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Full Length Research Paper

Milk hygienic practices and occurrence of *Staphylococcus aureus* and *Escherichia coli* O157:H7 in small-scale dairy farms in São Paulo, Brazil

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The objective of this study was to assess the relationship between somatic cell counts (SCC), the use of different milking practices, and the occurrence of *Staphylococcus aureus* and *Escherichia coli* O157:H7 in 42 small-scale dairy farms located in the state of Sao Paulo, Brazil. *S. aureus* and *E. coli* O157:H7 were isolated in the milk from dairy cows with low (< 200,000 cells/ml) and high SCC (>200,000 cells/ml), although no effect of SCC ($p > 0.05$) was observed on the incidence of the bacteria in raw milk. The use of disposable gloves during milking reduced *S. aureus* counts in milk ($p < 0.05$), but did not affect the occurrence of *E. coli* O157:H7. The other milking practices evaluated (closed milking system, use of pre- and post-dipping, mastitis diagnosis by strip cup test, and disinfection of teat cups) did not affect ($p < 0.05$) the occurrence of *S. aureus* or *E. coli* O157:H7 in raw milk. Results indicate the need for effective educational programs addressed to prevent the contamination of milk with *S. aureus* and *E. coli* O157:H7 in Brazilian small-scale dairy farms.

Key words: *Staphylococcus aureus*, *Escherichia coli* O157:H7, somatic cell counts (SCC), milk quality.

INTRODUCTION

Raw milk represents an ideal growth medium for several microorganisms that have been identified as foodborne pathogens or as causative agents of bovine mastitis, with *Staphylococcus aureus* and *Escherichia coli* most frequently isolated worldwide (Ksontini et al., 2011). *S. aureus* has an important role in foodborne diseases caused by milk and dairy products due to its high prevalence and the risk of toxin production in contaminated foodstuffs (Zecconi and Hahn, 2000). Another important agent of mastitis is *E. coli*, being some strains important foodborne pathogens, especially *E. coli* O157:H7. In humans, *E. coli* O157:H7 causes diarrhea and in 3 to 5% of human cases, hemolytic uremic syndrome (HUS) (Ksontini et al., 2011).

In dairy cattle, the inflammation of the mammary gland in response to bacterial attachment and growth leads to

increased somatic cell counts (SCC) (Zecconi and Hahn, 2000). It is generally accepted that healthy mammary glands show SCC lower than 200,000 cells/ml (Coldebella et al., 2004). Hence, udder quarters infected with common causative agents of mastitis are the main cause of milk contamination (Jayarao et al., 2004). Contaminated milking equipment and the hands of the milkers are also common sources of transmission (Fagundes et al., 2011). Although pasteurization kills *S. aureus* and *E. coli* cells, thermostable toxins from *S. aureus* generally retain their biological activity leading to potential health risks (Genigeorgis, 1989).

Dairy management techniques observed in Brazil in recent years has contributed to the increase in milk production, followed by investments to improve microbiological quality of milk in dairy farms, especially in large scale production. However, most of the dairy production in Brazil is either small or medium-scale farming with a mean daily output of up to 400 L of milk and 26 lactation dairy cows (Brito et al., 2004). The microbiological quality of the milk from those farms is

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Table 1. Prevalence of *S. aureus* and *E. coli* O157: H7 (number and percentage) in milk samples from individual cows with low and high somatic cell counts (SCC) in dairy farms of São Paulo, Brazil.

Microorganism	SCC in milk (cells/ml)				χ^2 value	P value
	< 200,000		> 200,000			
	N*	n (%)	N*	n (%)		
<i>S. aureus</i>	160	9 (5.6)	458	24 (5.2)	0.03	0.85
<i>E. coli</i> O157:H7	160	1 (0.6)	458	7 (1.5)	0.75	0.38

* Number of samples analyzed.

rather variable, mainly because of economical restrictions for investment, the absence of educational programs addressing the hygienic conditions during milking, milk preservation and transport, or unsuccessful attempts to train milkers on better hygiene practices (Fagundes et al., 2011).

There is little information in Brazil on the presence of mastitis-causing pathogenic bacteria in individual cow's milk on small dairy farms. Considering the different characteristics of milk production on the eastern central region of the state of São Paulo, the objective of the present study was to assess the relationship between SCC, the use of different milking practices, and the occurrence of *S. aureus* and *E. coli* O157:H7 on small-scale dairy farms.

MATERIALS AND METHODS

The study was conducted in 42 small-scale dairy farms located in the eastern central region of the state of São Paulo, Brazil. Seven farms per month were visited every other month from February, 2005 to March, 2006. Farms were chosen randomly and visited for sampling procedures only once during the entire study period. In each farm, milking management and hygiene conditions were evaluated by means of a questionnaire on the following items: milking equipment, milking management, handler hygiene habits, and measures used in mastitis diagnosis and prevention. The questionnaire was answered by the person responsible for milking the cows in each of the farms sampled. Based on the information obtained in the questionnaire, the following items were selected to evaluate the relationship of milking practices with the incidence of *S. aureus* and *E. coli* O157:H7 in raw milk produced in the farms: (1) closed milking system, (2) use of pre-dipping, (3) use of post-dipping, (4) use of disposable gloves during the milking procedure, (5) use of the strip cup test to diagnose clinical mastitis, (6) disinfection of teat cups between cows.

Individual milk samples were obtained from all cows showing signs of subclinical mastitis according to the California Mastitis Test (CMT). The number of lactating cows sampled in each farm varied between 9 to 20/farm, totaling 618 individual milk samples collected in the 42 dairy farms. Duplicate samples (100 ml) were aseptically collected during the first morning milking from the individual meters in the milking machine. At the end of the milking procedure, duplicate bulk milk samples (100 ml) were collected in sterile glass vials (Fagundes et al., 2011). Samples were stored and maintained under refrigeration in an isothermal container until analysis.

Isolation and identification of *S. aureus* were carried out according to American Public Health Association (1992). Aliquots of milk samples (25 ml) were cultured in Baird Parker agar supplemented

with 5% egg yolk saline suspension (1:1, w/w) with 1% potassium tellurite. Typical and atypical colonies on Baird Parker agar were identified as *S. aureus* using the following biochemical tests: catalase, coagulase, DNase, acetoin production, and maltose fermentation (without gas production).

For the isolation of *E. coli* O157:H7, dilutions of the samples (10^1 , 10^2 and 10^3) were cultured on plates containing Sorbitol-MacConkey agar + MUG (4-methylumbelliferyl- β -D-glucuronide, 50 mg/L), and incubated at 37°C. After 24 h, plates were examined for the identification of colonies that were sorbitol and MUG negative, that is, colonies that showed no changes in the color of the medium (sorbitol negative) and no fluorescence under UV light (glucuronidase negative), according to Massa et al. (1999). Confirmation was carried out in six typical colonies selected from each sample, enriched in casein-peptone soymeal-peptone broth for indol, citrate, methyl red, and Voges Proskauer (Silva et al., 2001). For all analyses, a standard *E. coli* O157:H7 strain, obtained from Adolf Lutz institute culture collection, was used as a positive control. After isolation and biochemical confirmation, strains were subjected to serology tests by slide agglutination to detect the presence of the O157 antigen using the anti-*E. coli* O157 serum. Results were considered positive when a complete and homogenous clot suspension was formed after two minutes of slight manual stirring (Edwards and Ewing, 1972).

Somatic cell counts were carried out automatically by flow cytometry (Somacount - Bentley Instruments Inc., US). Results were tabulated, and animals were classified in one of two groups for statistical analysis: low SCC (< 200,000 cells/ml of milk), which represented absence of subclinical mastitis; and high SCC (> 200,000 cells/ml of milk), which represented any degree of subclinical mastitis.

The dairy farms evaluated were divided into 2 categories based on the frequencies of positive and negative responses in the questionnaire regarding milking management and hygiene conditions items (closed milking system, use of disposable gloves, and measures used in mastitis diagnosis and prevention). The differences in the frequencies of *S. aureus* and *E. coli* in farms of each category were analyzed by chi-square test ($p < 0.05$) (SAS Institute, 2004).

RESULTS AND DISCUSSION

Prevalence of *S. aureus* and *E. coli* in the milk samples from cows with low and high SCC are presented in Table 1. The number of samples classified as high SCC (> 200,000 cells/ml) was greater than low SCC (< 200,000 cells/ml), indicating the presence of subclinical mastitis among the animals evaluated. There was no significant difference in SCC when the presence of either *S. aureus* or *E. coli* O157:H7 was detected in the same milk

Table 2. Prevalence of *S. aureus* in milk of individual cows as affected by various management practices on dairy farms of São Paulo, Brazil.

Milking practice	Yes		No		χ^2 value	P value
	N*	n (%)	N*	n (%)		
Closed milking system	447	21 (4.7)	171	12 (7.0)	1.31	0.25
Pre-dipping	414	18 (4.2)	204	15 (7.3)	2.44	0.11
Post-dipping	432	23 (5.4)	186	10 (5.3)	0.01	0.97
Disposable gloves	248	8 (3.2)	370	25 (6.7)	3.66	0.05
Strip cup test	434	20 (4.6)	184	13 (7.0)	1.54	0.21
Teat cup disinfection	147	6 (4.0)	471	27 (5.7)	0.60	0.43

* Number of samples analyzed.

Table 3. Prevalence of *E. coli* O157:H7 in milk of individual cows as affected by various management practices on dairy farms of São Paulo, Brazil.

Milking practice	Yes		No		χ^2 value	P value
	N*	n (%)	N*	n (%)		
Closed milking system	447	6 (1.3)	171	2 (1.1)	0.02	0.86
Pre-dipping	414	4 (1.0)	204	4 (2.0)	1.05	0.30
Post-dipping	433	4 (1.0)	186	4 (2.0)	1.53	0.21
Disposable gloves	248	3 (1.2)	370	5 (1.3)	0.02	0.87
Strip cup test	434	6 (1.4)	184	5 (1.0)	0.10	0.74
Teat cup disinfection	147	2 (3.0)	471	5 (1.0)	0.84	0.35

* Number of samples analyzed.

sample. In the condition of the present study, *S. aureus* and *E. coli* O157:H7 could be isolated both from healthy animals and those affected by subclinical mastitis. Although *S. aureus* was more frequently found in the milk (5.4%) than *E. coli* O157:H7 (1.3%), the occurrence of both pathogens in milk is of public health concern because raw, unprocessed milk is largely consumed by the Brazilian population.

In the present study, the determination of *E. coli* O157:H7 in raw milk was performed as recommended by Silva et al. (2001) for analysis of foods, selecting typical colonies in sorbitol-MacConkey agar supplemented with MUG. However, it was observed that when this technique is used for raw milk, other bacteria that do not ferment sorbitol and are typically found in milk, such as *Proteus* spp., *Aeromonas* spp., and other *E. coli* strains, are also able to grow. The excess of colonies on the plate made it difficult to isolate *E. coli* O157:H7 for confirmatory tests. Hence, it is possible that the rate of isolation of *E. coli* O157:H7 found in individual milk has been underestimated. Although in the present experiment *S. aureus* and *E. coli* O157:H7 have not been counted in milk samples, the detection limit of the methods used for each microorganism was 10^1 CFU/mL. This value is lower than the infectious dose for *S. aureus* (10^5 CFU), but within the infectious dose range for *E. coli* O157:H7 (10^0 to 10^2 CFU) as reported by Paton and Paton (1998),

which stresses the potential risk of *E. coli* O157:H7 poisoning transmission to consumers via raw, unprocessed milk.

Results on the characterization of the farms based on milking practices and the occurrence of *S. aureus* and *E. coli* O157:H7 in milk are presented in Tables 2 and 3, respectively. The use of disposable gloves during milking reduced *S. aureus* counts in milk ($p < 0.05$), but did not affect the occurrence of *E. coli* O157:H7. When disposable gloves are used correctly, they decrease the occurrence of cross-contamination between man and the animals, and between animals. According to several authors (Genigeorgis, 1989; Murray, 1998), humans are the most probable sources of primary contamination of food by *S. aureus*. This microorganism is part of the microbiota of the nose, neck, and hands of a large percentage of the human population, and when these people handle food, they may contaminate the raw product, equipment, and/or the final product. Our results are consistent with data reported by Tondo et al. (2000), who observed that 35.2% of the handlers from a dairy plant that did not use gloves could carry *S. aureus* into the milk and dairy products. In spite of these results, the Brazilian authorities and international organizations including the United Nations Food and Agriculture Organization do not consider the use of gloves among the mandatory procedures involved in hygienic milk

handling and processing (Food and Agriculture Organization, 2011).

The other milking practices evaluated (milking system, use of pre- and post-dipping, mastitis diagnosis by strip cup test, and disinfection of teat cups) did not affect ($p > 0.05$) the occurrence of either *S. aureus* or *E. coli* O157:H7 in raw milk. The fact that milking system did not affect the incidence of *S. aureus* or *E. coli* O157:H7 in raw milk was not surprising, since technological level of the milking procedure is not necessarily a guarantee of milk of better microbiological quality. In a study on the characterization of dairy farms in the state of Minas Gerais, Brazil, Guerreiro et al. (2005) observed that one of the three farms analyzed, which had a closed milking system, showed higher counts than the one obtained in a farm that used rudimentary manual milking. Hygienic procedures of the milking equipment are of particularly importance to avoid microorganism from environmental sources, such as *E. coli*, which can be spread everywhere, including the milking parlor. Thus, milking equipment may play a significant role in the contamination of milk with this microorganism, mainly when hygiene procedures are inadequate; or even during milking, by means of direct contact between milk and the surfaces of contaminated equipment.

Among milking practices, pre- and post-dipping are the better methods for avoid milk contamination and the occurrence of environmental mastitis in the milking routine. These two procedures are essential hygienic practices during milking to prevent the occurrence of mastitis among the animals (Hovinen and Pyorala, 2011). However, in our study, although the use of pre-dipping decreased the incidence of *S. aureus* in milk from 7.3 to 4.2%, there were no differences ($p > 0.05$) between the occurrence of *S. aureus* or *E. coli* O157:H7 in milk from farms using pre- and post-dipping.

Interestingly, it was observed that the strip cup test was used to determine SCC routinely in most of the animals (70% of 618 cows examined) from the 42 farms analyzed. This procedure is widely used by the farmers as a simple, effective indicator of clots in discard milk. There was no relationship between the use of strip cup test and the occurrence of *S. aureus* or *E. coli* O157:H7 in milk in the present study. Also, disinfection of teat cups with chlorine during the milking procedure did not affect ($p > 0.05$) the occurrence of *S. aureus* or *E. coli* O157:H7 in milk, which is consistent with previous reports showing that teat cup disinfection did not reduce significantly the number of contaminants, and that there was an increase in the number of microorganisms isolated after the disinfectant was used (Hovinen and Pyorala, 2011).

S. aureus and *E. coli* O157:H7 were isolated from the milk of healthy cows and those with subclinical mastitis. The use of disposable gloves during the milking procedure reduced the occurrence of *S. aureus* in milk.

However, the other milking management items analyzed (type of milking system, pre- and post-dipping, strip cup test, and teat cup disinfection), and somatic cell counts did not affect the occurrence of *S. aureus* or *E. coli* O157:H7 in milk from small-scale dairy farms in Brazil.

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