

ANTIMICROBIAL SUSCEPTIBILITY OF STRAINS OF COAGULASE-POSITIVE *STAPHYLOCOCCUS* ISOLATED FROM MASTITIC BOVINE MILK

(SUSCETIBILIDADE ANTIMICROBIANA DE CEPAS DE *STAPHYLOCOCCUS* COAGULASE-
POSITIVAS ISOLADOS DE LEITE BOVINO MASTITICO)

(SUSCEPTIBILIDAD ANTIMICROBIAL DE CEPAS DE *Staphylococcus*
COAGULASA-POSITIVOS AISLADOS EN LECHE BOVINA MASTÍTICA)

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SUMMARY

A total of 95 coagulase-positive *Staphylococcus* strains isolated from 635 samples of mastitic milk from 72 dairy farms, were characterized in relation to resistance to 15 antimicrobial drugs. Among the isolates, resistance to sulphonamide was most frequent (80.2%), followed by resistance to ampicillin (78.9%), penicillin (77.8%) and lincomycin (71.5%). Multidrug-resistant isolates were extremely common, and almost all isolates also showed resistance to two or more drugs; only one isolate showed resistance to a single drug. These results are probably a consequence of the intensive use of antimicrobial drugs.

KEY-WORDS: Coagulase-positive *Staphylococcus*. Mastitis. Multidrug resistance. Antibiotic.

RESUMO

Um total de 95 cepas de *Staphylococcus* coagulase-positivas foi isolado de 635 amostras de leite mastítico provenientes de 72 fazendas de leite. Estas cepas foram caracterizadas em relação à resistência a 15 drogas antimicrobianas. Entre as cepas obtidas, a resistência à sulfonamida foi a mais frequente (80,2%), seguida pela resistência à ampicilina (78,9%), à penicilina (77,8%) e à lincomicina (71,5%). Cepas apresentando resistência múltipla foram extremamente comuns e praticamente todas as cepas mostraram resistência a duas ou mais drogas, sendo que somente uma cepa apresentou resistência a uma única droga. Esses resultados são provavelmente uma consequência da pressão devido ao uso intensivo de drogas antimicrobianas.

PALAVRAS-CHAVE: *Staphylococcus* Coagulase-positivos. Mastite. Resistência múltipla. Antibiótico.

RESUMEN

Un total de 95 cepas de *Staphylococcus* coagulasa-positivos fueron aisladas de 635 muestras de leche mastítica provenientes de 72 haciendas lecheras, estas cepas fueron caracterizadas en relación a la resistencia a 15 drogas

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antimicrobianas. Entre las cepas obtenidas las resistencia a sulfonamida fue la más frecuente (80,2%), seguida por la resistencia a ampicilina (78,9%), penicilina (77,8%) y lincomicina (71,5%). Cepas que presentaron resistencia múltiple fueron extremadamente comunes y prácticamente todas las cepas mostraron resistencia a dos o más drogas siendo que solamente una cepa presentó resistencia a una única droga. Estos resultados son, probablemente, una consecuencia de la presión, debido al uso intensivo de antimicrobianos.

PALABRAS CLAVE: *Staphylococcus* coagulasa-positivos. Mastitis. Resistencia múltiple. Antibiótico

INTRODUCTION

Bovine mastitis has been defined as an inflammation of the mammary gland, usually as a consequence of microbial infection. A wide variety of microorganisms (137 species belonging to 15 genera) have been implicated as agents causative of intramammary infection (IMI) of lactating females; they differ in virulence, frequency and persistence in the mammary gland (WATTS, 1988).

The coagulase-positive *staphylococci* are the most frequently isolated microorganisms from IMI (DEVRIESE, 1979, WATTS, 1988), they presently include *Staphylococcus aureus*, *S. intermedius* and some strains of *S. hycus* (ROBERSON et al., 1996).

In cows IMI due to *Staphylococcus*, which account for 25-30% of total IMI (ANDERSON, 1983) are in general subclinical. Nonetheless, IMI cause considerable economic losses, in special of milk, ranging from 10-25% of the total yield according to the intensity of the inflammation and the stage of lactation when the infection occurs (HOBLET et al., 1991).

Extensive and often indiscriminate use of antimicrobial drugs in medicine and in animal ration has a selective effect on the emergence and maintenance of drug resistance in these bacteria (FRANKLIN, 1999). The involvement of plasmids and transposons in *Staphylococcus* drug resistance has been clearly established (LYON e SKURRAY, 1987, NOVICK, 1989).

There is increasing concern about the problem of antimicrobial resistance and about variability of antibiotic susceptibility patterns for bacteria isolated from cows (BEZEK, 1998). Therefore, the purpose of this work was to characterize strains of coagulase-positive *Staphylococcus* (CPS) isolated from cattle in Brazil in terms of their resistance to antimicrobial drugs.

MATERIAL AND METHODS

Samples and Bacteriological Examinations: Milk samples (635) from cows in eleven Brazilian states (Ceará, Goiás, Mato Grosso do Sul, Minas Gerais, Paraíba, Paraná, Pernambuco, Rio de Janeiro, Rio Grande do Sul, Santa Catarina e São Paulo), were collected aseptically between February 2000 and February 2003. Teat ends were cleaned using alcohol (70%)-moistened swabs and allowed to dry.

After discarding the first few streams, milk samples (2-4mL) were collected into sterile 10 mL glass flasks and submitted to the California Mastitis Test (CMT) according to the method proposed by Schalm e Noorlander (1957) on a scale of 1-5 (KLAstrup, 1975). CMT-positive samples were refrigerated to about 4°C and immediately delivered to the laboratory. Samples were analyzed in the laboratory of Genetics of the Morphology, Estomatology and Physiology Department, São Paulo University-Campus Ribeirão Preto, first of all they were plated on bovine blood agar (Columbia agar, CM 331 supplemented with 5% sterile bovine blood) and also on Baird Parker agar (Oxoid) and incubated for 24-48h at 37 °C. Gram-positive, catalase-positive, rabbit plasma coagulase-positive cocci were confirmed biochemically as *Staphylococcus* when producing acid from sucrose, maltose, mannitol and trehalose and not from xylose (GAHRN-HANSEN et al., 1987).

Drug susceptibility testing: Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method with Mueller Hinton agar plates (CRAIG, 1993), using the following antimicrobial impregnated disks: amoxicillin (30µg); ampicillin (10µg); bacitracin (40U); kanamicin (30µg); cefalexin (30µg); erythromycin (15µg); streptomycin (10µg); gentamicin (10µg); neomycin (30µg); lincomycin (10µg); oxacillin (1µg); penicillin (10U); sulphonamide (300µg); sulfamethoxazole/trimethoprim (25µg); tetracycline (30µg). Zones of growth inhibition were evaluated according to NCCLS standards (1997).

Plasmid: Plasmid DNA was prepared according to a previously described modification of the alkaline lysis procedure (SCHWARZ e NOBLE, 1994). Plasmids were separated electrophoretically in 0.8% (w/v) agarose gels at 8v/cm using TAE (SAMBROOK et al., 1989) as the running buffer.

RESULTS

CPS was isolated from 14.9% of the milk samples (95 isolates). Coagulase-negative *Staphylococcus* also showed a high rate of isolation 27.5% (Table1).

Antimicrobial resistance testing revealed that all 95 CPS isolates were resistant to at least one antimicrobial drug, and 2 isolates were resistant to all of them. Between

the 95 CPS, 76 isolates (80.2%) were resistant to sulphonamide, the most common type of resistance, followed by ampicillin (78.9%), penicillin (77.8%) and lincomycin (71.5%), these results are summarized on Table 2.

Antimicrobial phenotype diversity between the CPS isolates was extremely high, as shown on Table 3 for 17 randomly selected isolates. Multidrug resistance was very common and almost all isolates showed resistance to two or more drugs (Figure 1).

By using agarose gel electrophoresis a DNA plasmid was found in 80.0% of the isolates (results not shown). The CPS were plated on Baird Parker agar; among these colonies, 12 (12.7%) did not present the expected typical morphology and were characterized as atypical colonies (results not shown).

DISCUSSION

Coagulase-positive *staphylococci* are the most frequently isolated microorganism from IMI worldwide (WATTS, 1988), and so also in Brazil (NADER FILHO et al., 1984; COSTA et al., 1986). Roberson et al. (1996) examined 487 coagulase-positive *staphylococci* isolates from mastitic cattle and reported that 82.1% were *Staphylococcus aureus*, 17.7% *S.hycus* and 0.2% *S.intermedius*. In the present work, CPS represented 14.9% of the isolates from milk samples and most of them should be *S.aureus*. Coagulase-negative *staphylococci* were also isolated from 27.5% milk samples, in agreement with reports by Baba et al. (1980 cited in WATTS, 1988).

Bacterial resistance to antimicrobial drugs varies widely (BEZEK, 1998) and depends on country, use of drugs and infection control practices. However, the extensive use of antimicrobial drug has led to a heavy increase in antimicrobial resistance in animal production worldwide (MCKELLAR, 1998).

Amongst the 95 isolates of CPS investigated in this study, drug resistance to sulphonamide was most

Table 1 - Pathogenic organisms isolated from 635 bovine mastitic milk samples from 72 dairy herds in 11 states of Brazil, between February 2000 to February 2003.

Agent	Number of isolates (%)
<i>Staphylococcus sp</i> coagulase-positive	95 (14.9)
<i>Staphylococcus sp</i> coagulase-negative	175 (27.5)
other bacteria	302 (47.5)
fungi/yeast	63 (9.9)
Total	635

Table 2 - Results of antimicrobial susceptibility testing on *Staphylococcus coagulase-positive* strains isolated from milk samples obtained from cows with mastitis in Brazil, between February 2000 to February 2003.

Antimicrobial agent	No	%
Ampicillin	75/95	78.94
Amoxicillin	48/95	50.52
Bacitracin	60/95	63.16
Cefalexin	34/95	35.79
Erythromycin	56/95	58.94
Gentamicin	18/95	18.95
Kanamycin	40/95	42.11
Lincomycin	68/95	71.58
Neomycin	45/95	47.37
Oxacillin	33/95	34.74
Penicillin	74/95	77.89
Sulfamethoxazole/trimethoprim	23/95	24.21
Sulphonamide	76/95	80.21
Streptomycin	41/95	43.16
Tetracycline	61/95	64.22

No = Number of isolates resistant/number of isolates;
%= percentage of resistant isolates.

frequently detected (80.2%), followed by resistance to ampicillin (78.9%) and penicillin (77.8%). Resistance to penicillin in animal CPS isolates is a problem which has persisted since the mid-1950s; resistance to other antibiotics has been less common (SMITH, 1971, FRANCIS e CARROL, 1986). Lange et al. (1999) in a study with 66 isolates of *S.aureus* obtained from milk samples of dairy cows suffering from subclinical mastitis in southern Brazil observed predominant resistance to penicillin and ampicillin in 43.9% of the isolates, either alone or in combination with resistance to others antimicrobials. Pereira e Siqueira-Junior (1995) also reported a high level of penicillin resistance (80.4%) in strains of *S .aureus* isolated from healthy cattle in Brazil

Aarestrup et al. (1995) working with 105 isolates of *S.aureus* from bovine mastitis in Denmark, verified that 81.0% of the isolates were susceptible to all of 11 antibiotics tested. Fitzgerald et al. (2000) isolated 102 *S. aureus* strains from intrammary infection of Irish dairy herds; and 42 strains from the USA, both of them showed high rates of susceptibility to neomycin, cephalothin,

Tables 3 - Patterns and phenotypes of antimicrobial drug resistance amongst 17 random selected isolates between 95 isolates of *Staphylococcus* coagulase-positive from mastitic milk from 72 dairy farms from Brazil (2000-2003).

Patterns	Phenotypes
Resistant to 07 drugs (5)	Amp, Bac, Lin, Neo, Pen, Sul, Tet (1) Amo, Amp, Bac, Ery, Pen, Sul, Tet (1) Amp, Bac, Cef, Oxa, Sul, Sut, Tet (1) Bac, Kan, Cef, Str, Lin, Sul, Tet (1) Amp, Bac, Lin, Pen, Sul, Sut, Tet (1)
Resistant to 08 drugs (5)	Amp, Bac, Kan, Lin, Neo, Pen, Sul, Tet (1) Amo, Amp, Bac, Lin, Oxa, Pen, Sul, Tet (1) Amp, Bac, Cef, Ery, Str, Lin, Pen, Sul (1) Amo, Amp, Bac, Str, Lin, Oxa, Pen, Sul (1) Amo, Amp, Kan, Cef, Str, Oxa, Pen, Tet (1)
Resistant to 12 drugs (3)	Amo, Amp, Bac, Cef, Ery, Lin, Neo, Oxa, Pen, Sul, Sut, Tet (1) Amo, Amp, Bac, Kan, Cef, Ery, Str, Gen, Lin, Neo, Pen, Sul (1) Amo, Amp, Bac, Kan, Cef, Str, Lin, Neo, Pen, Sut, Tet, Sul (1)
Resistant to 13 drugs (4)	Amo, Amp, Bac, Kan, Cef, Ery, Str, Gen, Lin, Neo, Oxa, Pen, Sul (2) Amo, Amp, Bac, Cef, Ery, Str, Lin, Neo, Oxa, Pen, Sul, Sut, Tet (1) Amp, Bac, Kan, Cef, Ery, Str, Gen, Lin, Neo, Oxa, Pen, Sul, Sut (1)

Amp: Ampicillin; Amo: Amoxicillin; Bac: Bacitracin; Kan: Kanamycin; Cef: Cefalexin; Ery: Erythromycin; Str: Streptomycin; Gen: Gentamicin; Lin: Lincomycin; Neo: Neomycin; Oxa: Oxacillin; Pen: Penicillin; Sul: Sulphonamide; Sut: Sulfamethoxazole/trimethoprim; Tet: Tetracycline

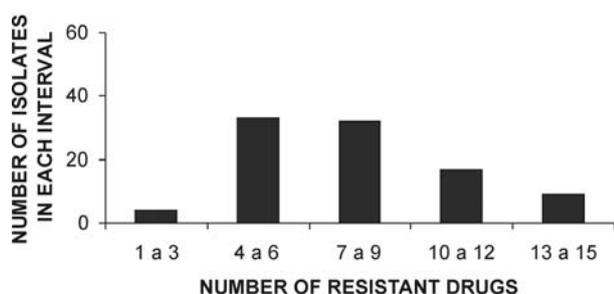


Figure 1 - Distribution of multidrug resistance to 15 antimicrobial drugs among 95 isolates of coagulase-positive *Staphylococcus* isolated from milk samples obtained from cows with mastitis in Brazil (2000-2003)

cloxacillin, tetracycline and erythromycin; only penicillin resistance was high (57.3% Irish isolates, 41.0% USA). Tollersrud et al. (2000) reported the isolation of 86 strains of *S. aureus* from cows with mastitis in Norway and showed that 78% of the isolates were susceptible to all of the 5 antibiotics tested.

One hundred and one coagulase-positive *staphylococci* isolated from bovine mammary secretion in the central dairy area of Argentina were tested against 8 antimicrobial drugs; a high level of resistance (47.5%) was shown against penicillin and ampicillin (CALVINHO et al., 2002). Cardoso et al. (2000) isolated 127 *S. aureus* strains from bovine milk and reported resistance to ampicillin (62.7%), penicillin (64.9%) and polymyxin

(72.3%). Oliveira et al. (2002) isolated 110 strains of *Staphylococcus sp* from bovine subclinic mastitis and reported resistance to penicillin (92.8%) and tetracycline (87.3%). Schoken-Iturrino et al. (1996) reported the isolation of 22 strains of coagulase-positive *Staphylococcus* from 17 cows positive to CMT test in the region of Jaboticabal, State of São Paulo. The authors analysed the susceptibility of the 22 strains against 16 antimicrobial drugs. Active drugs presenting highest and lowest efficiency, were nitrofurantoin (68.2%) and penicillin (0.0%). The resistance rates reported by the authors match those described in this work excepting oxacillin and gentamicin that were higher (Table 2). However, the authors did not discuss any result about multidrug resistance strains. The results described by Schoken-Iturrino et al. (1996) for the penicillin resistance agree with those described by Nader Filho et al. (1982, 1986, 1992) and Schoken-Iturrino e Nader Filho (1984) for cows from the State of São Paulo, Brazil.

The antimicrobial resistance in Europe and USA was extremely low when compared to Brazilian results. In the present study the level of resistance to antimicrobial drugs detected was extremely high (Table 2) constituting a reason for concern.

Resistance to streptomycin and tetracycline has been reported (BAUMGARTNER et al., 1984, DODD et al., 1987, AARESTRUP et al., 2000, CARDOSO et al., 2000, OLIVEIRA et al., 2002) during local investigations, which is not surprising since resistance may appear as a response to the extensive and indiscriminate use of antimicrobial drugs, as proved by Cardoso e Schwarg (1992) who reported that among 217 strains of *S. aureus* isolated from subclinic bovine mastitis in dairy herds with a low antibiotic use, only 5.0% of the isolates showed resistance to chloramphenicol.

Molecular population genetic analysis of *S. aureus* strains recovered from cows (KAPUR et al., 1995), showed an alarming heterogeneity of circulating strains, even within a given herd. However, in the study of Fitzgerald et al. (1997) only 12 RAPD types were identified among bovine *S. aureus* isolates from Ireland and the USA. In our study (Table 3) we found high antimicrobial phenotype diversity between the CPS isolates agreeing with the heterogeneity reported before (KAPUR et al., 1995).

The occurrence of multi-resistant strains may be a response to the selective pressure caused by the abusive use of antibiotics in animal practice (FRANKLIN, 1999). In the present study an alarming level of multidrug resistance was detected (Figura 1) when compared with results of other authors. Tollersrud et al. (2000) among 86 isolates of *S. aureus* reported only 2 isolates resistant to 3 antibiotics. Oliveira et al. (2002) and Cardoso et al. (2000) did not report any *Staphylococcus* isolate with multi-resistance. Pereira e Siqueira-Junior (1995) reported that

multi-resistant isolates among the 46 *S. aureus* strains were uncommon. Only Lange et al. (1999) reported multi-resistance among the *S. aureus* isolates from bovine mastitis in Brazil (43.9%). In the present study almost 100.0% of the isolates were multi-resistant with 33.6% of the isolates showing multi-resistance to 7-9 antimicrobial drugs (Figura1).

In *Staphylococcus* most of the genes associated with antibiotic resistance were present in plasmids (NOVICK, 1989). Plasmid profiling was the first DNA-based technique to be applied on *S. aureus*, and has shown a high typability (TENOVER et al., 1994). Baumgarten et al. (1984) found plasmid profiling to be useful in epidemiologic studies of bovine *S. aureus*, despite of 25.0% of the strains examined, not containing any plasmid. Aarestrup et al. (1995) also found a high number of *S. aureus* isolates from bovine mastitis without plasmids (77.0%). Lange et al. (1999) found that 53.1% of the *S. aureus* isolates from cases of bovine mastitis in Brazil have plasmids. Pereira e Siqueira-Junior (1995) also reported that many isolates of *S. aureus* from healthy cattle contain plasmids, and also described cases of plasmids inheritance of resistance to penicillin, streptomycin and tetracycline among the isolates, associated to the plasmid loss upon the return to susceptibility to specific antimicrobial drugs. In the present work, 80.0% of the CPS isolates showed a plasmid; this could explain the high level of antimicrobial drug resistance found, as well as the high number of isolates showing multidrug resistance.

Typical colonies of *S. aureus* on Baird Parker are 1-1.5 mm diameter, with an opaque halo surrounded by a 2-5mm clearing zone. The halo is a result of lipase activity and the clearing zone is due to proteolytic action (BENNETT e LANCETE, 1995, DEVRIESE, 1984, JABLONSKY e BOHACH, 1997). A typical colony may occur, whose frequency varies according to the type of food. According to Benett e Lancete (1995), nonlipolytic strains may be frequent in milk samples coming from mastitic animals. The frequency of atypical colonies detected in this study was lower than that reported by Wilson et al. (1994) and by da Silva et al. (2000). However, the exact meaning of these results remains uncertain (BENNETT e LANCETTE, 1995) and the study of atypical colonies is still under way (da SILVA et al., 2000).

In this study almost 100.0% of the CPS isolates showed multi-resistance to the antimicrobial drugs tested; the rates of resistance among the isolates was very high, a serious cause of concern. The constant monitoring of resistance patterns of isolates is necessary to chose suitable drugs for therapeutic use, in order to avoid the selection of multidrug-resistant strains.

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