

Methicillin-resistant *Staphylococcus aureus* in Human Milk

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We collected and analyzed 500 samples of human milk, from five Brazilian cities (100 from each) to detect methicillin-resistant strains of Staphylococcus aureus (MRSA) producing enterotoxins. We found 57 strains of MRSA, and the mecA gene, responsible for resistance, was detected in all of them using a specific molecular probe. We examined 40 strains for the presence of four enterotoxins, after selecting a subset that included all strains from each region, except for the largest sample, from which 10 were randomly selected. Among these two presented enterotoxin B, and growth in human colostrum and trypticase soy broth. After 5 h of incubation at 37°C, population sizes were already higher than 9.4×10^5 UFC/ml and enterotoxin was released into culture medium and colostrum. Our results stress the importance of hygiene, sanitary measures, and appropriate preservation conditions to avoid the proliferation of S. aureus in human milk.

Key words: human milk methicillin-resistant - *Staphylococcus aureus* - enterotoxin - Brazil

In recent years, *Staphylococcus aureus* has emerged as one of the most important human pathogens in community and hospital settings (Kloos & Bannerman 1995). Community infections by methicillin-resistant *S. aureus* (MRSA) strains were reported as early as 1981 (Layton et al. 1995). Nettleman et al. (1991) reported that 50% of MRSA strains isolated from Iowa City Medical Center (USA) originated from the community. Among these, 33% originated from inpatients in homes for the aged, 9% in patients transferred from other hospitals, and 58% were from patients arriving at the Center directly from home (Nettleman et al. 1991). In another study, involving five tertiary care hospitals in Canada, over the 1990-1992 period, 63% of the isolates had a community origin, and 81% were from local residents, referred to the community hospitals. The authors of the study suggested that the indiscriminate use of antibiotics on an empirical basis promotes the transmission and emergence of these organisms (Embil et al. 1994). In large university hospitals of the United States, the frequency of MRSA among *S. aureus* isolates from hospital infections has increased from 8% in 1986 to 40% in 1992 (Boyce et al. 1994).

Asymptomatic carriers play an important role in the maintenance and spread of these microorganisms, especially when the carriers have professional activities related to public health (Soares et al. 1997). The natural habitat of *S. aureus* is the body surface of mammals (Williams 1963), which accounts for its widespread presence in nature, its transmission to foodstuffs by asymptomatic carriers, and in animals, particularly cattle (Maschohshvili et al. 1991).

The presence of *S. aureus* in samples of human milk (HM) can be accounted for by secondary contamination from skin, breasts and the nasal cavity of milk donors and health care professionals, or alternatively, to unsatisfactory conditions of the utensils (Almeida et al. 1998). Pereira et al. (1995) demonstrated the presence of these microorganisms in 19 samples of HM, 9 of which were free of mastitis symptoms, while the remaining 10 had mastitis. In such samples, *S. aureus* counts ranged from 10^2 to 10^4 CFU/ml (Pereira et al. 1995).

Furthermore, inadequate handling of milk can contribute to the increase in staphylococcal numbers up to the moment of pasteurization (Brasil 1998), allowing for the production of thermostable toxins, which resist temperatures as high as 100°C for 30 min. Pasteurized milk, which may be free of microorganisms, but not of enterotoxins (Maschohshvili et al. 1991), can be given to premature babies, which are highly dependent on it for survival (Almeida et al. 1998). In the study referred above, with mastitis-afflicted and mastitis-free

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women from Brazil, Pereira et al. (1995) identified enterotoxin-producing strains in four samples from mastitis patients, as well as in four samples from mastitis-free women, with the help of the Optimum-Sensitivity-Plate (OSP) method. In addition, they identified two additional enterotoxin-producing strains with the help of the more sensitive Reversed Passive Latex Agglutination (RPLA) technique (Pereira et al. 1995).

Enterotoxins are among the extracellular proteins made by *S. aureus* that play a major role in the pathogenesis of staphylococcal disease (ICMSF 1978). They are classified in five large serological groups, termed A to E (Lebeau et al. 1994). These toxins present, in addition to their well-known emetic actions, the ability to bind to Class II molecules coded by the major histocompatibility complex, and to act as superantigens triggering polyclonal responses in target T lymphocytes, and resulting in large-scale secretion of cytokines, including tumor necrosis factor (TNF) and several interleukins (Bethley & Harries 1994).

The goal of this study was to evaluate the frequency of enterotoxin-producing strains of MRSA in samples of HM, as well as their ability to produce enterotoxin in human colostrum.

MATERIALS AND METHODS

Sample collection - Samples of HM were obtained in five Brazilian cities, from 500 different registered and instructed regular donors, in the following HM banks: "Banco de Leite da Maternidade Januário Cicco", in Natal, State of Rio Grande do Norte; "Banco de Leite da Maternidade Escola Assis Chateaubriant", in Fortaleza, State of Ceará; "Banco de Leite do Instituto Fernandes Figueira", Rio de Janeiro, State of Rio de Janeiro; "Banco de Leite da Universidade de Brasília", Brasília, Federal District; and "Banco de Leite da Universidade Estadual do Paraná", Londrina, State of Paraná. Approximately 5 ml of milk from the donated flasks were collected under aseptic conditions and transported frozen, by airplane, to Rio de Janeiro where the microbiological analyses were carried out.

Staphylococcus aureus counts - We inoculated 0.1 ml of HM samples, in duplicate, at the appropriate dilutions, on Baird-Parker Agar, and incubated at $35 \pm 1^\circ\text{C}$ for 24-48 h before colony counting. Colonies were black, punctiform, surrounded by a white opaque halo, and with a transparent external layer (Speck 1996).

Isolation of methicillin-resistant S. aureus - We added 0.5 ml of milk to 5 ml of trypticase soy broth (TSB, Difco), with NaCl 7.5% and 10 $\mu\text{g/ml}$ methicillin (Sigma), and incubation was carried out at $36 \pm 1^\circ\text{C}$ for 24-48 h. Samples that presented

growth were inoculated on trypticase soy agar (TSA, Difco) and incubated at $35 \pm 1^\circ\text{C}$ for 24 h. After growth, five colonies with morphology typical of *Staphylococcus* were inoculated in TSB. Cultures were incubated at $35 \pm 1^\circ\text{C}$ for 18 h, under vigorous stirring and used for detection of MRSA, by assessing resistance to 25 $\mu\text{g/ml}$ of methicillin, which was used to isolate pure cultures. Strains that were positive for mannitol, Gram staining, catalase, coagulase and thermonuclease were classified as *S. aureus* and kept frozen at -80°C , in TSB with glycerol at a 10% final concentration.

Detection of mecA genes - Hybridization with a molecular probe specific for the *mecA* gene, which accounts for methicillin-resistance, was carried out by the dot-blot method. The preparation of bacterial DNA and of the *mecA* probe, as well as the hybridization technique were those described in Soares et al. (1997).

Enterotoxin detection - Assays for enterotoxins A, B, C and D were carried out with a SET-RPLA TD900 kit (Difco). Forty of the 57 MRSA strains were assayed. These included all the MRSA isolates from four cities and ten from Natal. Technical conditions recommended by the manufacturer were used for running the assays.

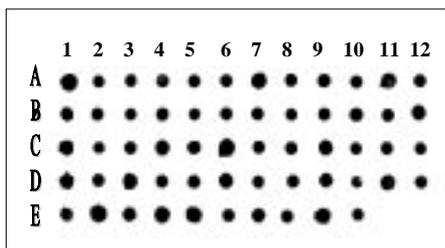
Analysis of microbial growth - Two different enterotoxin B-producing *S. aureus* strains (066 and 255) were inoculated in duplicate, into 40 ml of TSB or into 40 ml fresh human colostrum. Enterotoxin-producing strains were previously activated in 2 ml of TSB, incubated at 37°C for 24 h, centrifuged at $800 \times g$ under refrigeration at 4°C for 5 min and washed with TSB three times to remove preformed toxin. The number of microorganisms in the inoculum was estimated by turbidimetry at 560 nm, and diluted to approximately 5.0×10^2 CFU, in 1 ml of human colostrum or TSB. Growth was evaluated in screw-cap flasks incubated at 37°C , to simulate the summer temperatures in Rio de Janeiro. and population densities were determined at 0, 1, 2, 3, 4, 5, 8, 12 and 24 h by colony counts on Baird-Parker Agar, as described above.

RESULTS

Isolation of S. aureus from HM samples - A total of 171 strains of *S. aureus* were isolated from the 500 HM samples, with the following distribution: Natal, 52/100; Fortaleza, 38/100; Brasília, 23/100; Rio de Janeiro, 29/100 and Londrina, 29/100.

Determination of methicillin-resistance - A total of 57 *S. aureus* strains showing resistance to 25 μg of methicillin/ml were isolated from the 500 HM samples; and had the following distribution: Natal, 27/100; Fortaleza, 10/100; Brasília, 6/100; Rio de Janeiro, 8/100 and Londrina, 6/100.

Detection of the *mecA* gene - The presence of the methicilin-resistance gene was confirmed in all methicilin-resistant cultures with a specific *mecA* gene probe (Fig. 1).



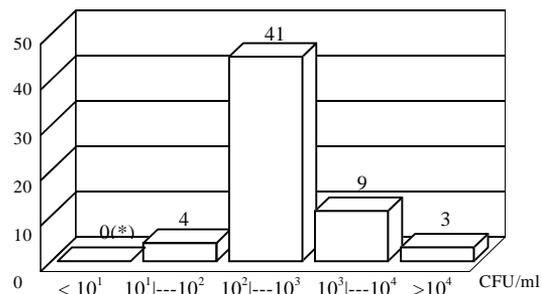
A1 - Positive control (Col) B4-B9 - Brasília
 A2-A9 - Rio de Janeiro B10-C7 - Fortaleza
 A10-B3 - Londrina C8-E10 - Natal

Fig. 1: Dot-blot hybridization of *Staphylococcus aureus* chromosome DNA with a specific *mecA* probe. Positive control, methicillin resistant *S. aureus* strain designated as Col (Soares et al. 1997).

Counting of *S. aureus* in samples containing methicilin-resistant strains - Seventy two percent of the 57 MRSA strains had counts in the range of 10^2 - 10^3 CFU/ml (Fig. 2)

Enterotoxin-production - Two EEB-producing strains were detected, among the 40 methicillin-resistant strains assayed with the SET-RPLA kit.

Growth curves of *S. aureus* strains in HM - Bacterial growth in human colostrum was slower (generation time 42 min) during the initial 5 h of culture, than that observed in culture medium (generation time 33 min; Fig. 3). After this period, microorganisms inoculated in colostrum presented an increased growth rate, comparable to that observed in culture medium, but did not reach the same final density. After 5 h incubation at $36 \pm 1^\circ\text{C}$, the population size was seen to increase from 5×10^2 CFU/ml to 9.4×10^5 . Enterotoxin production in both culture media was observed for both of the EEB-producing strains.



(*) below the sensitivity of the assay.

Fig. 2: colony forming units per milliliter detected in 57 human milk presenting methicillin resistant *Staphylococcus aureus* contamination.

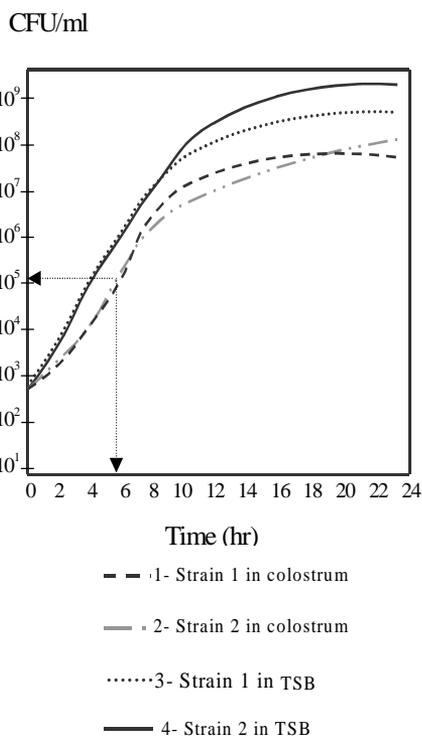


Fig. 3: growth of two EEB-producing *Staphylococcus aureus* strains in human colostrum and trypticase soy broth (TSB).

DISCUSSION

There was a high frequency of MRSA from HM and the *mecA* gene was present in all MRSA strains, as reported previously by De Lencastre et al. (1991). Concern over multiresistant, enterotoxin-producing *S. aureus* strains in hospital settings is justified by the report of Kluytmans et al. (1995), which described a MRSA hospital infection outbreak in Rotterdam, the Netherlands, transmitted through food prepared by a carrier with an MRSA isolate in these nostrils and oropharynx (Kluytmans et al. 1995). These reports indicate that healthy people may contribute to the dissemination of enterotoxin-producing MRSA clones, which produce and release toxins under conditions favoring growth. These toxins remain in contaminated food even after processing and the addition of antibiotics as preservatives.

In the present study, the fraction of MRSA that produced enterotoxin was much lower than that reported by other investigators working with clinical samples (Casman et al. 1967, Sourek et al. 1979, Melonian et al. 1983, Adesiyun et al. 1986, Humphreys 1989, Coia et al. 1992), and also lower than that reported by Pereira et al. (1995) for HM. However, those reports addressed enterotoxin-producing strains in the total *S. aureus* population present in the clinical samples. In contrast, we lim-

ited our study to methicillin-resistant strains, and to samples unrelated to outbreaks of toxi-infection. On the other hand, a low occurrence of enterotoxin production in cow's milk was observed in a study of 201 strains, in which only one EEB-producing strain was detected, amounting to 0.5% of the total (Freitas & Magalhães 1990).

According to Freitas and Magalhães (1990), enterotoxin-producing *S. aureus* strains are highly variable with regard to the frequency of isolation and to the type of enterotoxin produced. Pereira et al. (1991), in a study of intoxication induced by *S. aureus* in Belo Horizonte, Brazil, detected only production of EEB strains, and stressed the importance of such strains in Brazil (Pereira et al. 1991). Since, as proposed by Bohach et al. (1989), the gene for enterotoxin B is located on mobile genetic elements, which undergo integration into bacterial chromosomes (Bohach et al. 1989), it is possible that the presence of such elements accounts for the clonal diversity observed in EEB-producing strains (Soares et al. 1997). On the other hand, Renoud et al. (1994), studying 39 samples of EEB-producing *S. aureus* originating from clinical samples, classified them in three different clonal types, one of which was MRSA.

We believe the detection of toxins under our experimental conditions was mainly dependent on three factors: (a) the utilization of fresh culture, which may have rapidly adapted to the novel growth conditions; (b) the use of methodology able to detect 0.5 ng/ml which can detect enterotoxins even from poorly producing strains (Carmo & Bergdoll 1990); and (c) absence of competitors (Mitsuoka 1992).

MRSA growth in human colostrum was slower than that observed in culture medium during the first 5 h. This can probably be ascribed to antimicrobial factors associated to human colostrum, which include antistaphylococcal factor, lysozyme, macrophages and neutrophils (Wold & Adlerberth 1998). Growth in colostrum was faster after 5 h possibly due to exhaustion of these factors but the cells did not reach the same final density as in culture media, which could be related to differences in access to nutrients under these different conditions (Brock et al. 1994).

The data reported here illustrate a hazard associated with storage of HM under inappropriate conditions, especially at high environmental temperatures favoring growth of *S. aureus*. We suggest that implementation of stricter procedures for collection, processing, storage and distribution of HM, especially in tropical regions, would help restrict contamination by *S. aureus*. Refrigeration or, preferably, freezing, is likely to be a good control measure, because if *S. aureus* grow at low tem-

perature is not associated toxin release (Carmo & Bergdoll 1990).

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