloxacin, enrofloxacin, cloxacillin, gentamycin, neomycin, amoxicillin, ampicillin, oxytetracycline, penicillin, erythromycin and trimethoprim+sulphamethoxazole were 4 %, 10 %, 10.4 %, 18.8 %, 40 %, 40.8 %, 42 %, 45.6 %, 48 %, 50.4 % and 60.4 %, respectively. β-lactamase was produced by 65.1 % of S. aureus isolates and 29.8 % of coagulase negative staphylococci. The most effective antibiotics for β-lactamase producing isolates were amoxicillin+clavulanic acid, danofloxacin, enrofloxacin and cloxacillin.

Key words: antibiotic susceptibility, β-lactamase, bovine, mastitis, staphylococci

INTRODUCTION

Although it is well-known that various microorganisms cause mastitis in cows, the major microorganism is *Staphylococcus aureus* (Francis 1989, Francis and Carroll 1986, Waage *et al.* 1999, Watts 1989). Treatment of mastitis due to *S. aureus* is quite difficult in veterinary practice and may result in failure. The presence of strains resistant to antibiotics; the prevention of diffusion of the antibiotics

to the infection area by hyperplasia of the alveolar epithelium and the massive accumulation of inflammation cells; the ability of staphylococci to survive in the leucocytes of the host defence system, to form microabcesses and to produce the enzyme β-lactamase have been indicated as important factors influencing the treatment process (Craven *et al.* 1986, Francis 1989, Watts and Salmon 1997, Yancey *et al.* 1991).

β-lactam antibiotics (penicillin, ampicillin, cephalosporins, etc.) are generally used in bacterial mastitis treatments in veterinary practice. β-lactamase, which is an extracellular enzyme produced by *S. aureus* as well as some other bacteria, leads to inactivation of this group of antibiotics by hydrolyzing the C-N bonds in their β-lactam rings (Francis 1989, Watts and Salmon 1997). Many reports have indicated the presence of high rates of β-lactamase production by staphylococci in clinical mastitis cases in cows (Craven *et al.* 1986, De Oliveira *et al.* 2000, Francis and Carroll 1986, Jones and Heath 1985, Sezen *et al.* 1986, Watts and Salmon 1997).

There has been no particular study on the causative agents of mastitis cases in cows in the province of Burdur even though the dairy cow population is high and an intensive dairy industry is localized in the area. Thus, we aimed in this study to determine the pathogens responsible from mastitis cases in cows in Burdur, the production of β -lactamase by staphylococci isolated from clinical and subclinical mastitic milk samples and the pattern of antibiotic sensitivity to several antibiotics used for treatment of mastitis in veterinary practice.

MATERIAL AND METHODS

Sampling: A total of 131 dairy farms were visited and 1180 lactating cows were examined for clinical mastitis (by inspection and palpation) and for subclinical mastitis by the California Mastitis Test. From 249 mastitic cows 496 milk samples were collected aseptically, placed in a cooler and transported to the Microbiology Laboratory in the Faculty of Veterinary Medicine at Akdeniz University within 2 hours.

Microbiological examination: From each milk sample, 0.1 ml was plated on each of three 7 % sheep blood agar (Oxoid Ltd, Hampshire, England) plates and one MacConkey agar (Oxoid) and one sabouraud dextrose agar (Oxoid) plate. One of the blood agar plates and the MacConkey agar plate were incubated at 37 °C for 1-4 days in air. The other blood agar plates were incubated at 37 °C for 1-4 days in microaerophilic and anaerobic conditions. The sabouraud dextrose agar plate that was plated for fungus and yeast isolation was incubated at 25 °C for 7 days.

After presumptive identification based on colony morphology and microscopic morphology, biochemical and growth characteristics of the isolates were determined. To classify whether the presumptively identified staphylococci produced coagulase or not, the tube coagulase test with rabbit plasma (Bactident Coagulase, Merck KGaA, Darmstadt, Germany) was applied to each of them. In addition, coagulase positive isolates were identified based on their haemolytic activity, the asetoin test and the oxidation-fermentation test for mannitol, maltose and glucose (Koneman *et al.* 1988).

Susceptibility testing: The following commercial antibiotic disks were used for testing for antibiotic susceptibility: penicillin G (Oxoid, 10 iu), ampicillin (Oxoid, 10 μg), amoxicillin (Oxoid, 25 μg), cloxacillin (Oxoid, 5 μg), amoxicillin+clavulanic acid (Oxoid, 30 mg), oxytetracycline (Oxoid, 30 μg), trimethoprim+ sulphamethoxazole (Oxoid, 25 μg), enrofloxacin (Oxoid, 5 μg), danofloxacin (Mast Diagnostics, Mast Group Ltd., Merseyside, U.K., 5 μg), gentamycin (Oxoid, 10 μg), neomycin (Oxoid; 30 μg) and erythromycin (Oxoid,15 μg).

Antibiotic susceptibilities of the staphylococci were determined by the disk diffusion method on Mueller Hinton agar (Oxoid) plates (Bauer *et al.* 1966). An aliquot (0.2 ml) of pure culture of each isolate previously incubated at 37 °C for 6-8 hours in trypticase soy broth (Oxoid) was transferred on to the Mueller Hinton agar and spread evenly by a sterile glass baget. After drying the inoculum at room temperature for 5-10 minutes, the antibiotic disks were placed on the agar and plates were incubated at 37 °C for 24 hours. Finally, the growth inhibition zone diameter of each antibiotic disk was measured. The decision about whether the test isolate was resistant or susceptible to the antibiotic was made by comparing the measured zone diameters (in millimeters) with a standard zone chart.

β - *lactamase activity:* Commercial b - lactamase identification sticks (Oxoid, BR66A) were used to differentiate the b - lactamase producing staphylococci from other staphylococci. The colonies in the inhibition zone of the penicillin disk on the Mueller Hinton agar were touched gently with the sticks and the sticks were incubated at room temperature in humid conditions for one hour. The colour changes were recorded at 5 minutes, 15 minutes and 1 hour during incubation. A dark pink-red colour indicated β-lactamase activity in the isolate. The sticks with no colour change were incubated for 3 more hours under the same conditions and then a final decision was made. The sticks with no colour change were classified as non-β-lactamase producing staphylococci. (Anon 1995, Jones and Heath 1985).

RESULTS

Pathogenic microorganisms were isolated from 335 (67.5 %) out of the 496 milk samples collected in this study. *S. aureus* (33.16 %) was found to be the most common pathogen in the bovine mastitic milk samples in Burdur province. Coagulase negative staphylococci followed *S. aureus* at a high rate (31.10 %). The pathogens isolated are listed in Table 1.

Table 1: The number and prevalence of microorganisms isolated from bovine mastitic milk samples

Genus / Species	No of isolates	%
Staphylococcus aureus	129	33.16
Coagulase negative staphylococci	121	31.10
Escherichia coli	45	11.57
Streptococcus uberis	25	6.43
Streptococcus dysgalactiae	21	5.39
Streptococcus agalactiae	15	3.86
Streptococcus spp.	15	3.86
Candida spp.	5	1.29
Pseudomonas aeruginosa	4	1.03
Bacillus spp.	3	0.77
Citrobacter freundii	2	0.51
Proteus vulgaris	2	0.51
Actinomyces pyogenes	1	0.26
Enterobacter aerogenes	1	0.26
Total	389	100

β-lactamase activity was detected in 84 (65.1 %) out of 129 *S. aureus*, and 36 (29.8 %) out of 121 coagulase negative staphylococci isolates(Table 2).

Table 2: β-lactamase test results for *S. aureus* and coagulase negative staphylococci

	β -lactamase positive			β -lactamase negative	
	N	n	%	n	%
S. aureus	129	84	65.1	45	34.9
Coagulase negative staphylococci	121	36	29.8	85	70.2
Total	250	120	48	130	52

N: total isolate numbers, n: β - lactamase positive and negative isolate numbers

The antibiotic susceptibility test results for 250 staphylococci in this study are shown in Table 3. It was found that amoxicillin+clavulanic acid, danofloxacin,

Table 3: Susceptibility of the *S aureus* and coagulase negative staphylococci isolates to different antibiotics

Antibiotics	*S. aureus*								Coagulase negative staphylococci								Total n: 250			
	b -lactamase positive n: 84				b -lactamase negative n: 45				b -lactamase positive n: 36				b -lactamase negative n: 85							
	R		S		R		S		R		S		R		S		R		S	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Penicillin	58	69	26	31	8	17.8	37	82.2	27	75	9	25	33	38.8	52	61.2	126	50.4	124	49.6
Amplicillin	54	64.3	30	35.7	8	17.8	37	82.2	22	61.1	14	38.9	30	35.3	55	64.7	114	45.6	136	54.4
Amoxicillin	49	58.3	35	41.7	6	13.3	39	86.7	22	61.1	14	38.9	28	32.9	57	67.1	105	42	145	58
Cloxacillin	20	23.8	64	76.2	5	11.1	40	88.9	7	19.4	29	80.6	15	17.6	70	82.4	47	18.8	203	81.2
Amoxicillin+ clavulanic acid	3	3.6	81	96.4	1	2.2	44	97.8	0	0	36	100	6	7.1	79	92.9	10	4	240	96
Oxytetracycline	40	47.6	44	52.4	20	44.4	25	55.6	22	61.1	14	38.9	38	44.7	47	55.3	120	48	130	52
Trimethoprim+ Sulphamethox.	51	60.7	33	39.3	28	62.2	17	37.8	22	61.1	14	38.9	50	58.8	35	41.2	151	60.4	99	39.6
Enrofloxacin	10	11.9	74	88	5	11.1	40	88.9	4	11.1	32	88.9	7	8.2	78	91.8	26	10.4	224	89.6
Danofloxacin	8	9.5	76	90.5	6	13.3	39	86.7	4	11.1	32	88.9	7	8.2	78	91.8	25	10	225	90
Gentamycin	33	39.3	51	60.7	20	44.4	25	55.6	12	33.3	24	66.7	35	41.2	50	58.8	100	40	150	60
Neomycin	32	38.1	52	61.9	15	33.3	30	66.7	17	47.2	19	52.8	38	44.7	47	55.3	102	40.8	148	59.2
Erythromycin	51	60.7	33	39.3	23	51.1	22	48.9	25	69.4	11	30.6	47	55.3	38	44.7	146	58.4	104	41.6

R:resistant, S: sensitive, n: isolate number

enrofloxacin and cloxacillin were the most effective antibiotics for β-lactamase producing staphylococci. The susceptibility rates of *S. aureus* to these antibiotics were; 96.4 % for amoxicillin+clavulanic acid, 90.5 % for danofloxacin, 88 % for enrofloxacin and 76.2 % for cloxacillin. On the other hand, all coagulase negative staphylococci were susceptible to amoxicillin+clavulanic acid (100%), 88.9 % of them were susceptible to both danofloxacin and enrofloxacin, and 80.6 % were susceptible to cloxacillin.

DISCUSSION

It has been reported in several studies that *S. aureus* is a major microorganism isolated from mastitic milk and infected mammary glands of cows (Francis and Carroll 1986, Nazer and Tavakoli 1994, Waage *et al.* 1999, Watts 1989). In our study, *S. aureus* was found to be the most prevalent pathogen also. On the other hand, we could not isolate any pathogenic microorganisms from 32.5 % of the milk samples from mastitic cows. The reason might be elimination of the pathogens from milk of the cows with chronic mastitis (Yancey *et al.* 1991), and the presence of antibiotic residues in milk (Waage *et al.* 1999).

In this study, staphylococci were found to be resistant to several antibiotics (penicillin, ampicillin, amoxicillin, oxytetracycline, trimethoprim+sulphamethoxazole, gentamycin, neomycin and erythromycin) at high rates. These rates were similar to those found in other studies done in Turkey (Akay 1986, Hadimli *et al.* 2001, Ulusoy *et al.* 1985), but they were higher than the rates reported abroad (Craven *et al.* 1986, De Oliveira *et al.* 2000, Francis and Carroll 1986, Teale and David 1999, Watts and Salmon 1997). These high rates can be attributed to the random selection of antibiotics for treatment of mastitis in the field.

Several studies (Craven *et al.* 1986, De Oliveira *et al.* 2000; Francis and Carroll 1986, Hadimli *et al.* 2001, Jones and Heath 1985, Sezen *et al.* 1986, Watts and Salmon 1997) reported the presence of β -lactamase activity in *S. aureus* isolates at different rates ranging between 25 % and 100 %. In this study, 65.1 % of *S. aureus* isolates and 29.8 % of coagulase negative staphylococci were determined as β - lactamase producing staphylococci. As Hadimli *et al.* (2001) stated, we also detected β - lactamase activity in the coagulase negative staphylococci.

The resistance/susceptibility of the β - lactamase producing staphylococci to β - lactam antibiotics has been investigated by several research teams (Craven et al. 1986, De Oliveira *et al.* 2000, Francis and Carroll 1986, Hadimli *et al.* 2001, Jones and Heath 1985, Sezen *et al.* 1986, Watts and Salmon 1997), and penicillin and ampicillin were found to be the most affected antibiotics (Nazer and Tavakoli 1994, Sezen *et al.* 1986, Teale and David 1999, Watts and Salmon 1997). Our results supported this conclusion and we found resistance to amoxicillin in addition to penicillin and ampicillin. We also found a higher rate of resistance to cloxacillin in staphylococci than in the reports of some researchers (Craven *et al.* 1986, Francis and Carroll 1986, Hadimli *et al.* 2001). This high rate may have arisen from the high percentage of cloxacillin resistant isolates obtained from the same farms. Another factor might be differences among the isolates of staphylococci isolated in different geographical regions. On the other hand, high rates of resistance to the antibiotics were also determined in β - lactamase negative isolates. We consider that the development of resistance to β - lactam antibiotics in staphylococci may

be due to the random use of antibiotics for clinical / subclinical mastitis and dry period treatments, the resulting selection of bacteria resistant to antibiotics and mutations in the bacterial genome in addition to the production of β - lactamase enzyme.

The combination of β - lactam antibiotics with β -lactamase inhibitors, such as clavulanic acid and sulbactam, increases the activity of antibiotics against bacteria (Francis 1989). In the present study, we did not detect any resistance to amoxicillin + clavulanic acid by β - lactamase producing coagulase negative staphylococci. We only found resistance to this antibiotic in 3 β - lactamase positive *S. aureus* isolates. This finding confirmed earlier statements that β - lactamase inhibitors increase the effect of these antibiotics on staphylococci (Francis 1989, Hadimli *et al.* 2001, Teale and David 1999).

As a result of this study, we found that *S. aureus* is the most prevalent pathogen causing mastitis in cows in the Burdur province. We found β -lactamase activity at a high rate in staphylococci and we considere that this enzyme might be the main factor in the development of resistance to the β -lactam antibiotics like penicillin, ampicillin and amoxicillin. We also revealed that amoxicillin+clavulanic acid, danofloxacin, enrofloxacin and cloxacillin are the most effective antibiotics against staphylococci.

Acknowledgements

Support for this work was provided by the Akdeniz University Research Fund (Project No: 20.01.0122.13). We are also thankful to the veterinarians in the Department of Agriculture at Burdur for their assistance during sampling visits.

Address for correspondence:
Turutoglu H
Department of Microbiology,
Faculty of Veterinary Medicine,
Akdeniz University, 15100, Burdur - Turkey
E-mail: hulyaturogly@hotmail.com

REFERENCES

1. *Akay O.* 1986, Mastitisli inek sutlerinden izole edilen Staphylococcus aureus suslarinin cesitli antibiyotiklere karsi duyarliliklari uzerine bir arastirma. *AU Vet Fak Derg*, 33, 1-11.
2. *Anon.* 1995, The Oxoid Manuel, 7th Edition, Unipath Limited, Hampshire, England.
3. *Bauer AW, Kirby WMM, Sherris JC, Turck M.* 1966, Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*, 45, 493-6.
4. *Craven N, Anderson JC, Jones TO.* 1986, Antimicrobial drug susceptibility of *Staphylococcus aureus* isolated from bovine mastitis. *Vet Rec*, 118, 290-1.
5. *De Oliveira AP, Watts JL, Salmon SA, Aarestrup FM.* 2000, Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and United States. *J Dairy Sci*, 83, 855-62.
6. *Francis PG.* 1989, Update on mastitis. III. Mastitis therapy. *Br Vet J*, 145, 302-11.
7. *Francis PG, Carroll PJ,* 1986, Antibiotic resistance patterns of *Staphylococcus aureus* strains isolated from clinical bovine mastitis. *Vet Rec*, 118, 361-3.
8. *Hadimli HH, Ates M, Guler L, Kav K, Oncel T.* 2001, Mastitisli sut ineklerinden izole edilen stafilokoklarin β-laktamaz aktiviteleri ve antibiyotiklere duyarliliklari. *Vet Bil Derg*, 17, 21-5.
9. *Jones TO, Heath PJ.* 1985, β-lactamase production in *Staphylococcus aureus* isolated from bovine mastitic milk. *Vet Rec*, 117, 340.
10. *Koneman EW, Allen SD, Dovell VR, Janda WM, Sommers HM, Winn WC.* 1988, *Color Atlas and Textbook of Diagnostic Microbiology*, 3rd ed., JP Lippincott Comp, Philadelphia.

11. *Nazer AHK, Tavakoli AR.* 1994, Prevalence of antibiotic resistance and beta-lactamase production by bacteria isolated from cases of bovine mastitis. *J App Anim Res*, 6, 167-76.
12. *Sezen IY, Erganis O, Corlu M, Ates M.* 1986, Mastitisli inek sutlerinden izole edilen *Staphylococcus aureus* 'larda penisilin rezistansi ile bazi enzim karakterleri ve biyokimyasal testler uzerine calismalar. *Selcuk Univ Vet Fak Derg*, 2, 91-101.
13. *Teale CJ, David GP.* 1999, Antibiotic resistance in mastitis bacteria. *Proceedings of the British Mastitis Conference*, 24–9.
14. *Ulusoy E, Izgur M, Akay O, Diker KS, Aydin N, Arda M.* 1985, Mastitisli inek sutlerinden izole edilen mikroorganizmalarin identifikasyonlari ve antibiyotiklere duyarliliklari uzerine bir arastirma. *AU Vet Fak Derg*, 2, 358-70.
15. *Waage S, Mork T, Roros A, Aasland D, Hunshamar A, Odegaard SA.* 1999, Bacteria associated with clinical mastitis in dairy heifers. *J Dairy Sci*, 82, 712-9.
16. *Watts J L.* 1989, Etiological agents of bovine mastitis. *Vet Microbiol*, 16, 41-66.
17. *Watts JL, Salmon SA.* 1997, Activity of selected antimicrobial agents against strains of *Staphylococcus aureus* isolated from bovine intramammary infections that produce β-lactamase. *J Dairy Sci*, 80: 788-91.
18. *Yancey RJ, Sanchez MS, Rzepkowski RA, Chester ST, Barnes RE, Ford CW.* 1991, Therapy of chronic staphylococcal mastitis. *Flem Vet J Suppl* 1, 62, 187-204.

OSETLJIVOST NA ANTIBIOTIKE I ZASTUPLJENOST β LAKTAMAZE U STAFILOKOKAMA IZOLOVANIM IZ MLEKA KRAVA SA MASTITOM

TURUTOGLU H, MUDUL S i PEHLIVANOGLU F

SADRŽAJ

Ova studija je imala za cilj ispitivanje bakterijskih uzročnika mastitisa krava, aktivnosti β laktamaze u njima i osetljivosti na antibiotike.

Ukupno je ispitano 1180 mlečnih krava na 131 farmi. Od tog broja mastitis je dokazan kod 249 krava od kojih je uzeto 496 uzoraka. *Staphylococcus aureus* je bio zastupljen sa 33.16 %, koagulaza negativne stafilokoke sa 31.10 %, *Escherichia coli* sa 11.57 %, *Streptococcus uberis* sa 6.43 %, *Streptococcus dysgalactiae* sa 5.39 %, *Streptococcus agalactiae* i *Streptococcus spp.* sa 3.86 %, *Candida spp.* sa 1.29 %, *Pseudomonas aeruginosa* sa 1.03 %, *Bacillus spp.* sa 0.77 %, *Citrobacter freundii* i *Proteus vulgaris* sa 0.51 %, *Actinomyces pyogenes* i *Enterobacter aerogenes* sa 0.26.%

Stepen rezistencije za stafilokoke prema amoksicilinu+klavulinskoj kiselini, danofloksacinu, enrofloksacinu, kloksacilinu, gentamicinu, neomicinu, amoksicilinu, ampicilinu, oksitetraciklinu, penicilinu, eritromicinu i trimetoprim +sulfametokszolu je bio 4 %, 10 %, 10.4 %, 18.8 %, 40 %, 40.8 %, 42 %, 45.6 %, 48 %, 50.4 % i 60.4 %. Enzim β-laktamazu je stvaralo 65.1 % *S. aureus* izolata i 29.8 % koagulaza negativnih stafilokoka. Najefikasniji antibiotici za izolate koji stvaraju β-laktamazu su amoksicilin+klavulinska kiselina, danofloksacin, enrofloksacin i kloksacilin.