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Characterization of *Staphylococcus aureus* isolates in milk and the milking environment from small-scale dairy farms of São Paulo, Brazil, using pulsed-field gel electrophoresis

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ABSTRACT

This research aimed to evaluate the occurrence of *Staphylococcus aureus* isolates in milk and in the milking environment of 10 small-scale farms (<400 L/d) located in the regions of Franca and Ribeirão Preto, state of São Paulo, Brazil. Two-hundred twenty samples of milk were collected from individual cows, along with 120 samples from bulk tank milk, 389 samples from milking equipment and utensils (teat cups, buckets, and sieves), and 120 samples from milkers' hands. Fifty-six *Staph. aureus* strains were isolated from 849 analyzed samples (6.6%): 12 (5.5%) from milk samples of individual cows, 26 (21.7%) from samples of bulk tank milk, 14 (3.6%) from samples collected from equipment and utensils, and 4 (3.3%) from samples from milkers' hands. Pulsed-field gel electrophoresis typing of the 56 *Staph. aureus* isolates by *Sma*I restriction enzyme resulted in 31 profiles (pulsotypes) arranged in 12 major clusters. Results of this study indicate a low incidence, but wide distribution of *Staph. aureus* strains isolated from raw milk collected from individual cows and surfaces of milkers' hands and milking equipment in the small-scale dairy farms evaluated. However, the high percentage of bulk milk samples found with *Staph. aureus* is of public health concern because raw, unprocessed milk is regularly consumed by the Brazilian population.

Key words: *Staphylococcus aureus*, environment, pulsed-field gel electrophoresis, bulk milk

INTRODUCTION

Staphylococcus aureus is an important foodborne pathogen that causes several diseases transmitted between humans and animals, including infections of the mammary gland of milk-producing animals (Asperger and Zangerl, 2001). In dairy cattle, *Staph. aureus* is frequently associated with clinical and subclinical mastitis (Fagundes et al., 2010), which is a route for the pathogen to contaminate milk and other dairy products (Jørgensen et al., 2005). *Staphylococcus aureus* produces several virulence factors, including enterotoxins and toxic shock syndrome toxin (TSST-1; Asperger and Zangerl, 2001). Although pasteurization kills *Staph. aureus* cells, thermostable enterotoxins generally retain their biological activity in pasteurized milk and dairy products (Roberson et al., 1994). A large number staphylococcal food poisoning (**SFP**) cases have been reported worldwide (Rosec et al., 1997; Akineden et al., 2001; Rizek et al., 2011). Although in Brazil the exact number of SFP outbreaks is unknown, previous studies indicated that raw milk and dairy products manufactured from raw milk play important roles in SFP outbreaks in humans (Gilmour and Harvey, 1990; Tondo et al., 2000), which reinforces that contamination of milk by *Staph. aureus* is a public health problem (Cardoso et al. 1999; Fagundes and Oliveira, 2004; Oliveira et al., 2011).

Staphylococcus spp. may be found on skin and mucous membranes of healthy warm-blooded animals, as well as in soil, air, and water (Asperger and Zangerl, 2001). This pathogen easily spreads in the environment, requiring careful procedures during milking and sanitization to avoid the transmission among cows, equipment, and utensils (Akineden et al., 2001). Human-to-bovine transmission of *Staph. aureus* has been demonstrated by molecular studies that have shown

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that similar strains may be isolated from handlers and the milk of cows with mastitis (Jørgensen et al., 2005). Human handlers, milking equipment, the environment, and the udder and teat skin of dairy animals are possible sources of bulk milk contamination (André et al., 2008; Dufour et al., 2012). However, differences in management systems and factors such as the presence of flies capable of transmitting *Staph. aureus* may account for the observed variation in its prevalence as mastitis-causing agent (De Vliegher et al., 2012).

The incidence of *Staph. aureus* as a mastitis-causing agent in Brazilian dairy farms is generally high, ranging from 17 to 50% (Lange et al., 1999; da Silva et al., 2003; Fagundes et al., 2010), based on the diagnostic of individual cows in each farm. However, little is known about the occurrence of *Staph. aureus* in raw, refrigerated milk on dairy farms, and about the main sources of contamination on the farms, especially in small-scale production, which contributes with almost 30% of the total milk produced in Brazil. Small-scale farms generally sell the raw milk to dairy factories, which ultimately may contribute to the bacterial load of bulk tank milk in the industry. Moreover, these farms also sell raw milk directly to the public; although this practice is prohibited, informal milk in Brazil is almost 20% of the total milk produced in the country. Despite these facts, investigations on the epidemiology of important pathogens and actions aiming to improve the microbiological quality of milk in dairy farms have been addressed mostly in medium and large commercial operations. Improvement and monitoring of raw milk quality is a major issue for the development of the milk production chain worldwide (Oliveira et al., 2011). In this context, this study aimed to investigate the occurrence of *Staph. aureus* in milk and the milking environment of dairy farms in the state of São Paulo, Brazil.

MATERIALS AND METHODS

Sampling

The study was conducted on 10 dairy farms with a mean daily output of up to 400 L of milk: 5 (A to E) located in the region of Franca and 5 (F to J) in the region of Ribeirão Preto (state of São Paulo, Brazil) from August 2010 to January 2011. The dairy farms were commercial operations, and represented a convenience sampling, with the purpose of getting a gross estimate of results related to a research subject (Carrillo et al., 2012). In the present study, dairy farms were randomly selected by drawing among members of a dairy cooperative located in those 2 regions, which are important milk regions in São Paulo.

Individual milk samples were obtained from at least 5 cows from each dairy farm and were collected aseptically, directly from all quarters. At the end of the milking procedure, duplicate bulk tank milk samples (500 mL) were also collected in sterile milk containers. Samples from the milking equipment (internal surfaces of teat cups), utensils (sieves and buckets), and hands of milkers in each farm were also collected with sterile dry swabs (Pro-Lab Diagnostics, Round Rock, TX), previously wet and stored in peptone water, according to da Silva et al. (2003). Sampling procedures were performed after the cleaning process of milking equipment and utensils, and before the sanitization step. Swab samples of milkers' hands were collected during the milking procedure. Samples were collected once per month for 6 mo, totaling 220 samples of milk from individual cows, 120 samples of bulk tank milk, 120 samples from milkers' hands, and 389 samples from milking equipment and utensils. Samples were transported to the laboratory in coolers with ice (5.0 ± 1.2°C), and analyzed on the same day.

Staphylococcus aureus Count and Molecular Analysis

Isolation and identification of *Staph. aureus* were performed according to the Compendium of Methods for the Microbiological Examination of Foods (Lancette and Bennett, 2001). Serial dilutions of 2 replicates of each sample were plated onto Baird Parker agar (Oxoid Ltd., Hampshire, UK) supplemented with egg yolk and tellurite emulsion (1%; Oxoid Ltd.), and incubated at 35°C for 48 h. Colonies suggestive of *Staph. aureus* were submitted to the following tests: Gram staining, catalase reaction, clotting of rabbit plasma, presence of clumping factor (Staphyclin test; Laborclin, Pinhais, Brazil), and acetoin production. Suggestive colonies were gray to black (potassium tellurite reaction) surrounded by clear zones (egg yolk reaction; Capurro et al., 1999).

All *Staph. aureus* strains were subjected to chromosomal DNA and restriction endonuclease digestion strictly following the procedures as described by McDougal et al. (2003). Pulsed-field gel electrophoresis (**PFGE**) was run using a CHEF-DR III system (Bio-Rad Laboratories Inc., Hercules, CA). Running parameters were as follows: 200 V (6 V/cm); temperature, 14°C; initial switch, 5 s; final switch, 40 s; and length, 19 h. After the electrophoresis run was completed, the gel was stained with 50 μL of ethidium bromide solution (10 mg/mL; Sigma-Aldrich, St. Louis, MO) in 500 mL of distilled water for 20 min in a covered container, and destained in 500 mL of fresh distilled water for 30

Table 1. Incidence of *Staphylococcus aureus* in small-scale dairy farms from Franca and Ribeirão Preto, São Paulo, Brazil

	No. of positive samples/total no. of samples tested (%)		
Sampling site	Franca (farms A–E)	Ribeirão Preto (farms F–J)	Total
Milkers' hands	0/60 (0)	4/60 (6.7)	4/120 (3.3)
Milking equipment[1]	6/169 (3.6)	8/220 (3.6)	14/389 (3.6)
Raw milk			
Individual cows	3/52 (5.8)	9/168 (5.4)	12/220 (5.5)
Bulk tanks	9/60 (15.0)	17/60 (28.3)	26/120 (21.7)
Total	18/341 (5.3)	38/508 (7.5)	56/849 (6.6)

[1]Surfaces of milking machine (internal surface of teat cups) and utensils (sieves and buckets). No differences ($P > 0.05$) existed between the percentages within the row.

min. Gel Doc XR (Bio-Rad Laboratories Inc.) was used to capture the images under UV light. A molecular weight marker (Lambda Ladder PFG Marker; BioLabs, São Paulo, Brazil) was used in the analysis, with an effective range from 48.5 to 727.5 kb (PFGE). Bands above or below were not included in the analysis.

Isolates of *Staph. aureus* were placed in groups of identical or related strains by comparing banding patterns by computer analysis (BioNumerics, version 6.1; Applied Maths Inc., Austin, TX). Banding patterns were marked using several different stained gel images, in addition to curves provided by BioNumerics. A known strain of *Staph. aureus* (N315) was included in the macrorestriction analysis by PFGE; therefore, this strain could work as a control for the efficiency of the *Sma*I restriction enzyme. A dendrogram was generated by the unweighted pair group method with arithmetic mean (UPGMA), and Dice coefficients were used to define the groups based on 80% similarity (McDougal et al., 2003).

Statistical Analysis

The chi-squared test was used to compare the frequency of samples positive for *Staph. aureus* in the 2 regions studied (Franca and Ribeirão Preto), using $\alpha = 0.05$ (Gacula and Singh, 1984).

RESULTS

Incidence of Staph. aureus

The incidence of *Staph. aureus* on dairy farms of the state of São Paulo is shown in Table 1. Among all samples of the milk of individual cows, bulk tank milk, swabs from milking equipment, and milkers' hands (n = 849), 56 (6.6%) *Staph. aureus* strains were isolated. Dairy farms from the Franca region (dairy farms A to E) showed 18 (5.3%) positive samples, whereas dairy farms from Ribeirão Preto (F to J) showed 38 (7.5%) positive samples. No differences ($P > 0.05$) existed between the percentages of positive samples for *Staph. aureus* in dairy farms located in the Franca or Ribeirão Preto regions, except for raw milk samples collected from bulk tanks, which showed a tendency to higher percentages ($P = 0.076$) in samples from Ribeirão Preto farms (28.3%; n = 17).

Molecular Characterization by PFGE

Molecular characterization of *Staph. aureus* strains by PFGE is shown in Figure 1. The *Sma*I macrorestriction fragment profiles of 56 *Staph. aureus* isolates showed great genetic diversity among the strains isolated from different samples from the same farm, and from different farms throughout the sampling period. Thirty-one profiles could be defined, from all *Staph. aureus* strains typed, with 80% similarity, and the dendrogram revealed 12 major clusters of isolates.

The genotype clusters of isolates by sample source are presented in Table 2. Seven clusters (A, B, E, F, G, K, and L) were isolated only from different sites in dairy farms from Ribeirão Preto, whereas 3 clusters (D, I, and J) were from samples of milk collected in farms from Franca. However, a large number of different strains were found in cluster C and 2 strains in cluster H isolated from different regions and points of sampling. In particular, cluster C strains were found in raw milk from individual cows and bulk tank in dairy farms from the Ribeirão Preto and Franca regions, and at different times of sample collection.

DISCUSSION

The incidences of *Staph. aureus* isolates in swab samples from milkers' hands, milking equipment, samples of raw milk from individual cows, and bulk tanks were 3.3% (n = 120), 3.6% (n = 389), 5.5% (n = 220), and 21.7% (n = 120), respectively. Higher results were reported by D'Amico and Donnelly (2010) in raw milk

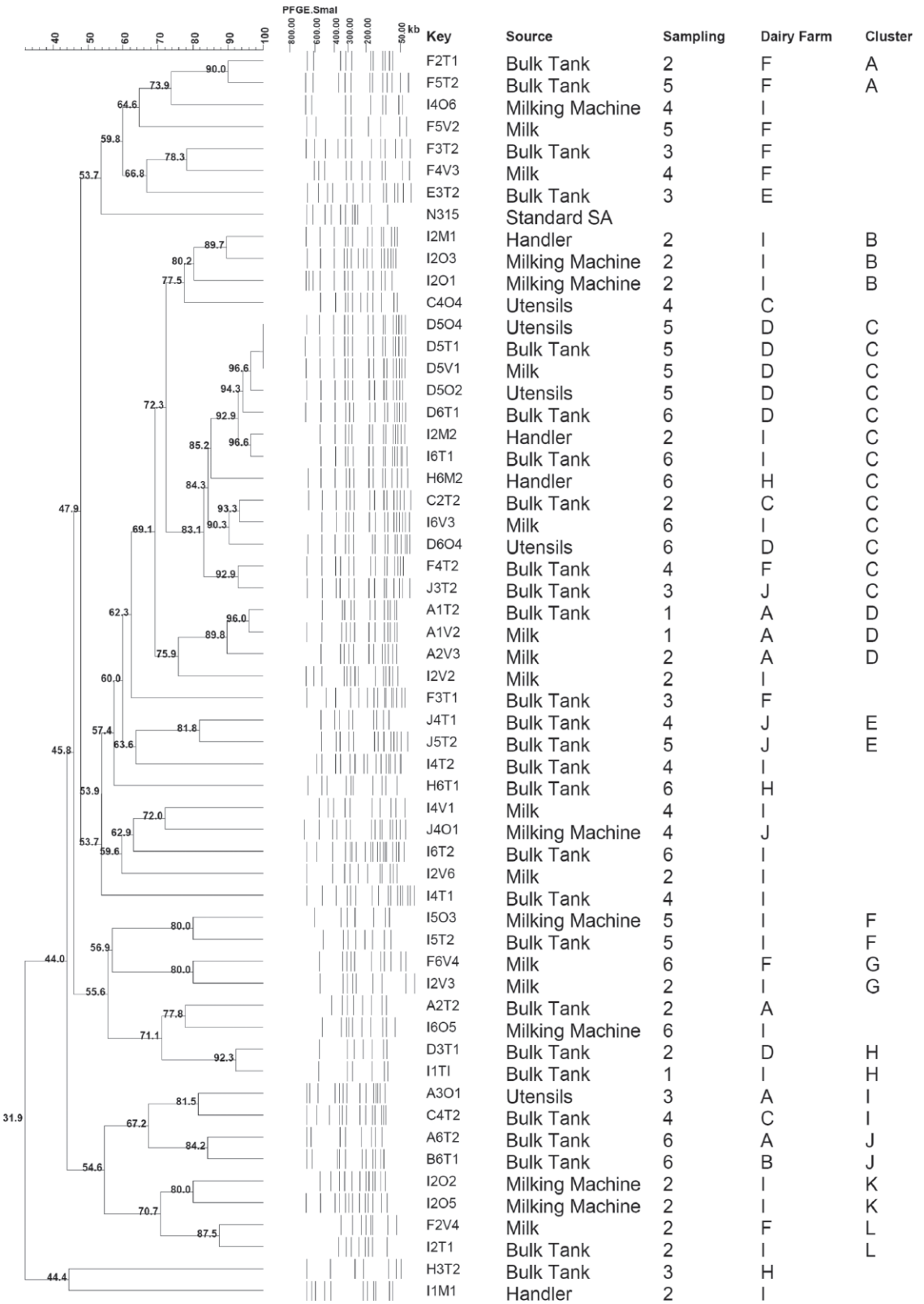

Figure 1. Dendrogram showing *Sma*I restriction enzyme pulsed-field gel electrophoresis (PFGE) patterns of genomic DNA of *Staphylococcus aureus* isolated from the milking environment (Standard SA = *Staph. aureus* N315). Isolates were cluster generated based on an 80% Dice similarity cutoff value of the unweighted pair group method with arithmetic mean (UPGMA) clustering method (0.5% optimization; 1.25% tolerance).

Table 2. *Staphylococcus aureus* genotype clusters (A–L) by sample source in small-scale dairy farms from Franca (F) and Ribeirão Preto (RP), São Paulo, Brazil

	Milkers' hands		Milking equipment[1]		Raw milk			
					Individual cows		Bulk tanks	
Staph. aureus cluster	F	RP	F	RP	F	RP	F	RP
A								2
B		1		2				
C		2	3		1	1	3	3
D					2		1	
E								2
F				1				1
G						2		
H							1	1
I			1				1	
J							2	
K				2				
L						1		1
Total	0	3	4	5	3	4	8	10

[1]Surfaces of milking machine (internal surface of teat cups) and utensils (sieves and buckets).

samples used for small-scale artisan cheese production in Vermont in the United States. Those authors performed repeated sampling of farms over a seasonal time frame and found that 14 farms (67%) were positive for this pathogen, which was detected in 38% of samples at an average level of 20 cfu/mL. Similarly, *Staph. aureus* was also detected in 33 (66%) of small- and medium-scale dairy farms in the northeastern region of Brazil, with this incidence being associated with the premilking teat wash procedure and postmilking teat dip (Oliveira et al., 2011). These findings are related to different levels of adoption of good practices at raw milk handling. Indeed, a comparison of the results of the present study and those reported by other authors is difficult, because the occurrence of *Staph. aureus* as a causative agent of subclinical mastitis varies according to the area, animal management practices, and hygiene conditions during milking, among other factors (Fagundes et al., 2010). The low percentage of *Staph. aureus* observed in the milk of individual cows may also be a consequence of the recent Brazilian investments in dairy cattle management that aimed at improving the microbiological quality of milk. However, from the 120 samples of bulk milk analyzed in our study, 26 (21.6%) were positive for *Staph. aureus*, hence indicating a potential route of SFP transmission to consumers via contaminated milk. This would be particularly important in small-scale farms from Ribeirão Preto, where the incidence of *Staph. aureus* in bulk milk was higher, although the isolates were not screened in the present study for toxin genes or enterotoxin production.

Pulsed-field gel electrophoresis typing of 56 *Staph. aureus* isolates by *Sma*I restriction enzyme resulted in 31 pulsotypes arranged in 12 major clusters. Similar strains (clusters C and H) were found in farms from both regions of Franca and Ribeirão Preto, which had no direct link between them, indicating the possibility of wide dissemination of some strains over vast geographic areas. In a similar study carried out in a different environment (a dairy plant in the state of Goiás, Brazil), André et al. (2008) also found high diversity among the strains, demonstrating lack of predominance of an endemic clone of *Staph. aureus* in the environment. Similar findings were reported in Sweden, with *Staph. aureus* isolates with genotypes indistinguishable from those found in milk also dominated in extramammary sites within the dairy herds studied (Capurro et al., 2010). Data presented in the current study and previous works indicate a huge variety of *Staph. aureus* strains either on dairy farms or in dairy plant environments, which makes the identification of a more precise relationship of multiple routes of contamination of milk difficult.

The large number of patterns observed in the present study indicated that great genetic heterogeneity among *Staph. aureus* strains isolated from raw milk in the farms studied. High variability in genotypic patterns may be due the diversity of locations in which *Staph. aureus* may be found. Similar results were found in a dairy plant in the south of Brazil, where 19 unrelated strains of *Staph. aureus* were isolated from raw milk (Tondo et al., 2000). To control the spread of staphylococcal infections, the sources of contamination and mechanisms of transmission must be identified. Jørgensen et al. (2005) indicated that *Staph. aureus* from udders may contaminate bulk milk and, subsequently, raw milk products. Association between dairy cows and their handlers, in addition poor sanitary practice, may result in the inter-

change of staphylococcal strains and contribute to the poor microbiological quality of the milk (Adesiyun et al., 1998), which could be observed in cluster C. In the present study, PFGE was used as a tool for identifying the diversity of *Staph. aureus* strains among different sites inside the dairy farms, and how they contribute to contamination of the bulk milk. Although PFGE would be useful to screen for staphylococci in milk, it remains to be determined if the cost-benefit relationship of such technique would be appropriate for dairy operations in Brazil.

In the present study, *Staph. aureus* isolates from milkers, utensils, milk from individual cows, and bulk tank milk were epidemiologically related, indicating that it may not only be transmitted between cows on the same farm, but also disseminated among farms and different, unrelated regions. Pulsed-field gel electrophoresis identification of clusters and pulsotypes at different periods of sampling may show the persistence of the strain in the environment. However, further studies are needed to confirm this hypothesis, as it possible that the *Staph. aureus* strains isolated were just common strains present in the environments evaluated.

Pasteurization of milk intended for human consumption is mandatory in dairy factories in Brazil. However, improper refrigeration of raw milk in farms and transportation still exists all over the country and this problem allows the growth of pathogens such as *Staph. aureus* and the production of thermostable enterotoxins prior to pasteurization of raw milk. The variability of *Staph. aureus* strains found in samples of raw milk and surfaces stresses the need for stringent control strategies, including regular monitoring of the sanitizing procedures, to prevent the dispersion of *Staph. aureus* into the milking environment of small-scale dairy farms.

CONCLUSIONS

Our findings indicate a high incidence of *Staph. aureus* strains in raw milk from bulk tanks, and a low incidence in raw milk collected from individual cows and the surfaces of milkers' hands and milking equipment on small-scale dairy farms in the state of São Paulo, Brazil. Pulsed-field gel electrophoresis analysis showed high diversity among the strains of *Staph. aureus* isolated from raw milk and the milking environment. The fact that a large percentage of bulk milk samples tested positive for *Staph. aureus* is of public health concern because unprocessed milk is regularly consumed by the Brazilian population because of cultural habits and lack of information on health issues.

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