

High Incidence of Enterotoxin D Producing *Staphylococcus* spp. in Brazilian Cow's Raw Milk and Its Relation with Coagulase and Thermonuclease Enzymes

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Abstract

In this study, the enterotoxigenic potential of *Staphylococcus* strains ($n = 574$) isolated from raw milk samples ($n = 140$) was determined for their capacity to produce staphylococcal enterotoxins. In addition, the relationship between the presence of enterotoxins, coagulase, and thermonuclease (Tnase) was assessed. The results showed that 19% of *Staphylococcus* was enterotoxigenic, being able to produce at least one of the staphylococcal enterotoxins (A, B, C, and D). Most of the strains were able to produce enterotoxin D (68.8%), whereas 12.8% of the *Staphylococcus* strains were able to produce staphylococcal enterotoxin A. Besides, the production of more than one type of enterotoxins by the same strain was observed. Tnase was considered the best marker for enterotoxigenic potential of isolates, although some of them were negative for coagulase and Tnase but positive for enterotoxin production. Therefore, either the use of Tnase to assess *Staphylococcus* enterotoxigenic potential or the use of simple and easy screening tests for enterotoxin production should receive more attention when evaluating the pathogenic potential of foodborne *Staphylococcus* strains. Due to the association of both coagulase positive *Staphylococcus* and coagulase negative *Staphylococcus* with foodborne disease outbreaks, regulators and industries should pay more attention to enterotoxigenic *Staphylococcus* rather than focusing only on *S. aureus* or coagulase positive *Staphylococcus*. Finally, data found here suggest a high risk of staphylococcal intoxication with the consumption of raw milk or dairy products made from raw milk.

Introduction

STAPHYLOCOCCUS spp. ARE widespread microorganisms that play an important role in foods as beneficial microorganisms (Irlinger, 2008; Zell *et al.*, 2008; Coton *et al.*, 2009) or as agents of foodborne diseases (Miwa *et al.*, 2001; Do Carmo *et al.*, 2002, 2003; Colombari *et al.*, 2007; López *et al.*, 2008). Foodborne diseases associated with enterotoxigenic *Staphylococcus* are caused by the consumption of foods contaminated with preformed enterotoxins (Le Loir *et al.*, 2003). Among staphylococcal enterotoxins, type-A enterotoxin is responsible for almost 80% of outbreaks (Balaban and Rasooly, 2000).

The ability of *Staphylococcus* to produce coagulase is used to indicate enterotoxigenic potential of isolates, which are called coagulase positive *Staphylococcus* (CPS). Due to this, they have received more attention by both governments and industries when establishing regulations, microbiological specifications,

or methods. Despite this, the ability of coagulase negative *Staphylococcus* (CNS) to produce enterotoxins (Vernozy-Rozand *et al.*, 1996; Udo *et al.*, 1999; Zell *et al.*, 2008) and their association with foodborne disease outbreaks have been reported (Do Carmo *et al.*, 2002; Veras *et al.*, 2008). The presence of CNS in foods is of concern, as they are commonly found in raw material (e.g., as milk) and may be used as starter cultures for production of fermented foods (Zell *et al.*, 2008). Since analytical methodology assumes that enterotoxigenic *Staphylococcus* must produce coagulase (Lancette and Bennett, 2001), the presence of CNS is overlooked and, thus, implications for food safety may arise. Therefore, testing the potential of *Staphylococcus* strains to produce enterotoxins using easy and inexpensive methods (Robbins *et al.*, 1974) may be a better choice so as to report on the enterotoxigenic potential of *Staphylococcus* strains. This study aimed at determining the enterotoxigenic potential of *Staphylococcus* strains isolated from

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Brazilian raw milk and estimating the relationship between production of enterotoxins and coagulase and thermonuclease (Tnase) enzymes.

Materials and Methods

Raw milk sampling

Raw milk samples (200 mL) of nine different and randomly selected milk producers and a pool of them were collected during 10 months. These samples ($n = 140$) were sent by milk producers to a cooperative dairy plant located in the city of Campinas, State of São Paulo (Brazil), for milk and dairy production. Samples were collected under aseptic conditions, transported, and maintained at 4°C. Microbiological analysis was performed at the same day of sampling.

Enumeration and identification of *Staphylococcus* spp.

The enumeration and identification of *Staphylococcus* spp. in raw milk samples were performed as proposed by Lancette and Bennett (2001). An aliquot of samples (10 mL) was homogenized in 90 mL of 0.1% sterile peptone water. Decimal dilutions were prepared in tubes containing 9 mL of this diluent and inoculated onto Baird Parker agar (Oxoid, Basingstoke, United Kingdom) plates. The inoculum was spread over the surface of the agar using sterile rods. Plates were kept in an upright position until liquid was absorbed. Baird Parker agar plates were inverted and incubated at 37°C for 48 h. Characteristic colonies of *Staphylococcus* spp. (circular, smooth or rough appearance, convex, gray-black surrounded by opaque and clear zones or not) were counted. About 5–10 colonies of each sample were picked up for confirmation. Confirmation at genera level was performed by using Gram's staining, production of catalase, coagulase, heat stable nuclease (Tnase), and anaerobic utilization of mannitol tests (Lancette and Bennett, 2001). Coagulase was performed using the tube method as described by Lancette and Bennett (2001), whereas Tnase production was assessed by the plate technique with toluidine blue-deoxyribonucleic acid agar (Lachica, 1976). Anaerobic fermentation of mannitol was used when isolates were negative for coagulase and Tnase tests to differentiate *Staphylococcus* spp. from *Micrococcus* spp. Since

Micrococcus is a strict aerobe, mannitol fermentation indicated that an isolate was within *Staphylococcus* genus (Schleifer, 1986). An isolate was considered within *Staphylococcus* genus when presented as gram-positive cocci in grape-like clusters, catalase, and mannitol fermentation positive. A 3+ or 4+ clot formation was considered as a positive reaction for *Staphylococcus*, whereas isolates presenting 1+ and 2+ clot types were only considered positive when Tnase test was also positive. The number of isolates that presented positive and negative results in coagulase and Tnase tests was recorded. Isolates presenting positive or negative reactions for coagulase and positive reaction for Tnase were investigated for their ability in producing enterotoxins.

Enterotoxin production and detection

For enterotoxin production, each of the *Staphylococcus* isolates were inoculated in brain-heart infusion (BHI) broth (Difco, Sparks, NV). Tubes were incubated overnight at 37°C. BHI Agar plates were covered with membrane disks made from Spectra/membrane dialysis tubing, 6000–8000, 100 mm flat width (Thomas Scientific, Philadelphia, PA). Then, 0.5 mL of each isolate grown in BHI broth was inoculated in membranes, and the plates were incubated at 37°C for 24 h. Membranes were washed twice with 1.5 and 1 mL, respectively, of 0.02 M NaHPO₄. Then, cultures were centrifuged and culture supernatants were used for enterotoxin detection (Robbins *et al.*, 1974; Pereira *et al.*, 1996).

For enterotoxin detection, the optimum-sensitivity plate (OSP) method was used (Robbins *et al.*, 1974). Noble agar (1.2%) prepared in 0.02 M phosphate-buffered saline, pH 7.4 was put in 50 mm diameter Petri dishes. Then, seven wells (five with 8.3 mm and two with 6.7 mm diameter) were cut in the agar. The antiserum, control enterotoxin, and samples were placed in the central, in the two smaller and in the four larger outer wells, respectively. Petri dishes were closed and stored in a humidified box at 37°C for 18 h. The union of the precipitation line produced by isolate fluids with those formed by control indicated a positive reaction. To detect low-enterotoxin-producing strains, culture supernatant fluids were concentrated 10 times. A, B, C, and D *Staphylococcal* enterotoxins, together with their respective antiserum, were gently obtained

TABLE 1. PERCENTAGE OF ENTEROTOXIGENIC *STAPHYLOCOCCUS* SPP. IN RAW MILK

Milk producers	Enterotoxigenicity of <i>Staphylococcus</i> strains (%)		Total number of isolates tested
	Positive	Negative	
Pool	15.2 ^{a,b,A}	84.8 ^{a,b,B}	66
P2	18.4 ^{a,b,A}	81.6 ^{b,c,B}	87
P3	11.1 ^{a,b,A}	88.9 ^{a,b,B}	45
P4	15.6 ^{a,b,A}	88.4 ^{a,b,B}	64
P5	3.3 ^{c,A}	96.7 ^{a,B}	61
P6	24.1 ^{a,b,A}	75.9 ^{b,c,B}	54
P7	29.8 ^{a,b,A}	70.2 ^{b,c,B}	47
P8	14.6 ^{a,b,A}	85.4 ^{a,b,B}	48
P9	17.9 ^{a,b,A}	82.1 ^{a,b,c,B}	39
P10	39.7 ^{a,A}	60.3 ^{c,B}	63
Mean	19	81	574

Values in the same line with different capital letters indicate significant difference (95%) between enterotoxigenic and nonenterotoxigenic *Staphylococcus* strains for the same producer. Values in the same column with different small letters indicate significant difference (95%) between enterotoxigenic and nonenterotoxigenic *Staphylococcus* strains among producers.

TABLE 2. TYPES OF ENTEROTOXINS PRODUCED BY 109 RAW MILK *STAPHYLOCOCCUS* SPP. ISOLATES

Types of enterotoxins produced by <i>Staphylococcus</i> spp. isolated from raw cow milk (%)						
A	B	C	D	A-B	A-D	B-D
12.8	7.3	2.8	68.8	3.7	ND	4.6

ND, nondetected.

from M.S. Bergdoll (Food Research Institute, University of Wisconsin-Madison). Different dishes were used for testing enterotoxin production by raw milk *Staphylococcus* isolates.

Statistical analysis

Goodman's test was used for contrasts among multinomial proportions at 5% significance level (Sheskin, 2000).

Results and Discussion

In Brazil, *Salmonella* spp. is the first agent causing food-borne diseases, whereas enterotoxigenic *Staphylococcus* is the second most important microorganism associated with these diseases. *Staphylococcus* are blamed for more than 572 outbreaks from 1999 to 2007 (ANVISA, 2008). Several outbreaks caused by these microorganisms have been reported in the literature (Do Carmo *et al.*, 2002, 2003, Colombari *et al.*, 2007; Veras *et al.*, 2008), and some of them are linked to dairy products.

In this study, all samples presented presumptive *Staphylococcus* colonies, and counts of these microorganisms ranged around 8.9×10^3 – 1.5×10^5 colony forming units (CFU)/mL, which may be indicative of poor hygiene during milking or in temperature control during transportation, as the milk came from healthy cows. From 140 samples, 574 *Staphylococcus* strains were isolated and tested for enterotoxins production. Despite the significant difference between some milk producers (from 11.1% to 39.7%) according to Goodman's statistical test ($p < 0.05$), it is possible to see in Table 1 that the average rate for the incidence of enterotoxigenic *Staphylococcus* was 19% ($n = 109$). Although the percentage of enterotoxigenic strains cannot be considered high, it must be observed with concern by milk farmers and dairy processing plants. For milk

farmers, the results found here indicate that although programs on mastitis control may be successful, more care should be taken with hygiene and temperature control between milking and milk being received at the dairy plant for processing. Counts of *Staphylococcus aureus* higher than 10^6 CFU/g or mL in foods are reported as the critical level for these microorganisms to produce enterotoxins and to cause food-borne disease outbreaks (Balaban and Rasooly, 2000; Le Loir *et al.*, 2003). However, even low counts such as 10^3 CFU/g reached by *S. aureus* may be enough for this microorganism to produce enough amount of enterotoxin (1 ng/g) to cause intoxication (Pereira *et al.*, 1991). This reinforces the need of dairy processing plants working together with milk farmers so as to improve the microbiological quality of raw milk. Besides being a source of contamination by *Staphylococcus* in the processing environment, the presence of enterotoxins in raw milk samples may lead to the production of high-risk dairy products. This is because staphylococcal enterotoxins are highly stable to the steps used in dairy processing, mainly milk pasteurization (Modi *et al.*, 1990; Schwabe *et al.*, 1990).

According to Table 2, *Staphylococcus* spp. isolated from Brazilian raw milk samples were mainly producers of type D enterotoxin (68.8%), with less than 10% of the strains producing two different types of enterotoxins. The percentage of isolates producing enterotoxin type A was low. Currently, more than 20 types of staphylococcal enterotoxins are known (Balaban and Rasooly, 2000; Pereira *et al.*, 1991; Munson *et al.*, 1998; Orwin *et al.*, 2001; Omoe *et al.*, 2005; Thomas *et al.*, 2006; Ono *et al.*, 2008). However, enterotoxin A is the most commonly staphylococcal enterotoxin associated with foodborne outbreaks, followed by enterotoxin D (Balaban and Rasooly, 2000). Data obtained here indicated that enterotoxin D may play an important role in staphylococcal food poisoning in

TABLE 3. CORRELATION BETWEEN ENTEROTOXIN PRODUCTION, COAGULASE, AND THERMONUCLEASE BY RAW MILK *STAPHYLOCOCCUS* SPP. ISOLATES

Producer	Tnase (+) Coag (+) (%)	Tnase (–) Coag (–) (%)	Tnase (+) Coag (–) (%)	Tnase (–) Coag (+) (%)	Number of cultures tested
Pool	20 ^{c,A}	30 ^{a,A}	40 ^{b,c,A}	10 ^A	10
P2	87.5 ^{a,b,A}	0.0	12.5 ^{c,B}	0.0	16
P3	20 ^{c,B}	0.0	80 ^{a,A}	0.0	05
P4	100	0.0	0.0	0.0	10
P5	100	0.0	0.0	0.0	02
P6	38.5 ^{b,c,A}	0.0	61.5 ^{a,A}	0.0	13
P7	35.7 ^{b,c,A}	0.0	64.3 ^{a,A}	0.0	14
P8	42.8 ^{b,c,A}	28.6 ^{a,A}	28.6 ^{b,c,A}	0.0	07
P9	85.7 ^{a,b,A}	0.0	14.3 ^{b,c,B}	0.0	07
P10	92 ^{a,b,A}	0.0	8.0 ^{c,B}	0.0	25

Values in the same line with different capital letters indicate significant difference (95%) regarding coagulase and Tnase production by enterotoxigenic *Staphylococcus* strains for the same producer. Values in the same column with different small letters indicate significant difference (95%) regarding coagulase and Tnase production by enterotoxigenic *Staphylococcus* strains among producers.

Tnase, thermonuclease; Coag, coagulase.

Brazil, as this enterotoxin and type A enterotoxin have been commonly associated with outbreaks in which *Staphylococcus* strains have been implicated (Do Carmo *et al.*, 2002, 2003; Veras *et al.*, 2008). However, which type of staphylococcal enterotoxins is more associated with foodborne disease outbreak seems not to be relevant. This is because staphylococcal enterotoxins have very similar structure and function (Balaban and Rasooly, 2000) and will cause known symptoms if they are found in foods in enough amount to cause food poisoning.

OSP method has been chosen to screen staphylococcal enterotoxins, as it requires an easy sample preparation and setup, simple reagents, and has a sensitivity of 0.5 mg/mL (Su and Wong, 1997). Table 3 shows the correlation between enterotoxigenic *Staphylococcus* strains and their ability to produce coagulase and Tnase. Pools P3, P6, and P7 presented higher percentages of isolates within Tnase (+) and coagulase (−) group, whereas a significant difference between enterotoxin and enzymes production was only found in P3 ($p < 0.05$). It can be noticed that in pool and P8 milk producers, up to 30% of isolates were enterotoxigenic and even presented negative results for Tnase and coagulase tests ($p > 0.05$). Isolates from P4, P5, P9, and P10 milk producers presented the higher percentages of enterotoxin production when Tnase and coagulase were positive ($p < 0.05$). Among all milk producers, only one strain (10%) of 10 tested in pool, presented enterotoxigenic potential and Tnase (−) and coagulase (+). Staphylococcal isolates from pool and P3 milk producers yielded the lower percentages of correlation (20%) between enterotoxin and coagulase/Tnase production ($p < 0.05$). This research found that a high percentage of enterotoxigenic *Staphylococcus* isolates were coagulase negative. Danielsson and Hellberg (1977) also reported that CNS isolated from food handlers produced SE. Additionally, data indicated that the Tnase test is more faithful to assess pathogenic potential of foodborne *Staphylococcus*. So, either the use of Tnase to assess *Staphylococcus* enterotoxigenic potential or the use of simple and easy screening tests for enterotoxin production such as OSP (Robbins *et al.*, 1974) could be considered. Due to the association of both CPS and CNS with foodborne disease outbreaks, regulations and food industries should pay more attention to enterotoxigenic *Staphylococcus* rather than focusing only on *S. aureus* or CPS. Finally, data found here suggest a high risk of staphylococcal intoxication by raw milk or dairy products made from raw milk due to the enterotoxigenic potential of the strains isolated.

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Disclosure Statement

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