Characterization of *Staphylococcus aureus* Isolates Recovered from Bovine Mastitis in Rio de Janeiro, Brazil

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ABSTRACT

Phenotypic characteristics, antimicrobial susceptibility, and genetic relationships were analyzed in 107 *Staphylococcus aureus* isolates recovered from cows with subclinical mastitis in Southeastern Brazil. Thirteen different biochemical patterns were detected among isolates. A predominant pattern represented by about 54% of the isolates was distributed among several herds. Isolates of distinct phenotypic profiles were also detected within a herd. Susceptibility to ampicillin, cefotaxime, cephalotin, chloramphenicol, erythromycin, gentamycin, kanamycin, nitrofurantoin, norfloxacin, ofloxacin, oxacillin, penicillin, rifampin, sulfamethoxazole-trimethoprim, tetracycline, trimethoprim, and vancomycin, determined by the disk diffusion method, was observed in 44.9% of isolates. On the other hand, 55.1, 7.4, and 2.8% of the strains were resistant to ampicillin/penicillin, tetracycline, and erythromycin, respectively. Genetic diversity was analyzed by pulsed-field gel electrophoresis using *Sma*I as the restriction enzyme. All isolates could be typed by pulsed-field gel electrophoresis, which identified 16 types and 24 subtypes. Type A and its subtypes comprised 54.2% of all isolates and were recovered from 6 of the 9 herds analyzed. Other types and subtypes were also found in multiple herds. Although multiple types and subtypes were found within a specific herd, a predominant type was frequently observed.

(Key words: *Staphylococcus aureus*, bovine mastitis, antimicrobial resistance, genetic diversity)

Abbreviation key: PFGE = pulsed-field gel electrophoresis.

INTRODUCTION

Mastitis is the most prevalent infectious disease among dairy cattle, and is responsible for severe fi-

nancial losses for farmers worldwide (Esslemont and Kossaibati, 1997; Fourichon et al., 2001). The majority of the losses are attributed to subclinical mastitis that is only detectable with the use of diagnostic tests applied to the milk (Bramley, 1992).

Staphylococcus aureus is one of the most important causes of bovine subclinical mastitis (Wilson et al., 1997; Brito et al., 1999). Although some studies (Zadoks et al., 2002a; Sommerhauser et al., 2003) have shown it is not possible to eradicate intramammary infections caused by this microorganism, adoption of control practices reduces the incidence and prevalence to acceptable levels. Current control practices include hygiene measures at milking time (particularly post-milking disinfection), appropriate operation of milking machine, culling of chronically infected animals, segregation of infected animals, and suitable antimicrobial therapy.

Antimicrobial therapy plays a role in mastitis control by reducing the levels of herd infection and by preventing new infections. However, bacteriological cure rate against *Staph. aureus* for antimicrobial therapy is relatively low due to pathogen characteristics such as the ability to survive inside the host cell and pathological changes induced in chronic infections (Bramley, 1992). Moreover, *Staph. aureus* isolates resistant to antimicrobial agents have been reported in studies carried out in different countries (Owens et al., 1997; Aarestrup et al., 1998; Lange et al., 1999; de Oliveira et al., 2000), which may also contribute to treatment failures. Hence, antimicrobial resistance monitoring among bacterial isolates from herds will be important for the knowledge of resistance profiles, which might enable the establishment of a more effective therapy for staphylococcal udder infections.

Molecular characterization of *Staph. aureus* isolates associated with bovine mastitis can be helpful in the development of more effective control practices of this disease. Some studies are consistent with the hypothesis that bovine mastitis is caused by a few specialized strains that have a broad dissemination (Matthews et al., 1994; Fitzgerald et al., 1997; Zadoks et al., 2000), whereas other studies have suggested that strains are

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more likely to be restricted to a single herd (Joo et al., 2001). However, all those studies have reported predominant strains responsible for bovine mastitis within herds (Matthews et al., 1994; Fitzgerald et al., 1997; Zadoks et al., 2000; Joo et al., 2001). A variety of methods has been applied for genetic characterization of bovine *Staph. aureus* (Matthews et al., 1994; Fitzgerald et al., 1997; Zadoks et al., 2000). Pulsed-field gel electrophoresis (PFGE) has been considered one of the most reliable and reproducible typing procedures, allowing for the detection of a high degree of DNA polymorphism (Bannerman et al., 1995).

Data on phenotypic and genotypic characteristics of *Staph. aureus* isolates recovered from milk of cows with mastitis in Brazil are still very limited. Therefore, the purpose of the present study was to survey susceptibility to antimicrobial agents so as to both provide data that may help in the choice of more appropriate drugs in the prevention and treatment of bovine mastitis caused by *Staph. aureus* and to determine whether mastitis is caused by groups of genetically related *Staph. aureus* strains in Brazil as observed in other countries. This knowledge will certainly be relevant in the development of mastitis control strategies directed to these specific strains. In addition, biochemical patterns of the bacterial isolates were determined to better characterize those strains.

MATERIALS AND METHODS

Bacterial Isolates and Phenotypic Characterization

This study included 107 bovine *Staph. aureus* isolates collected from 9 dairy herds in Rio de Janeiro, Brazil, between June 2001 and June 2002. The bacterial isolates were obtained from individual quarter milk samples from cows whose California Mastitis Test score was trace, 1, 2, or 3 according to the National Mastitis Council guidelines (1999). The milk samples were collected after cleaning the teats, discarding a few streams of milk, and scrubbing the teat ends with cotton balls moistened with 70% alcohol. Subsequently, milk samples were frozen and transported to the laboratory within 24 to 48 h. Bacteria were cultured from milk samples and identified according to the National Mastitis Council guidelines (1999). Complete identification was carried out with the API STAPH (bioMérieux, Marcy-l'Etoile, France).

Antimicrobial Susceptibility

The antimicrobial susceptibility of the staphylococcal isolates was determined by disk diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (1999, 2003). The

drugs tested were ampicillin (10 µg), cefotaxime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), ofloxacin (5 µg), oxacillin (1 µg), penicillin G (10 IU), rifampin (5 µg), sulfamethoxazole-trimethoprim (1.25/23.75 µg), tetracycline (30 µg), trimethoprim (5 µg), and vancomycin (30 µg). Antimicrobial disks were obtained from CECON (São Paulo, Brazil).

PFGE

Bacteria grown on blood agar plates were suspended in 500 µL of buffer (10 mM Tris-HCl, 1.0 M NaCl, pH 7.6), adjusted to match the 2 McFarland standards and mixed with an equal volume of 2% low-melting agarose (Nu Sieve GTG Agarose, FMC BioProducts, Rockland, ME) to prepare agarose plugs. The plugs were incubated overnight at 37°C in lysis solution (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.2% sodium deoxycholate, 0.5% sodium laurylsarcosine, 0.5% Brij 58) with 0.5 mg/mL lysozyme and 20 µg/mL lyso-staphin, and then incubated twice at 50°C for 24 h in ES solution (0.5 M EDTA, 1% sodium laurylsarcosine) containing 0.1 mg/mL proteinase K. After at least 6 washings with Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6), plugs were incubated in 250 µL of *Sma*I buffer for 1 h at 25°C and then treated overnight at 25°C with 20 U of *Sma*I (Boehringer Mannheim, Indianapolis, IN). Macrorestriction DNA fragments were separated in a 1% agarose gel (Seaken GTG agarose, FMC BioProducts) using a CHEF DR III (Bio-Rad Laboratories, Hercules, CA) with 0.5× Tris-borate-EDTA (1 M Tris, 0.01 M EDTA, 1 M boric acid) as running buffer. Pulse marker of 50- to 1000-kb (Sigma Chemical Co., St. Louis, MO) was used as a molecular weight marker. Running parameters were as follows: initial switching time, 2 s; final switching time, 35 s; run time, 21 h; 6 V/cm; 120° angle; 13°C (dos Santos et al., 2001). The gels were stained with 0.5 µg/mL ethidium bromide.

Macrorestriction patterns were analyzed both visually and using a computer-aided method. Visual interpretation of banding patterns was done following guidelines suggested by Bannerman et al. (1995). Isolates with identical restriction patterns in size and number of bands were considered the same type, and were designated by a capital letter. Isolates that differed from main types by 1 to 3 band shifts were assigned subtypes, indicated with a numeric suffix. Isolates with more than 3 such differences were considered different types. Banding patterns were analyzed by software Image Analysis System using the program Molecular Analyst Fingerprinting Plus version 1.12

Table 1. Distribution of biochemical patterns of 107 *Staphylococcus aureus* isolates recovered from bovine subclinical mastitis in Rio de Janeiro, Brazil.

Pattern	No. of isolates (%)	Biochemical reactions ¹								
		TRE	MAN	NIT	RAF	XYL	MDG	NAG	ADH	URE
A	58 (54.1)	+	+	–	–	–	–	+	–	+
B	5 (4.7)	+	+	+	–	–	–	+	–	+
C	21 (19.6)	+	+	+	–	–	–	+	+	+
D	1 (0.9)	+	+	–	–	–	–	+	+	+
E	1 (0.9)	+	+	–	–	–	–	+	+	–
F	6 (5.6)	+	–	+	–	–	–	+	+	+
G	1 (0.9)	–	+	+	–	–	–	+	–	+
H	1 (0.9)	–	+	+	–	–	–	+	–	–
I	2 (1.9)	+	+	–	–	–	–	+	–	–
J	1 (0.9)	+	+	–	–	–	–	–	–	+
L	2 (1.9)	–	–	+	–	–	–	+	+	+
M	7 (6.5)	+	+	–	–	–	+	+	–	+
N	1 (0.9)	+	+	–	+	+	+	+	–	+

¹TRE = Trehalose fermentation; MAN = mannitol fermentation; NIT = nitrate reduction; RAF = raffinose fermentation; XYL = xylose fermentation; MDG = α -methyl-D-glucosidase fermentation; NAG = N-acetyl-glucosamine fermentation; ADH = arginine dihydrolase production; URE = urease production.

(Bio-Rad Laboratories). Dice coefficient was used to calculate the similarity matrix and the unweighted pair group method using arithmetic averages to generate a dendrogram.

RESULTS

Phenotypic Characterization

Based on the phenotypic characteristics using API STAPH, *Staph. aureus* isolates were classified into 13 biochemical patterns each represented by a capital letter (A to N). Database of the API STAPH did not permit identification of 8 isolates although they have been previously identified as *Staph. aureus* according to the National Mastitis Council guidelines. Biochemical patterns of those isolates were assigned M and N in API STAPH identification. All isolates fermented glucose, fructose, mannose, maltose, lactose, and sucrose but not xylitol and melibiose. They were able to produce alkaline phosphatase and acetyl-methyl-carbinol. Isolates varied in the following biochemical capacities: fermentation of trehalose, mannitol, raffinose, xylose, α -methyl-D-glucoside and N-acetyl-glucosamine, reduction of nitrate to nitrite, production of arginine dihydrolase and urease (Table 1). The biochemical pattern A was predominant, being observed in 58 isolates (54.1%) and distributed in 7 of the 9 herds analyzed. Isolates of the patterns B, C, and F were also recovered from more than 1 herd. Isolates of distinct patterns were found in 6 herds with up to 5 patterns observed in the same herd (Table 2).

Antimicrobial Susceptibility

Susceptibility to all drugs tested was observed among 48 isolates (44.9%). The remaining 59 isolates

(55.1%) were resistant to at least 1 antimicrobial class. Only resistance to ampicillin (55.1%), erythromycin (2.8%), penicillin (55.1%), and tetracycline (7.4%) was observed among *Staph. aureus* strains. Five resistance profiles were observed among the isolates: no resistance (Pattern I); ampicillin/penicillin resistance (Pattern II: 45.8%); ampicillin/penicillin/erythromycin resistance (Pattern III: 1.9%); ampicillin/penicillin/tetracycline resistance (Pattern IV: 6.5%); and ampicillin/penicillin/erythromycin/tetracycline resistance (Pattern V: 0.9%). Most of the staphylococci isolates belonged to patterns I (6 herds) and II (all herds). Isolates of patterns III, IV, and V were recovered from 1, 3, and 1 herd, respectively. In addition, 2 or 3 resistance profiles were found in each of the herds analyzed (Table 2).

PFGE

Visual analysis of PFGE yielded 16 types and 24 subtypes among the 107 *Staph. aureus* isolates recovered from 9 herds. Figure 1 shows a gel with some of the banding patterns observed. Seven types and 9 subtypes were represented by 2 or more isolates and represented 77.6% of all isolates. Two types (A and C) and 5 subtypes (A1, A2, A4, A12, and C2) were found in multiple herds and corresponded to 39.3% of isolates. The remaining isolates (60.7%) were of types and subtypes each isolated from only 1 herd. Most of those strains were represented by 1 to 3 isolates and, in spite of being found in only 1 herd, closely related strains were often found in the same and in other herds, as shown in Table 2. Type A and its subtypes represent the majority of the isolates ($n = 58$) and were found in 6 of the 9 herds analyzed. By computer

Table 2. Distribution of pulsed-field gel electrophoresis (PFGE), biochemical, and resistance patterns of 107 *Staphylococcus aureus* isolates recovered from bovine subclinical mastitis in Rio de Janeiro, Brazil.

Herd	No. of isolates	Patterns			Herd	No. of isolates	Patterns		
		PFGE ¹	Biochemical ²	Resistance ³			PFGE	Biochemical	Resistance
1	1	C	F	II	5	1	C	F	IV
1	1	C3	F	I	5	1	C1	C	IV
2	1	A1	A	II	5	1	C2	F	IV
2	3	E	C	I	5	1	K	H	II
2	1	E1	C	I	5	1	L2	A	II
2	1	E2	C	I	5	1	P	C	IV
2	2	F	C	I	6	7	A	A	II
2	1	H	B	II	6	3	A	B	II
3	7	A	A	II	6	1	A1	A	I
3	1	A	A	III	6	1	A1	J	II
3	1	A2	A	II	6	1	A1	I	I
3	1	A3	A	II	6	1	A2	A	II
3	1	A3	A	III	6	1	A13	A	II
3	1	A4	A	II	6	1	A14	A	II
4	1	A	A	II	6	1	A15	I	II
4	1	A2	A	II	6	1	N	C	I
4	1	A2	A	IV	7	1	A	A	II
4	2	A4	A	II	7	1	C	F	IV
4	1	A5	A	I	7	1	C2	F	V
4	1	A6	B	I	7	1	O	G	II
4	8	A7	A	I	8	2	G	C	I
4	1	A7	A	II	8	1	G1	C	I
4	1	A9	A	I	8	1	M	C	II
4	1	A10	D	II	9	1	A1	M	I
4	1	A12	A	II	9	2	A8	M	I
4	1	C	L	II	9	2	A11	M	I
4	1	C	L	IV	9	1	A11	N	I
4	1	J	E	I	9	2	A12	M	I
4	1	L	A	I	9	13	B	A	I
4	1	L1	A	II	9	5	D	C	II
					9	1	D1	C	II

¹PFGE types were represented by capital letter (A to P) and subtypes represented by a numeral suffix (e.g., A1 to A15).

²*Staphylococcus aureus* isolates were classified in biochemical patterns due to differences in the capacity of fermentation of trehalose, mannitol, raffinose, xylose, α -methyl-D-glucosidase, and N-acetyl-glucosaminase, and production of arginine dihydrolase and urease. These patterns were represented by capital letter (A to N).

³Resistance patterns: no resistance to tested antimicrobial agents (I), ampicillin/penicillin (II), ampicillin/penicillin/erythromycin (III), ampicillin/penicillin/tetracycline (IV), and ampicillin/penicillin/erythromycin/tetracycline (V).

analysis, all isolates were grouped together at 52% pattern similarity. Isolates of type A and its subtypes were grouped together at 78% pattern similarity, as shown by dendrogram in Figure 2. However, isolates of other types were found within the same cluster. These isolates were considered other types by visual analysis because they differed from type A by more than 3 bands shifts but they may have been grouped in the same cluster because they differed by less than 3 bands shifts from some subtypes of type A. A similar observation was also noticed in the clusters of type C and E, which had each one about 80% pattern similarity.

In general, *Staph. aureus* strains of different types were recovered within a herd although 1 type and its

subtypes were predominant. Only 2 of the 9 herds had a single type with its subtypes (herds 1 and 3).

As shown in Table 2, multiple biochemical patterns were observed only to PFGE type A and C and their subtypes. However, each of those types had a predominant biochemical pattern. About 74% of the type A and its subtypes strains were of the biochemical pattern A whereas 64% of the type C and its subtypes strains were of biochemical pattern F. Multiple resistance patterns were found for type A, C, and L, and their subtypes.

DISCUSSION

The API STAPH method proved successful in identifying the most of the bovine bacterial isolates (92.5%).

Figure 1. Example of pulsed-field gel electrophoresis *Sma*I macrorestriction patterns of *Staphylococcus aureus* strains isolated from bovine subclinical mastitis in Rio de Janeiro, Brazil. Molecular weight marker (M) is in lanes 1 and 20. Lanes 2 to 19 contain representative types.

Even though it did not identify some isolates, they were identified as *Staph. aureus* according to the National Mastitis Council guidelines and had PFGE banding patterns similar to other *Staph. aureus* strains. A diversity of biochemical patterns was observed among tested strains. Curiously, most of the strains had no capacity to reduce nitrate or to produce arginine dihydrolase and differed from other reports in the literature, suggesting some strains have atypical biochemical patterns (Lange et al., 1999; Tollersrud et al., 2000b). Understanding of atypical biochemical patterns is important so that isolates are not misidentified. Misidentification could lead to a decrease in the true number of isolates in a specific study. Therefore, isolates with atypical patterns should be only excluded from studies after use of other identification methods as molecular methods.

Susceptibility to several antimicrobial classes is usually observed among bovine isolates (Owens et al., 1997; Lange et al., 1999; Tollersrud et al., 2000b). In the present study, 44.9% of *Staph. aureus* strains were susceptible to all antimicrobial agents tested, a susceptibility rate similar to that found by other Brazilian researchers (Lange et al., 1999) when studying *Staph.*

aureus strains recovered from cows with subclinical mastitis in Southern Brazil.

Antimicrobial-resistant *Staph. aureus* strains isolated from cows have been reported in different geographic areas (Aarestrup et al., 1998; Lange et al., 1999). In the present study, a high rate of resistance to penicillin/ampicillin (55.1%) was observed among *Staph. aureus* strains. In Lange's study, a rate of 43.9% of resistance to these 2 drugs was observed among *Staph. aureus* strains (Lange et al., 1999). Resistance to ampicillin and penicillin was also predominant in studies carried out in other countries (Aarestrup et al., 1998; de Oliveira et al., 2000). The predominance of resistance to those drugs may be related to their general use. They are among the most commonly used antibiotics in veterinary medicine worldwide. Countries with a policy of prudent use of antimicrobial agents in veterinary practices as Denmark and Norway have shown lower levels of resistance compared with other countries (Aarestrup et al., 1998; Aarestrup, 1999; Grave et al., 1999; de Oliveira et al., 2000). In Ireland, England, and Finland, high rates of β -lactamase-producing *Staph. aureus* strains are observed, whereas in Denmark and Norway, very low

Figure 2. Dendrogram showing the level of similarity among *Sma*I macrorestriction patterns of 107 strains of *Staphylococcus aureus* recovered from bovine subclinical mastitis in Rio de Janeiro, Brazil.

rates of β -lactamase-producing strains are found; consequently, those drugs are still effective for mastitis treatment (de Oliveira et al., 2000). Data from the present study indicate a low rate of erythromycin or tetracycline resistance among *Staph. aureus* strains isolated from milk, as already observed in studies carried out by other authors (Owens et al., 1997; Aarestrup et al., 1998; Lange et al., 1999; Tollersrud et al., 2000b; Erskine et al., 2002). However, in contrast to results from the current study, resistant isolates to other antibiotics (cefazetril, chloramfenicol, kanamycin, lincomycin, neomycin, nitrofurantoin, oxacillin, and streptomycin) were recovered from other Brazilian herds (Lange et al., 1999). Makovec and Ruegg (2003) reported a decrease and an increase of *Staph. aureus* isolates resistant to penicillin and erythromycin, respectively. Resistance to more than 1 drug class was observed in approximately half of the resistant staphylococci isolates in the present study. Although resistance typing may provide important information toward the development of effective prevention and treatment strategies for this disease, eradication of *Staph. aureus* mastitis has not been possible. Mastitis control measures as suitable herd management and therapy of infected cows can only reduce the prevalence and incidence at an acceptable level (Zadoks et al., 2002a). Therefore, the spread of isolates with susceptibility to several antimicrobial drugs indicates that other strains characteristics could be important to the persistence of the intramammary infections caused by this pathogen.

Genetic diversity among *Staph. aureus* isolates recovered from cases of bovine mastitis was studied by PFGE. This methodology has been extensively used to study genetic relationships among *Staphylococcus* strains recovered from both human and bovine infections (Bannerman et al., 1995; Zadoks et al., 2000; dos Santos et al., 2001; Joo et al., 2001). In current study, it was able to type all isolates and identify different profiles. Although different types have been identified, some types were prevalent among and within herds. Type A and its subtypes were represented by 54.2% of isolates analyzed and were recovered from the majority of herds, being predominant in 3 of them. Other types were also found in multiple herds. Nine of the 16 types were represented by only 1 isolate among all isolates analyzed. Our results were consistent to those from other authors who have reported that few types of *Staph. aureus* were predominant in bovine mastitis (Matthews et al., 1994; Fitzgerald et al., 1997; Zadoks et al., 2000). Matthews et al. (1994) observed that 52% of isolates from different geographic regions belonged to 2 types when analyzed by random amplified polymorphic DNA PCR. The major type identified by

multilocus enzyme electrophoresis associated with bovine mastitis in the United States was also the predominant type in Ireland according to Fitzgerald et al. (1997). Those authors used other techniques such as ribotyping, besides random amplified polymorphic DNA PCR, which identified distinct combinations also found in both countries. However, there is some controversy about whether techniques used in those and other studies failed to discriminate among certain types. In the study carried out by Joo et al. (2001), PFGE was used to type the isolates due to its higher discriminatory power. Their results suggested that *Staph. aureus* types from bovine mastitis were more probably restricted to a single herd. Otherwise, in other studies using the same method, the types were found in multiple herds (Zadoks et al., 2000, 2002b).

Although isolates of type A and its subtypes were susceptible to most of the antimicrobial agents tested, they were prevalent in the herds analyzed in this study. Their prevalence within and among herds leads to speculation that those strains have a greater capacity for infection and persistence that may be associated with production of a range of virulence factors. Therefore, detection of genetic determinants of putative virulence factors and comparison among predominant and minor strains may identify which factors are associated with higher prevalence and thereby provide a rational basis for the development of an effective vaccine for bovine mastitis. Moreover, the study of virulence factors may identify which of those factors would play a role in pathogenesis of the mastitis. Several studies have investigated putative virulence factors among *Staph. aureus* strains isolated from bovine mastitis but few studies examined a large number of factors (Fitzgerald et al., 2000). Most of the studies examined only one specific factor (e.g., capsule) or one restricted group of factors (e.g., superantigenic exotoxins) (Cardoso et al., 1999; Tollersrud et al., 2000a; Vasudevan et al., 2003). It is likely that a number of factors act in combination during the infective process and so those studies did not allow for analysis of combinations of factors more involved in pathogenesis of mastitis. Moreover, new potential virulence factors reported recently (e.g., new enterotoxins and the protein Bap involved in biofilm formation) need to be evaluated in association with known virulence factors. (Munson et al., 1998; Cucarella et al., 2001; Orwin et al., 2003). In Brazil, the studies concerning the virulence factors produced by *Staph. aureus* recovered from bovine mastitis are scarce and mainly deal with enterotoxins and toxic shock syndrome toxin (Freitas and Magalhães, 1990; Cardoso et al., 1999).

Therefore, our results call for the investigation of potential virulence factors that would benefit predomi-

nant clones in dairy herds with particular characteristics and management conditions. The study of such factors could help to identify potential targets for development of vaccines and antibacterial drugs more effective for the control of mastitis.

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