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Methicillin-Resistant *Staphylococcus aureus* from Brazilian Dairy Farms and Identification of Novel Sequence Types

C. J. B. Oliveira[1,2], N. Tiao[3], F. G. C. de Sousa[1], J. F. P. de Moura[1], L. Santos Filho[4] and W. A. Gebreyes[2,3]

[1] Department of Animal Science, Centre for Agricultural Sciences, Federal University of Paraiba, Areia, PB, Brazil
[2] Veterinary Public Health and Biotechnology Global Consortium (VPH-Biotec), The Ohio State University, Columbus, OH, USA
[3] Department of Preventive Veterinary Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA
[4] Department of Pharmacy, Center for Health Sciences, Federal University of Paraiba, João Pessoa, PB, Brazil

Impacts

- Methicillin-resistant *Staphylococcus aureus* (MRSA) and strains with diverse antimicrobial resistant patterns are prevalent in milk from semi-extensive dairy cows in north-eastern Brazil.
- *Staphylococcus aureus* from milk were clonally associated with bovine staphylococci lineage strains, which have been found in other regions of Brazil and worldwide.
- Although MRSA was also isolated from milkers' hands, its low frequency and distinct genotype from those MRSA found in milk suggest the possibility of the farmers contaminating the milk with resistant isolates, particularly MRSA.

Keywords:
Methicillin-resistant *Staphylococcus aureus*; oxacillin resistance; oxacillin resistant *Staphylococcus aureus*; milk

Correspondence:
W. A. Gebreyes. Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, OH 43210, USA.
Tel.: +1 614 292 9559;
Fax: +1 614 292 4142;
E-mail: gebreyes.1@osu.edu

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Summary

The aim of this study was to investigate the phenotypic and genotypic diversity and anti-microbial resistance among staphylococci of dairy herds that originated from Paraiba State, north-eastern Brazil, a region where such studies are rare. Milk samples (n = 552) were collected from 15 dairy farms. Isolates were evaluated for anti-microbial susceptibility by Kirby–Bauer disc diffusion method. Confirmation of methicillin-resistant *Staphylococcus aureus* (MRSA) was performed using multiplex PCR targeting *mecA* and *nuc* genes in addition to phenotypic assay based on PBP-2a latex agglutination. Clonal relatedness of isolates was determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) genotyping. Staphylococci were detected in 269 (49%) of the samples. Among these, 65 (24%) were *S. aureus*. The remaining 204 isolates were either coagulase-negative staphylococci (n = 188; 70%) or coagulase positive other than *S. aureus* (n = 16; 6%). Staphylococci were cultured in seven (35%) of the 20 hand swab samples, from which five isolates were *S. aureus*. The isolates were most commonly resistant against penicillin (43%), ampicillin (38%) and oxacillin (27%). The gene *mecA* was detected in 21 *S. aureus* from milk and in one isolate from a milker's hand. None of the isolates were resistant to vancomycin. PFGE findings showed high clonal diversity among the isolates. Based on MLST, we identified a total of 11 different sequence types (STs 1, 5, 6, 83, 97, 126, 1583, 1622, 1623, 1624 and 1625) with four novel STs (ST1622-ST1625). The findings show that MRSA is prevalent in milk from semi-extensive dairy cows in north-eastern Brazil, and further investigation on its extent in various types of milk production systems and the farm-to-table continuum is warranted.

Introduction

Staphylococci are ubiquitous bacteria in mammalian species associated with different types of infections in humans, companion animals and livestock. *Staphylococcus aureus* is a major pathogen causing skin infections in humans as well as clinical and subclinical mastitis in cows and other milk-producing animals. Its occurrence in primary food

sources and role as a foodborne pathogen have not been elucidated.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been widely known as a major nosocomial pathogen [often referred as hospital-associated (HA) MRSA] and also implicated as a major source of community-associated infections in recent years (Gray, 2004; Crum et al., 2006). MRSA strains express an alternative penicillin binding protein (PBP2a), which has a low affinity to β-lactam agents (Hartman and Tomasz, 1984). This protein is encoded by the *mecA* gene usually found in large mobile genetic elements named staphylococcal cassette chromosome *mec* (SCC*mec*), which can also confer resistance to anti-microbial classes other than β-lactam agents making the infections more difficult to be treated (Mombach Pinheiro Machado et al., 2007).

There is a worldwide increase of community-acquired MRSA infections (CA-MRSA) (Simor et al., 2010). Community-acquired MRSA have been reported increasingly in the last two decades and in some cases have been shown to be different from HA-MRSA (Vandenesch et al., 2003). Although staphylococcal food poisoning outbreaks caused by milk and dairies consumption have been reported worldwide (Yamane, 2006), the role of livestock and the overall significance of MRSA as a foodborne pathogen remains to be investigated. The first reported CA-MRSA infection in Brazil was found to be associated with ST30-SCCmecIV (Rozenbaum et al., 2009), while the majority of HA-MRSA infections is associated with the clone ST239-MRSA-IIIA (Oliveira and De Lencastre, 2002). Overall, studies on MRSA in Brazil have often been limited to the more temperate southern regions. There is paucity of data on the status of CA-MRSA in the more tropical north-eastern as well as Amazonian regions.

Methicillin-resistant *Staphylococcus aureus* was first reported in milking cows in 1972 (Devriese and Hommez, 1975), and it has been reported from milk in other countries (Kwon et al., 2005; Haran et al., 2012). Despite the few reports, characterization of MRSA from the milk value chain is overall scarce. While MRSA detected in foodstuff do not often match the isolates associated with human infection episodes (Hata et al., 2008), some clonal complexes found in human staphylococcal intoxications, such as CC5 and CC30, have been detected in milk from mastitic cows (Rabello et al., 2007). In recent years, unique strains of MRSA, such as sequence type (ST) 398, commonly designated as livestock-associated (LA) MRSA, have been reported from pigs and other food animals in various parts of the world. The higher risk of harbouring MRSA ST398 has been associated with subpopulations that are in contact to food animals, particularly pigs (Van Loo et al., 2007). The genetic relatedness of MRSA from humans and animals strongly support that ST398 or clonal complex (CC) 398 is indeed a 'livestock associated' MRSA (LA-MRSA) (Khanna et al., 2008; Golding et al., 2010; Hasman et al., 2010; Huber et al., 2010; Loeffler and Lloyd, 2010).

Characterization of MRSA from animal-derived foods and investigations on the epidemiology of MRSA in animal production systems are essential to elucidate the role of livestock and associated environment in the persistence and dissemination of MRSA in community settings. The objective of the present study was to determine the occurrence, MRSA and genotypic characterization of staphylococci from smallholder semi-extensive milk production systems in the north-eastern Brazil. To our knowledge, this is among the few reports on the MRSA detection in livestock in this region.

Methods

Study design and origin of samples

A total of 15 dairy farms that were located within the State of Paraiba, north-eastern Brazil, were included in this study. The herd size of the farms ranged from 8 to 40 lactating cows. Farms were managed with a semi-extensive production system on natural pasture, and at different stages of lactation. The breeds included mixed Holstein (*Bos taurus*) and Gir (*Bos indicus*), a local zebu breed. Milk samples were collected after each teat was disinfected using 70% ethanol and dried with disposable paper towels. The first few streams of milk (foremilk) was discarded to avoid surface contamination, and 15 ml samples were collected using sterile glass tubes and kept on ice for transportation to the Federal University of Paraiba (UFPB) laboratory. Milkers' hand swabbing was performed after milking only from those who provided a written consent to participate in the study. Palms were swabbed with moistened cotton placed in Stuart's medium for transportation to the laboratory.

Staphylococci culture and isolation

A loopful of milk samples was streaked onto Baird–Parker agar (Oxoid Ltd, Basingstoke, UK) supplemented with egg yolk tellurite (Remel, Lenexa, KS, USA) and mannitol salt agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After incubation for 24 h at 37°C, colonies showing morphological characteristics of staphylococci were evaluated by Gram staining and catalase for genus identification. Cultivation on DNase agar (Oxoid Ltd) for 24 h at 37°C were used for initial screening and *S. aureus* and confirmed using latex agglutination-based kit for *S. aureus* (Staphytest; Probac do Brazil, São Paulo, Brazil).

Anti-microbial susceptibility testing

Anti-microbial susceptibility testing of isolates was performed using Kirby–Bauer disc diffusion method according

to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013; Vet01-S4 and Vet01-S2). Briefly, colonies were suspended in 0.7% saline and standardized to 0.5 McFarland. They were cultured onto Mueller–Hinton (MH) agar and incubated at 37°C for 18–24 h. Results were obtained after interpreting the zones of inhibition against nine antimicrobial drugs. The anti-microbials, abbreviations and disc potency were as follows: ampicillin (Am; 10 μg), ceftiofur (Cf; 30 μg), cephalothin (Ch; 30 μg), erythromycin (Er; 15 μg), oxacillin (Ox; 1 μg), penicillin (Pn; 10 U), pirlimycin (Pr; 2 μg), penicillin + novobiocin (Pv; 30/24 μg), tetracycline (Te; 30 μg) and vancomycin (Vc; 30 μg). Penicillin + novobiocin discs were made in the laboratory following the protocol by Thornsberry et al. (1997). Briefly, 20 μl of a 312.5 μg/ml penicillin solution, made from penicillin G potassium salt (Acros Organics, Fair Lawn, NJ, USA), was added to a 30/24 μg novobiocin disc. This resulted in 6.25 μg (10 U) of penicillin per 30 μg novobiocin disc. The discs were prepared fresh under aseptic conditions and used on the same day. Reference strains *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used (American Type Culture Collection, Manassas, VA, USA). Multidrug resistance (MDR) was defined here as resistance to three or more classes of antimicrobials.

PCR for *S. aureus* and MRSA confirmation

Genomic DNA was extracted using a commercial kit (DNeasy Blood and Tissue Kits, Qiagen, Valencia, CA, USA) according to the manufacturer's guidelines. Briefly, the lysis buffer contains a mixture of 20 mM pH 8.0 Tris–HCl (Fisher BioReagents, Fairlawn, NJ, USA), 2 mM sodium EDTA (Gibco, Grand Island, NY, USA), 1% Triton X-100 (Sigma, St. Louis, MO, USA) and 20 mg/ml lysozyme (Lysozyme Ultrapure; USB Corporation, Cleveland, OH, USA). Molecular biology grade water (Eppendorf, Hamburg, Germany) was used for the elution. DNA was stored at 4°C for further genotypic analyses. The genes *nuc* (*S. aureus* confirmatory gene) and *mecA* (gene that encodes methicillin resistance) were detected using a duplex PCR in conditions previously reported (Oliveira and De Lencastre, 2002). The primers used for *nuc* gene amplification included forward: 5′-GCGATTGATGGTGATACGGTT-3′ and reverse: 5′-AGCCAAGCCTTGACGAACTAAAGC-3′ with an expected amplicon size of 270 bp. The primers used for *mecA* amplification were as follows: forward: 5′-TCCAGATTACAACTTCACCAGG-3′ and reverse: 5′-CCACTTCATATCTTGTAACG-3′ with an expected amplicon size of 162 bp. All isolates were also tested for carriage of *blaZ* gene, a non-*mecA*-associated β-lactamase that is shown to encode for methicillin resistance without the accompanied wide spectrum of resistance to other anti-microbials. The primers for *blaZ* were forward 5′-TACAACTGTAATATCGGAGGG-3′ and reverse 5′-CA TTACACTCTTGGCGGTTTC-3′ (Nannini et al., 2003) with an expected amplicon size of 861 bp. PCRs were performed using beads (GE Healthcare Life Sciences, Piscataway, NJ, USA) in a final reaction volume of 25 μl containing 1 μl of template DNA. Thermocycling was performed using the following conditions: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 7 min. Aliquots of the PCR products (10 μl) were electrophoresed in 2% agarose gels containing ethidium bromide and visualized by ultraviolet transilluminator. The amplicons were visualized using Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA) and analysed by Quantity One software (Bio-Rad Laboratories).

Genotyping by pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed according to a modified protocol as recommended previously (Mulvey et al., 2001). Briefly, *S. aureus* isolates were grown overnight on MH agar (Becton, Dickinson and Company). The bacterial cell concentration was adjusted by diluting cells with sterile cell suspension buffer to the OD value 1.35 (range 1.3–1.4) at 610 nm wavelength. Agarose-embedded cells were lysed, and intact genomic DNA was digested with 25–40 U of SmaI restriction enzyme (New England Biolabs, Ipswich, MA, USA) for at least 2 hours at 25°C. The fragments were separated by CHEF DR-III system (Bio-Rad Laboratories) with the following conditions and reagents: 1% SeaKem Gold agarose (FMC BioProducts, Rockland, ME, USA) in 0.5× Tris-borate-EDTA buffer, temperature at 14°C, voltage at 6 v/cm, run time of 19 h with initial switch time of 5.3 and final switch time of 34.9 s. The PulseNet 'universal' standard marker strain *S. enterica serovar* Braenderup H9812 was used as a molecular reference marker. The gels were stained with ethidium bromide and visualized under UV trans-illumination, and images were captured using Gel Doc 2000 (Bio-Rad Laboratories). PFGE gels were analysed by Bionumerics software V. 4.61 (Applied Maths NV, Keistraat, Belgium) using Dice similarity index and unweighted pair group average (UPGMA) cluster analysis. Banding patterns were compared with 1% optimization and 1% band position tolerance.

Genotyping by multilocus sequence typing

Multilocus sequence typing (MLST) was performed as described before (Enright et al., 2000). Seven housekeeping genes were targeted, and all the primers used were as recommended by the MLST global database system (http://www.mlst.net). After DNA extraction, PCR amplification

was performed on the seven targeted housekeeping genes: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glk*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). PCRs were performed using beads (GE Healthcare Life Sciences) in a final reaction volume of 25 μl containing 1 μl of template DNA. Amplification was performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc, Watertown, MA, USA) with an initial 5-min denaturation at 95°C, followed by 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min and denaturation at 95°C for 1 min, followed by a final extension step of 72°C for 5 min. The amplified products were precipitated with 20% polyethylene glycol-2.5 M NaCl, resuspended in cold 70% ethanol and reprecipitated; the DNA sequences of both strand were determined using an automated sequencer (CEQ8000; Beckman Coulter, Fullerton, CA, USA) using 30 cycles comprised by a denaturation step at 96°C for 20 s, annealing at 50°C for 20 s and elongation at 60°C for 4 min. Allelic profiles of the seven genes were assigned by comparing the sequences at each locus to those of the known alleles in the *S. aureus* MLST database (http://www.mlst.net). Based on the allelic profiles for the seven genes for each of the isolates, a ST was assigned.

Statistical analysis

Descriptive statistical analysis was used to provide information about prevalence of *Staphylococcus*, and anti-microbial susceptibility profiling. Data were analysed using the Fisher's exact and the chi-square tests (SPSS 17.0; SPSS Inc., Chicago, IL, USA) to compare the proportion of the phenotypic and genotypic differences of staphylococcal isolates among the farms. Analyses were considered significant when P-value was < 0.05.

Results

Occurrence of *S. aureus* and other staphylococci. Of the total 552 milk samples, 269 (49%) were positive for staphylococci. The majority of these isolates (188; 70%) was identified as coagulase-negative staphylococci. A total of 65 (24%) *Staphylococcus aureus* and 16 (5.9%) coagulase-positive isolates other than *S. aureus* ($n = 16$) were also identified. At least one sample from all the 15 farms were found to be positive for *S. aureus* with farm level prevalence ranging between 5% and 25%. *S. aureus* was identified in 35% (7 of 20) of the hand swab samples, from which five isolates (71%) were *S. aureus*, one coagulase-negative staphylococci and one coagulase positive other than *S. aureus*.

Anti-microbial resistance phenotypes and occurrence of MRSA. Frequency of anti-microbial resistance among the staphylococci from milk samples is shown in Table 1. Of the 269 staphylococci isolates, two-third (67%) were resistant to at least one anti-microbial. About half (49%) of the *S. aureus* isolates and 42% of CoNS isolates were pan-susceptible. At the farm level, proportion of pan-susceptibility ranged from 0% to 65%. Isolates were most commonly resistant to β-lactams including penicillin (43%), ampicillin (38%) and oxacillin (27%); followed by tetracycline (14%) and the macrolide and erythromycin (11%). There was statistically significant difference in the proportion of resistance among the farms for ampicillin ($P = 0.05$), oxacillin ($P = 0.001$) and penicillin ($P = 0.006$) but not for the remaining anti-microbial agents. No vancomycin resistance was detected. MDR was found in 8.9% of the isolates (24 of 269), from which 16 were *S. aureus* and eight were coagulase-positive staphylococci other than *S. aureus*. There was a large variation in proportion of MDR among the 15 farms, and the differences were not statistically significant ($P = 0.368$).

Twenty-one isolates from milk (8% of all staphylococci) and one milkers' hand were also *mecA* positive and identi-

Table 1. Frequency of anti-microbial drug resistance in staphylococci cultured from milk samples collected from dairy farms in north-eastern Brazil

Farm	Samples	Am (%)	Er (%)	Ox (%)	Pn (%)	Te (%)
1	14	9 (64)	4 (28)	7 (50)	9 (64)	2 (14)
2	15	6 (40)	3 (20)	14 (93)	5 (33)	4 (27)
3	15	9 (60)	4 (27)	2 (13)	11 (73)	6 (40)
4	18	5 (28)	2 (11)	4 (22)	6 (33)	2 (11)
5	09	6 (67)	0	3 (33)	7 (78)	3 (33)
6	13	8 (61)	1 (8)	2 (15)	9 (69)	7 (54)
7	15	2 (13)	1 (7)	5 (33)	3 (20)	2 (13)
8	31	10 (32)	0	2 (6)	11 (35)	3 (37)
9	24	7 (29)	5 (21)	6 (25)	12 (50)	4 (17)
10	17	3 (18)	3 (18)	7 (41)	4 (23)	0
11	13	6 (46)	1 (8)	3 (23)	4 (31)	2 (15)
12	13	5 (38)	0	7 (54)	4 (31)	0
13	14	6 (43)	1 (7)	2 (14)	6 (43)	2 (14)
14	23	7 (30)	2 (9)	1 (4)	6 (26)	0
15	35	14 (40)	3 (8)	8 (23)	19 (54)	0
Total	269	103 (38)	30 (11)	73 (27)	116 (43)	37 (14)

Determined by Kirby–Bauer disc diffusion method using the following anti-microbials and their respective disc potency: ampicillin (Am; 10 μg), ceftiofur (Cf; 30 μg), cephalothin (Ch; 30 μg), erythromycin (Er; 15 μg), oxacillin (Ox; 1 μg), penicillin (Pn; 10 μg), pirlimycin (Pr; 2 μg), penicillin + novobiocin (Pv; 30/24 μg), tetracycline (Te; 30 μg) and vancomycin (Vc; 30 μg).

Not including minor resistance frequencies: resistance against Pv was observed in 1 (3%) isolate from farm 15; pirlimycin resistance was seen in single isolates from farms 11 (8%), 12 (8%) and 15 (3%); resistance to cephalothin was observed in 3 isolates from farms 4 (5%), 10 (6%) and 11 (8%) and resistance against ceftiofur seen in a single isolate from farm 3 (7%).

fied as MRSA based on phenotypic (PBP-2 latex agglutination) as well. Overall, 34 different anti-microbial resistance patterns were identified (Table 2). Not all MRSA isolates were MDR. AmPn R-type was the most predominant among *S. aureus* (n = 33), while Ox only R-type was the most common among CoNS species (n = 26) followed by AmPn (n = 11).

PCR for *mecA* and *blaZ* genes

The *mecA* gene was detected in 3% of the isolates, and the one *S. aureus* isolate identified from a milkers' hand also was *mecA* positive. Of 104 isolates tested for the *blaZ* gene, we found 23 (23%) to carry this gene. Of the 16 MDR strains of *S. aureus*, we found seven isolates carried both *mecA* and *blaZ*, 10 carried *mecA* but not *blaZ*, two carried *blaZ* but not *mecA*, and four were found to carry neither. Neither of the two isolates with *blaZ* but no *mecA* were found to be phenotypically oxacillin resistant. On the other hand, all the seven isolates that carried both *mecA* and *blaZ* were found to be multidrug resistant with resistance to at least three anti-microbial agents including methicillin.

Pulsed-field gel electrophoresis and multilocus sequence typing genotyping

Pulsed-field gel electrophoresis and MLST genotyping were conducted on 16 *S. aureus* isolates systematically selected

Table 2. Frequency of resistance patterns in *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci other than *S. aureus* (CoPS) cultured from bovine milk samples collected in north-eastern Brazilian farms

Resistance Patterns	*S. aureus* (n = 65)	%	CoPS other than *S. aureus* (n = 16)	%	CoNS (n = 188)	%	Total	%
AmPn	22	40.7	04	33.3	22	16.4	48	21.7
Pan-susceptible	11	20.4	01	8.3	30	22.4	42	19.0
Ox	01	1.9	0	0.0	28	20.9	29	13.1
AmPnTe	06	11.1	0	0.0	08	6.0	14	6.3
Pn	02	3.7	0	0.0	09	6.7	11	5.0
OxPn	0	0.0	0	0.0	10	7.5	10	4.5
Er	0	0.0	0	0.0	08	6.0	08	3.6
Am	02	3.7	01	8.3	03	2.2	06	2.7
AmOxPn	01	1.9	0	0.0	05	3.7	06	2.7
AmErPn	0	0.0	02	16.7	04	3.0	06	2.7
AmOxPnTe	03	5.6	0	0.0	02	1.5	05	2.3
OxTe	0	0.0	0	0.0	05	3.7	05	2.3
Te	01	1.9	02	16.7	02	1.5	05	2.3
ErOx	0	0.0	0	0.0	04	3.0	04	1.8
AmErOxPn	0	0.0	0	0.0	03	2.2	03	1.4
AmErPnTe	01	1.9	0	0.0	01	0.7	02	0.9
PnPrTe	01	1.9	0	0.0	01	0.7	02	0.9
AmCfPn	01	1.9	0	0.0	0	0.0	01	0.5
AmCh	0	0.0	01	8.3	0	0.0	01	0.5
AmChOx	0	0.0	0	0.0	01	0.7	01	0.5
AmErOx	0	0.0	0	0.0	01	0.7	01	0.5
AmErOxTe	0	0.0	0	0.0	01	0.7	01	0.5
AmOx	0	0.0	0	0.0	01	0.7	01	0.5
AmOxPnTe	01	1.9	0	0.0	0	0.0	01	0.5
AmOxTe	0	0.0	0	0.0	01	0.7	01	0.5
AmPnPr	0	0.0	01	8.3	0	0.0	01	0.5
ChPnTe	01	1.9	0	0.0	0	0.0	01	0.5
ErOxPnTe	0	0.0	0	0.0	01	0.7	01	0.5
ErOxTe	0	0.0	0	0.0	01	0.7	01	0.5
ErPnTe	0	0.0	0	0.0	01	0.7	01	0.5
OxPr	0	0.0	0	0.0	01	0.7	01	0.5
PnTe	01	1.9	0	0.0	0	0.0	01	0.5
Total	54	100	12	100	134	100.0	221	100.0

Determined by Kirby–Bauer disc diffusion method using the following anti-microbials and their respective disc potency: ampicillin (Am; 10 μg), ceftiofur (Cf; 30 μg), cephalothin (Ch; 30 μg), erythromycin (Er; 15 μg), oxacillin (Ox; 1 μg), penicillin (Pn; 10 μg), pirlimycin (Pr; 2 μg), penicillin + novobiocin (Pv; 30/24 μg), tetracycline (Te; 30 μg) and vancomycin (Vc; 30 μg).

based on their anti-microbial resistance profiles. We identified four major genotypic clusters at genetic relatedness cut-off of 70%. Based on MLST, we identified a total of 11 STs, four of which were identified for the first time (ST1623, ST1624, ST1625 and ST1626) (Fig. 1).

Discussion

In this study, we found high prevalence of *S. aureus* in dairy farms as compared to what has been reported in other parts of the world (Virgin et al., 2009). As *S. aureus* is a well-recognized agent of foodborne intoxications, the common occurrence of this organism in milk could be of public health concern particularly in this region of Brazil, where the habit of drinking raw milk is common. Sixteen different outbreaks of staphylococcal intoxication caused by consuming contaminated dairy products were reported in central Brazil within a 4-year period between 1998 and 2002 (Veras et al., 2008).

We found anti-microbial resistance commonly to β-lactams, tetracycline and erythromycin. This finding is similar to reports in other parts of the world. However, the findings in this study indicated higher frequency of resistance as compared to previous reports in Brazil (Lange et al., 1999; Aires-de-Sousa et al., 2007; Rabello et al., 2007). The isolates expressed diverse resistance patterns within farms and between farms. Resistance to penicillin and ampicillin has been commonly reported in *Staphylococcus* from livestock in Brazil (Pereira and Siqueira-Junior, 1995).

Our study showed that 22 of 65 (33%) of *S. aureus* were MRSA as confirmed by *mecA* PCR. This finding is higher than previous reports and could be of public health concern considering the high rate of raw milk consumption in the region. Previous reports showed a 1.3% (Lee, 2003) and 0.15% (Kwon et al., 2005) prevalence rates in Korea. Another study in South America (Gentilini et al., 2000) reported that no MRSA detected in a collection of 206 *S. aureus* isolates identified from mastitis cases in dairy herds, while 3.2% of CoNS showed to be methicillin resistant. No MRSA was detected in dairy farms in Finland (Pitkälä et al., 2004), Korea (Moon et al., 2007) and USA (Anderson et al., 2006). A recent study reported 4% herd prevalence of MRSA in bulk tank milk samples in Minnesota, USA (Haran et al., 2012); *mecA*-positive staphylococci in unpasteurized milk samples were reported at a rate of 7% in United Kingdom (McKay, 2008). However, direct comparison of prevalence of MRSA reported in different studies should be considered cautiously as considerable variations in the study designs exist.

The detection of MRSA in milk has triggered a major concern about the potential role of MRSA may play as a foodborne pathogen, especially after the detection of LA-MRSA strain in bulk tank milk (Paterson et al., 2012). It is also noteworthy to mention that the frequency of MRSA in livestock reported in the last decade might be

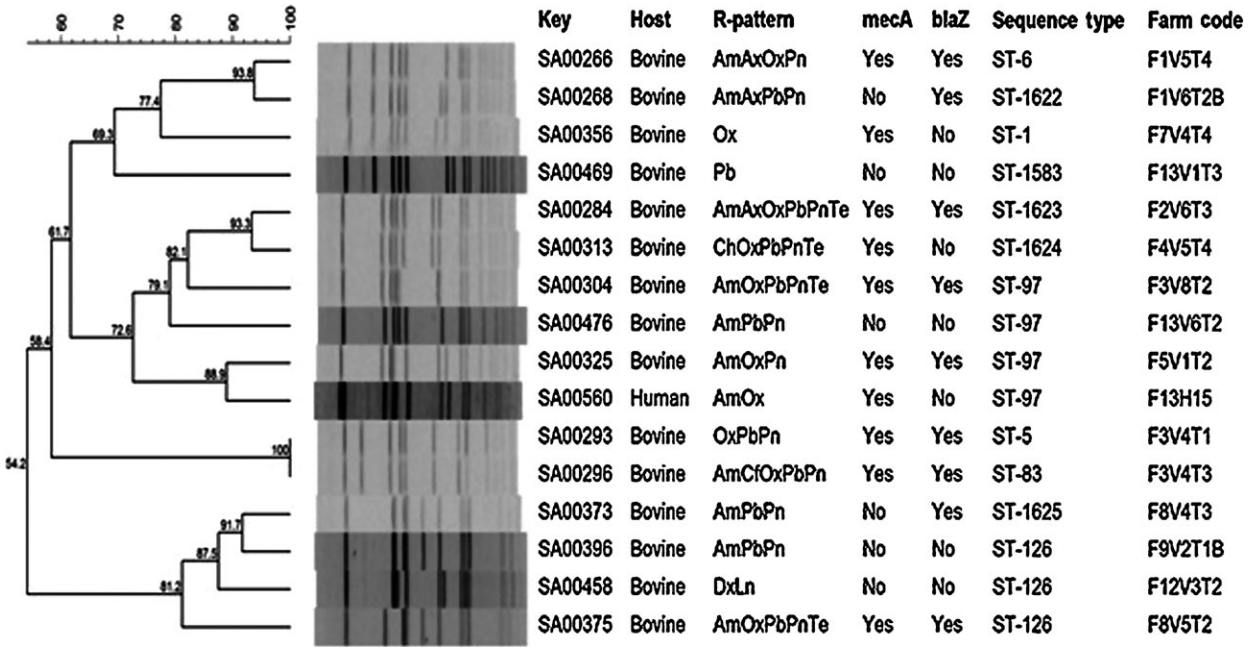

Fig. 1. Dendrogram showing the genotypic relatedness of 16 *S. aureus* (15 from bovine milk and one human hand swab) isolates from north-eastern Brazilian dairy farms using PFGE and MLST. PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing.

biased as novel mechanisms of methicillin resistance in *S. aureus*, such as *mecC*, have recently been reported in different countries, such as France (Laurent et al., 2012) and Sweden (Unnerstad et al., 2013).

Although the *mecA* gene is the most common mechanism for methicillin resistance, some of the isolates in the present study also harboured *blaZ* gene, responsible for β-lactamase production that could also play a role in oxacillin resistance. Our observations in the current study imply that isolates that carried both *mecA* and *blaZ* tend to have a higher spectrum of resistance and are often MDR in addition to oxacillin than those with only *mecA*.

The widespread usage of β-lactam agents in local dairy production for therapeutic and prophylactic purposes could be the driving force for the common occurrence of resistance to β-lactam agents such as ampicillin, penicillin and oxacillin. In the current study, a questionnaire data were collected in the farms and majority indicated the common usage of semisynthetic penicillins (benzathine cloxacillin) and cephalosporins (cephalonium dihydrate, cephapirin benzathine) in the dry-off treatments. Gentamicin bromhexine chlorhydrate, tetracycline and spiramycin with neomycin were commonly reported to be used for the treatment of clinical mastitis. A previous meta-analysis study reported a significant association between anti-microbials exposure and MRSA isolating from human patients (Tacconelli et al., 2008). In animals, antibiotic use mainly aminoglycoside has been considered to be a risk factor for colonization by MRSA in horses (Weese et al., 2006). Some reports have shown that anti-microbial resistance levels in organic farms are not different from those found in conventional animal production systems (Roesch et al., 2006; Thakur et al., 2007).

We found only one hand swab isolate collected from milkers' hand that was resistant to methicillin and the isolate harboured the *mecA* gene. However, based on MLST genotyping the ST, it was distinctly different from what was found for *S. aureus* isolated from milk samples from the same farm. This indicates independent source of contamination between the hand and the milk. Considering that hand swabs were collected after milking, a contamination originated from the cow cannot be disregarded. Although the limited number of observations does not allow us to take conclusion about the contamination routes, the fact that the human *S. aureus* isolate shared a common ST type and a similar PFGE pulsotype (Fig. 1) with one other bovine isolate from a different farm might indicate the existence of common source MRSA clone(s) in the region.

Overall, the frequency of MDR *Staphylococcus* in the current study was low as compared to previously reported in other studies on dairies (Roesch et al., 2006). In this study, no isolates were found to be resistant to vancomycin. Subclinical carriers of vancomycin-resistant staphylococci have been documented in Brazil (Palazzo et al., 2005).

The diverse phenotypic resistance profiles (R-types) could be linked to the genetic variability of the staphylococci isolates. Using MLST genotyping, we identified 11 different STs among 16 MRSA isolates. Of these, four STs were reported for the first time. PFGE discriminated the MRSA isolates into four major genotypic clusters at genetic similarity level of 70%. The high genotypic variability could be explained by the environment and management differences among the farms, as extensive or semi-intensive production systems are often expected to have diverse genotypes due to their exposure to various environmental sources. In a previous report about the characterization of *S. aureus* causing bovine mastitis in Rio de Janeiro (Rabello et al., 2007), the sequence types ST126, ST97 and ST1 were commonly observed. *Staphylococcus aureus* ST97 found in Brazilian herds has also been associated with dairy in USA (www.mlst.net). Indeed, *S. aureus* causing mastitis in cows that belong to clonal complex (CC) CC126 and CC97, found in this study, are also known to be clonally associated with bovine *S. aureus* lineage strains worldwide (Smith et al., 2005). According to a previous study performed in Rio de Janeiro State (28), most found STs (747, 740, 126 and 751) related to animal strains, except one isolate from buffalo (ST5), which has been also isolated from human infections indicating this is may be a common clone among humans and animals. In summary, the present study indicates a low prevalence of MRSA among dairy herds in Paraiba State, Brazil. Although the isolates showed genotypic diversity, the identification of a common clone to humans and animals warrants further investigations about the role of milkers in the epidemiology of MRSA in semi-extensive dairy production systems.

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