Antimicrobial-resistance and enterotoxin-encoding genes among staphylococci isolated from expressed human breast milk

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Resistance traits and the presence of enterotoxin-encoding genes were investigated in staphylococcus isolates obtained from expressed human breast milk. A total of 54 staphylococcal isolates identified as Staphylococcus epidermidis (53.6%) and Staphylococcus warneri (20.4%), Staphylococcus haemolyticus (13%) and Staphylococcus aureus (13%) were investigated. By using a disc-diffusion method, higher rates of resistance, including intermediate resistance, were observed for penicillin (87%) and erythromycin (59.3%). All strains were susceptible to clindamycin and vancomycin. Minimal inhibitory concentration (MIC) was determined by a macrodilution method for four clinically relevant antimicrobial drugs. High rates of resistance or intermediate resistance were observed for erythromycin, gentamicin and oxacillin. Additionally, three isolates showed reduced susceptibility to vancomycin (MIC, 8 µg ml⁻¹). Genetic determinants of resistance were detected by using PCR and the results showed good correlation with the macrodilution tests. Moreover, in four staphylococcus isolates, the presence of enterotoxin-encoding genes (seg, seh and sea) was identified. The results demonstrated that expressed human breast milk can be a reservoir of multiresistant staphylococci that may also harbour important virulent determinants.

INTRODUCTION

High rates of nosocomial infections among neonates, especially those premature, are a great concern among health professionals (Sohn et al., 2001; Edwards, 2002). Several critical factors have been shown to be associated with this problem including food as a potential vehicle for the dissemination of pathogens and microbial metabolites in the nosocomial setting. The ingestion of these organisms should not lead to subsequent infection since gastric acid and the microflora normally prevent colonization within the gastrointestinal tract. However, in immunocompromised patients taking antacids (gastroesophageal reflux therapy) or antibiotics, for example hospitalized infants, these physical barriers may be altered, allowing the gastrointestinal tract to be colonized by these organisms. Colonization and subsequent infection, particularly septicaemia in neonates, have been associated with the ingestion of contaminated infant formulas or expressed human breast milk (EHM) (Weir, 2002; Youssef et al., 2002). Contamination of EHM is likely to occur because of maternal infection and/or colonization by opportunistic pathogens or by improper expression, manipulation and storage procedures (Boo et al., 2001; Qutaishat et al., 2003). According to Novak et al. (2000b), the isolation of multiresistant bacteria in EHM could be associated with long-term hospitalization and abusive antimicrobial usage that commonly occur in Brazil, particularly because of the high number of caesarean sections, which increases the rate of the mother’s colonization by opportunistic pathogens.

Staphylococci have been recognized as potential pathogens with increasing rates of resistance to various antimicrobial drugs currently used in the nosocomial setting (NNIS, 2002). In addition to Staphylococcus aureus, coagulase-negative staphylococci (CNS) have been isolated from EHM and intestinal microflora of neonates (Goldmann, 1988; Novak et al., 2000b). The presence of staphylococci in EHM samples can be accounted for by secondary contamination from the skin and the nasal cavity of milk donors and health care professionals, or alternatively, by unsatisfactory conditions of the utensils (Gutierrez & Almeida, 1998). Furthermore, there is evidence of a significant association between gastrointestinal colonization by CNS and septicaemia and necrotizing enterocolitis in premature neonates (Ng et al., 1995).

In staphylococci, resistance to macrolides, aminoglycosides, β-lactams and glycopeptides usually compromises antimicrobial therapeutic choices. In addition to antimicrobial-resistance traits, these micro-organisms, particularly S. aureus, produce a variety of exoproducts, including...
enterotoxins, toxic shock syndrome toxin 1, exfoliative, haemolysin and coagulase. Staphylococcal enterotoxins (SE) are a major cause of food poisoning. To date, nine biochemically, genetically and serologically different SEs have been described (SEA, SEB, SEC, SEF, SEG, SEH, SEJ, SEK and SEI) (Novick et al., 2001). SEA is the most frequently implicated in food-borne outbreaks. The presence of enterotoxin-producing staphylococci in EHM has been reported elsewhere (Adekeye & Adesiyun, 1984; Novak et al., 2000a).

Considering the potential role of EHM in the dissemination of pathogens, known to be an important cause of nosocomial infections in neonatology units, the present study aimed to detect the presence of staphylococci in EHM samples as well as to examine phenotypic and genotypic markers of antimicrobial resistance and to detect genes encoding enterotoxin production.

**METHODS**

**Bacterial strains.** A total of 54 bacterial strains obtained from EHM were analysed. EHM samples were collected during a survey of 15 weeks (one sample per week) at the Hospital Universitário Pedro Ernesto, a tertiary-teaching hospital located in Rio de Janeiro, Brazil. Samples were collected from different mothers 24 h after expression. The EHM were collected into sterile vials, transported under refrigeration and frozen and non-pasteurized until the volume was enough to pour into a single EHM sample, it was possible to recover up to three colonies from each milk sample, it was possible to recover up to three distinct isolates. The different staphylococcus colonies recovered from the same EHM sample were counted as distinct isolates.

**Antimicrobial susceptibility testing by disc-diffusion.** Resistance to amoxycillin/clavulanic acid, ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, oxacillin, penicillin, rifampicin, sulfamethoxazole/trimethoprim, tetracycline and vancomycin was determined by a disc-diffusion method as recommended by the NCCLS (2000a). *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 were used as reference strains.

**Antimicrobial susceptibility testing by macrodilution (MIC).** MICs were determined by a macrodilution method using cation-adjusted Mueller–Hinton broth (Difco Laboratories) according to the recommendations of the NCCLS (2000b). The antimicrobial drugs tested were gentamicin, erythromycin, oxacillin and vancomycin. *S. aureus* ATCC 29213 was used as a quality control of the procedures.

**DNA extraction.** DNA was extracted by the phenol/chloroform method and further precipitated using ethanol as described elsewhere (Sambrook et al., 1989). After centrifugation, the dried pellet was suspended in 200 µl pyrogen-free water and diluted to a final concentration of 10 ng µl⁻¹.

**Detection of resistance genes.** The presence of genes involved in erythromycin, gentamicin and methicillin resistance in staphylococci was determined by using PCR. The amplification parameters used to detect *erm, aac(6’)/aph(2’)* and *mecA* followed the recommendations of Sutcliffe et al. (1996), Martineau et al. (2000) and York et al. (1996), respectively. The nucleotide primer sequences are described in Table 1.

**SE gene detection.** The following SE genes were analysed: *sea, seb, sec, sed, see, seh, sei, sei* and *sej*. The multiplex PCR was performed in a 50 µl reaction mixture containing: 1× Taq polymerase buffer, 4 mM MgCl₂, 300 nM of each primer, 400 µM of each dNTP and 5 µl DNA template. The PCR tubes were incubated for 10 min (95°C); after the initial 3 min, 5 U Taq polymerase was added to each reaction. DNA was amplified by 15 cycles of 95°C for 1 min, 68°C for 45 s and 72°C for 1 min and then 16 cycles of 95°C for 1 min, 64°C for 45 s and 72°C for 1 min and a final cycle of 72°C for 10 min. The nucleotide sequences are described in Table 1. Positive control strains FRI472 and FRI100 were generously provided by D. Doro and T. C. Oliveira from Universidade Federal de Londrina, Brazil.

**Electrophoresis.** PCR products were resolved by electrophoresis in 1.5% agarose gels (0.5× TBE) at 100 V, stained with ethidium bromide and photographed under UV light.

**RESULTS AND DISCUSSION**

The role of food is now recognized in the dissemination and maintenance of opportunistic pathogens in the nosocomial environment and the potential threat that it might represent to the treatment of patients at high risk such as preterm babies is also known.

In this study, a total of 54 staphylococcus isolates were recovered from 13 EHM samples. Conventional physiological tests identified 53-6, 20-4, 13 and 13% of the isolates as *S. epidermidis, Staphylococcus warneri, Staphylococcus haemolyticus* and *Staphylococcus aureus*, respectively. From a single EHM sample, it was possible to recover up to three different species. The different staphylococcus colonies recovered from the same EHM sample were counted as distinct isolates.

Disc-diffusion tests indicated that higher rates of resistance, including intermediate resistance, were observed for penicillin (87%) and erythromycin (59.3%). Strain-to-strain variation, among isolates of the same species as well as among isolates of different species was observed, even between isolates recovered from the same milk sample. Forty-seven isolates (87%) were resistant to at least two of the drugs tested and one single isolate was resistant to seven of the antimicrobial agents tested (erythromycin, gentamicin, oxacillin, penicillin, sulfamethoxazole/trimethoprim, ofloxacin and tetracycline). All isolates were susceptible to vancomycin and clindamycin (Table 2).

Both disc-diffusion and broth macrodilution methods showed that 24.1% of the isolates were resistant to gentamicin and 3.7% were included in the intermediate-resistance category. However, there were discrepancies between the two methods: three isolates were considered to be susceptible by disc-diffusion and included in the intermediate-resistance category by macrodilution, while another four strains were included in the intermediate-resistance category by disc-diffusion and considered to be susceptible by the macro-
dilution testing. MIC determination showed that three isolates identified as *S. epidermidis* and one isolate of *S. warneri* exhibited an MIC > 128 µg ml⁻¹ to gentamicin. MIC values are listed in Table 3.

Genotypic characterization of the resistance genes by PCR allowed the detection of the *aac(6')/aph(2')* gene in 18.5% of isolates. This gene was detected in all isolates showing MIC values >128 µg ml⁻¹ (Table 3). The *aac(6')/aph(2')* gene encodes the bifunctional aminoglycoside-modifying-enzyme AAC(6')/APH(2'), which confers concomitant resistance to gentamicin and the majority of aminoglycosides commonly used in medical practice. This gene is the most common aminoglycoside-resistance gene found in staphylococci. In this study, a good correlation between gentamicin resistance and the *aac(6')/aph(2')* gene among EHM isolates does not differ significantly from the distribution among isolates recovered from infections.

Disc-diffusion and MIC determination for erythromycin showed good correlation. The prevalence of erythromycin resistance recorded among all staphylococcus isolates was 25.9%. Another 33.3% isolates were included in the intermediate-resistance category. However, there were minor discrepancies between the two methods: 5.6% of the strains showed good correlation. The prevalence of erythromycin resistance among all staphylococci isolates was 72.3%, while only one of the *S. aureus* isolates showed intermediate resistance to erythromycin. MICs for erythromycin varied from 0.25 to >64 µg ml⁻¹ (Table 3).

Among the 35 (64.8%) isolates showing resistance or

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**Table 1. Nucleotide sequences of the primers used for amplification of resistance and enterotoxin staphylococci genes**

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<td>aac(6')/aph(2')</td>
<td>5’TATGTAATTTCTATGATGGAATTAA-3’</td>
<td>74</td>
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<tr>
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<td>mecA</td>
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<td>533</td>
<td>York et al. (1996)</td>
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<tr>
<td></td>
<td>5’CTGTATGTATGGAGGAATTAA-3’</td>
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</table>

Reference: 91 Martineau et al. (1996); 142 Monday & Bohach (1999); 364 York et al. (1996)
intermediate resistance to erythromycin, 4 (11.4%), 12 (34.3%) and 16 (45.7%) showed amplification products related to \( \textit{ermA}, \textit{ermB} \) and \( \textit{ermC} \), respectively. Three isolates (one \( \textit{S. epidermidis} \) and two \( \textit{S. warneri} \)) showed intermediate-resistance to erythromycin but were negative for amplification products of the \( \textit{erm} \) genes, shown by PCR. Westh et al. (1995) have reported the isolation of resistant strains that harboured neither the \( \textit{erm} \) genes nor the \( \textit{mrsA} \) gene, another erythromycin-resistance determinant that encodes efflux pumps, suggesting the occurrence of additional resistance mechanisms.

Considering the distribution of the \( \textit{erm} \) gene variants among the staphylococci species studied, \( \textit{ermA} \) was detected in all \( \textit{S. warneri} \) isolates with MIC \( \geq 64 \, \mu g \, ml^{-1} \) and in one of the \( \textit{S. haemolyticus} \) isolates with MIC of \( 1 \, \mu g \, ml^{-1} \); \( \textit{ermB} \) was detected in all \( \textit{S. epidermidis} \) and \( \textit{S. haemolyticus} \) with MIC \( \geq 32 \, \mu g \, ml^{-1} \) and in one \( \textit{S. warneri} \) isolate with MIC of \( 16 \, \mu g \, ml^{-1} \); and \( \textit{ermC} \) was present in \( \textit{S. epidermidis}, \textit{S. warneri} \) and \( \textit{S. aureus} \) isolates with MICs ranging from 0.5 to \( \geq 2 \, \mu g \, ml^{-1} \).

Erythromycin resistance in staphylococci is predominantly mediated by erythromycin-resistant methylases encoded by the \( \textit{erm} \) genes. The \( \textit{ermA} \) gene has been reported as having the most prevalent erythromycin resistance in staphylococci and is usually associated with inducible resistance. In human infections, \( \textit{ermA} \) and \( \textit{ermC} \) are the most commonly found methylase genes (Nicolae et al., 1998). In the present study, we observed a low frequency of the \( \textit{ermA} \) gene among CNS isolates; also, none of the \( \textit{S. aureus} \) isolates harboured this gene. This contrasts with the study of Martineau et al. (2000), in which \( \textit{ermA} \) was the most prevalent erythromycin-resistance determinant in \( \textit{S. aureus} \). Among the CNS isolates, except for \( \textit{S. warneri}, \textit{ermB} \) was the most prevalent, presenting high-level resistance (MIC \( \geq 32 \, \mu g \, ml^{-1} \)). The gene \( \textit{ermB} \), which is very similar to the \( \textit{Streptococcus} \) gene \( \textit{ermAM} \) and is also usually found in \( \textit{Enterococcus} \), has been associated with constitutive high-level erythromycin resistance (Westh et al., 1995; Sutcliffe et al., 1996). On the other hand, the \( \textit{ermC} \) gene was the most prevalent among staphylococcus isolates presenting MIC values ranging from 0.5 to 8 \( \mu g \, ml^{-1} \). Eady et al. (1993) described the distribution of erythromycin-resistance genes in a broad array of human and animal CNS. In that study, the \( \textit{ermC} \) gene was found to be widely distributed while the \( \textit{ermA} \) and \( \textit{ermB} \) genes were only detected in a minority of strains. Others have also reported a high incidence of erythromycin-resistant CNS isolates carrying \( \textit{ermC} \) (Martineau et al., 2000).

By using a macrodilution method, 53 (98.1%) of the isolates were found to be resistant to oxacillin (MIC \( \geq 0.5 \, \mu g \, ml^{-1} \) for CNS and MIC \( \geq 2 \, \mu g \, ml^{-1} \) for \( \textit{S. aureus} \)). Only one \( \textit{S. epidermidis} \) isolate (MIC \( = 0.06 \, \mu g \, ml^{-1} \)) was susceptible to this drug (Table 3). The detection of the \( \textit{mecA} \) gene by PCR is considered to be the gold standard method to test methicillin resistance, since phenotypical methods using oxacillin may be difficult to interpret. Additionally, some isolates do not express resistance unless selective pressure is applied. This has been frequently observed in heterogeneously resistant isolates (Killgore et al., 2000; Louie et al., 2000). Among the CNS showing resistance to oxacillin according to the macrodilution method (46 strains; MIC \( \geq 0.5 \, \mu g \, ml^{-1} \)), 37 (80.4%) were also \( \textit{mecA} \)-positive. Another nine isolates (7 \( \textit{S. epidermidis}, 1 \textit{S. haemolyticus} \) and 1 \( \textit{S. warneri} \)) that were negative for the presence of the \( \textit{mecA} \) gene showed MICs ranging from 0.5 to 2.0 \( \mu g \, ml^{-1} \) and should be classified as borderline oxacillin-resistant staphylococci. In general, CNS isolates with MICs from 0.5 to 2 \( \mu g \, ml^{-1} \) are \( \textit{mecA} \)-negative (Hussain et al., 2000). According to other authors the resistance to methicillin in these isolates may be associated with alternative resistance mechanisms, such as \( \beta \)-lactamase hyperproduction (Kolbert et al., 1998; Louie et al., 2000).

In this study, two isolates identified as \( \textit{S. haemolyticus} \) that were recovered from the same EHM sample were considered to be intermediate-resistant to vancomycin by MIC.
Table 3. MIC values, resistance and enterotoxin genetic determinants of staphylococcus isolates

ERI, Erythromycin; GEN, gentamicin; VAN, vancomycin; OXA, oxacillin; SE gene, staphylococcus enterotoxin gene.

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determination (MIC = 8 µg ml$^{-1}$). The occurrence of staphylococcus isolates, especially *S. aureus*, *S. haemolyticus* and *S. epidermidis*, with reduced susceptibility to vancomycin has been reported in Japan, North America, Europe and Brazil (Sieradzki et al., 1998; De’Alamo et al., 1999; Biavasco et al., 2000). To our knowledge, this is the first report of isolation of staphylococci strains expressing reduced susceptibility to vancomycin from EHM.

In addition to resistance markers, all isolates were tested for the presence of genes encoding SE by using multiplex PCR. Although *S. aureus* is considered to be the species with greater pathogenic potential, CNS strains have also been reported as enterotoxin producers (Novak et al., 2000a). In the present study, one *S. aureus* isolate was sea-positive, while three *S. epidermidis* isolates were seg- (two strains) and seh- (one strain) positive (Fig. 1). SEA is also the SE most frequently implicated in food-borne outbreaks (Balaban & Rasooly, 2000). Additionally, because of their role as superantigens, SEA, SEG and SEH have been linked to other staphylococcal syndromes such as toxic shock syndrome, staphylococcal scarlet fever and recalcitrant erythematous desquamating disorder (Sharma et al., 2000). The presence of enterotoxin-producing staphylococci in EHM has been reported elsewhere (Novak et al., 2000a).

The dosage of enterotoxin necessary to cause symptoms in humans is yet to be established. However, there is evidence to show that the presence of SE in food with concentrations as low as 1 ng g$^{-1}$ is enough to initiate an outbreak (Jay, 1992). Although one cannot ensure that the isolates harbouring genes encoding enterotoxins are actually able to express them, they have the potential to do so. If such strains find favourable conditions in the hospital environment, they might produce SEs resulting in critical situations, particularly considering the immune status of the population for which this food is destined.

At the hospital analysed in the present study, CNS infections are the major cause of bacteraemia in the neonatology unit (E. Castro, personal communication). The use of broad-spectrum antibiotic regimens may contribute to increasing isolation rates of multiresistant strains of CNS. Rigorous bacteriological investigation incorporated into Gram-positive, CNS identification schemes and the monitoring of their resistant features should properly detect and characterize these pathogens. The presence of staphylococci in EHM and their wide antimicrobial-resistance profiles as well as the presence of enterotoxin determinants are reasons for concern considering the immunological immaturity of the population that will receive the EHM. Additional studies are necessary to determine the source of contamination and the potential contribution of contaminated EHM in propagating multiresistant staphylococcus colonization and infection.

Our results bring attention to the necessity of quality-control programs, personnel training and continuous surveillance in order to reduce the risk that EHM might represent as a vehicle of dissemination of these pathogens. This in turn will

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Table 3. cont.
Resistance and enterotoxin genes in staphylococci

improve information in developing countries related to the epidemiological aspects of isolates obtained from different sources.

REFERENCES


