Isolation, molecular characteristics and disinfection of methicillin-resistant *Staphylococcus aureus* from ICU units in Brazil

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SUMMARY

The aim of the present study was to isolate *S. aureus* strains resistant to antibiotics, characterize the genotype profiles of resistance staphylococci, and evaluate the efficacy of antiseptic agents and disinfectants used in two public hospitals of Vitória da Conquista, Bahia, Brazil. Clinical samples were obtained from ICU environments and equipment surfaces in two public hospitals in Vitória da Conquista. Broth cultures were plated onto mannitol salt agar, and antimicrobial susceptibility testing was performed by the broth microdilution method according to CLSI. MRSA strains were submitted to PCR for detecting the *mecA* gene. PCR products were purified and sequenced for SCCmec type identification. Moreover, the strains were tested for efficacy of different disinfectant solutions. *S. aureus* were isolated from 31 and 67 sites in each hospital, respectively. Among the isolates from hospital 1, 07 (22.6%) were resistant to oxacillin while 28 (41.8%) were resistant in hospital 2. Thirty-one were positive for the *mecA* gene. All isolates showed SCCmec type III genotype characteristics of the Brazilian epidemic clone. In disinfectant tests, sodium hypochlorite (0.5, 1.0 and 2.0%), 2% chlorhexidine gluconate, quaternary ammonium, peracetic acid and formaldehyde were effective against the isolates tested. The strains showed higher resistance to vinegar (4% acetic acid), alcohol and glutaraldehyde. The findings of this study should assist in reducing the occurrence of nosocomial infections and therefore the morbidity, mortality and socio-economic burden caused by prolonged hospitalization.

KEY WORDS: MRSA, SCCmec type III, Environmental contamination, Disinfection

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INTRODUCTION

The Brazilian Ministry of Health (Brazil, 1998) defines Hospital Infection (HI) as an infection is acquired after admission and manifested in patients during hospitalization or after hospital discharge, being possibly related to hospitalization or hospital procedures. HI is presented as an issue of great epidemiological significance due to significant human and economic losses (Pereira *et al.*, 2005).

In recent decades, antimicrobial resistance has been increasing rapidly worldwide, particularly in hospitals. The genus *Staphylococcus* is among the pathogens undergoing significant changes in antimicrobial susceptibility over the years (Tavares, 2000). These microorganisms are gram cocci and catalase positive, about 1.0 mm in diameter, immobile, non-endospore-forming and usually non-encapsulated (Crossley *et al.*, 2009). Among the species of the genus *Staphylococcus*, the most interesting is *Staphylococcus aureus*, which can be found in normal flora of human skin and mucous (Giammarinaro *et al.*, 2005). *S.*

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aureus is associated with various infections, which occur mainly in nurseries and intensive care units (ICU) (Dos Santos *et al*., 2007). Due to the use of semi-synthetic penicillins and acquisition of the *mecA* gene, strains of Methicillin-resistant *Staphylococcus aureus* (MRSA) were isolated (Novak, 1999).

The analysis of MRSA isolates usually requires not only phenotypic characterization, but also genotypic Staphylococcal Chromosome Cassette mec (SCCmec) characterization. SCCmec is a mobile genetic element integrated into the chromosome of MRSA, of the *mecA* gene (Hanssen, 2004). Other genetic elements may also be present, such as genes for resistance to another β-lactam antibiotics and heavy metals. Six types of *SCCmec* have been identified (Appelbaum, 2007). Over the years, antimicrobial resistance has been studied. However, bacterial resistance against sanitizing products is still poorly understood and studied (Nuñez and Moretton, 2007). In clinical practice, antiseptics and disinfectants are used extensively in hospitals and other health environments for a variety of applications (McDonnell and Russel, 1999). Sanitizing and disinfectant agents are essential components in the practice of control and prevention of nosocomial infections. Some agents are more effective against gram-positive than gram-negative bacteria, necessitating an evaluation of their effectiveness (McDonnell and Russel, 1999).

The aim of the present study was to isolate *S. aureus* strains resistant to antibiotics, characterize the genotype profiles of resistance staphylococci, and evaluate the efficacy of antiseptic agents and disinfectants used in two public hospitals of Vitória da Conquista, Bahia.

MATERIAL AND METHODS

Sample collection and bacterial isolates

Clinical samples were obtained from intensive care unit environments and equipment surfaces in two public hospitals in Vitória da Conquista, Bahia, Brazil. Both institutions authorized this study. The sites were determined by following the routines of each hospital. We selected sites with a greater possibility of contamination and a total of 117 points were analyzed. The selected collection sites were: floors, hospital cots, hospital cot control panels, heart monitors, hospital ventilator control panels, infusion-pump control panels, blood-gas analyzer control panels, hospital incubators, telephones, scales, doors, tables, hospital beds, cabinets, emergency carts, medication carts, computers, air conditioners, faucets, handles, hospital countertops and prescription records.

Samples were collected from each surface using sterile swabs. The samples were transported in 3 ml of sterile modified culture medium BHI (Brain Heart Infusion) at 4°C. Broth cultures were plated onto mannitol salt agar. Cultures were incubated at 37°C for 48 hours. Suspect colonies, which revealed acidification of mannitol, were subjected to identification procedures. Colonies with coagulase-positive, Gram-positive cocci and catalase positive were selected as possible *S. aureus* and identified by PCR.

Susceptibility testing

Antimicrobial susceptibility testing was performed by the broth microdilution method, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2008). Oxacillin and vancomycin were obtained from the respective manufacturers, and the plates were prepared and used on the same day as testing. The strain was subcultured on mannitol salt agar at 37°C overnight. On the day of experiment, the bacterial suspension was prepared by sodium chloride 0.9% solution, and the inoculum was adjusted by spectrophotometer. Susceptibility results were interpreted according to CLSI document. The tests were read 24 h after incubation at 35°C. Quality control was performed by testing *S. aureus* ATCC 29213 and ATCC 43300. All experiments were performed in triplicate with three independent repetitions.

Genotypic characterization using *PCR and sequencing*

MRSA strains were submitted to DNA extraction by the boiling method described by Fan *et al*. (1995) The primers *mec*A1 (5'- GTA GAA ATG ACT GAA CGT CCG ATA A-3') and *mec*A2 (5'- CCA ATT CCA CAT TGT TTC GGT CTA A –3') were used in a PCR to detect the *mecA* gene (Perez-Routh *et al.*, 2001). After confirmation of the only band in the electrophoresis, PCR products were purified using Purelink™ PCR Purification kit

(Invitrogen). Purified PCR products were sent to a sequencing laboratory (Centro de Ciências Exatas e da Natureza, Departamento de Biologia Molecular, Universidade Federal da Paraíba, Brazil). Briefly, samples were sequenced according to the ABI 3100 protocol, using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, US). To determine the Staphylococcal Chromosome Cassette mec (SCCmec) type, the *mecA* gene sequences were compared to sequences deposited in the GenBank database using the BLASTn algorithm (Benson *et al.*, 2009)

Analysis of efficacy of different disinfectant solutions

The methodology followed the method of antimicrobial sensitivity of disinfectants recommended by the National Committee for Clinical Laboratory Standards (CLSI, 2008). This test was modified for use with a Steers replicator. Disinfecting agents used in the hospitals and chosen for this study included: sodium hypochlorite (0.5, 1.0 and 2.0%), 2.0% glutaraldehyde, 10.0% formaldehyde, ethanol at 70% p/p, 2.0% chlorhexidine gluconate, 2.0% peracetic acid, quaternary ammonium, and 100% white vinegar (4% acetic acid). The isolates were placed on Müller-Hinton agar and a Steers applicator was used to apply about 2 µL of each disinfectant to certain points on the agar. The contact time of the applicator on the plate was approximately 20 to 30 seconds. The plates were kept at room temperature to allow the moisture to be absorbed by the agar at the point of application and, after this process, were incubated at 35°C for 24 hours. We observed the presence or absence of an inhibition zone of visible growth (99.9% microbial death) on the surface of the agar where the disinfectant agent was applied. All experiments were performed in duplicate with three independent repetitions. The results were analyzed using equality proportions testing with continuity correction (R Project, Vienna, Austria).

RESULTS

***S. aureus* isolation and MRSA selection**

A total of 117 sites were analyzed in two public hospitals, 36 sites being in hospital 1 and 81 sites in hospital 2. *S. aureus* were isolated from 31 and 67 sites in each hospital, respectively (Tables 1 and 2). Among the isolates from hospital 1, 07 (22.6%) were resistant to oxacillin while 28 (41.8%) were resistant in hospital 2 (Tables 1 and 2).

Genotypic characterization

Thirty-five strains resistant to oxacillin were analyzed by PCR for the detection of the mecA gene. Thirty-one (88.6%) were positive. Detection of SCCmec was possible in isolates positive for mecA. The similarity of the isolate sequences was compared with the SCCmec sequence deposited in GenBank - accession number AB539727.1. All isolates showed SCCmec type III genotype characteristics of the Brazilian epidemic clone associated with nosocomial infection (Figure 1).

Analysis of efficacy of different disinfectant solutions

The isolates were used to test disinfectants and antiseptic solutions, with the aim of assessing the

TABLE 1 - *Oxacillin resistance profile of Staphylococcus aureus isolated in hospital 1 from Vitoria da Conquista, Bahia.*

Sites	*Isolates*	*Resistance*	
		n.	*%*
Refrigerator	01	0	0
Faucet	02	1	50.0
Infusion pump control panel	02	1	50.0
Hospital ventilator control panel	03	1	33.3
Scales	04	2	50.0
Telephone	03	0	0
Blood-gas analyzer control panel	01	0	0
Computer	01	0	0
Hospital cots	10	2	20.0
Hospital cot control panel	03	0	0
Floor	02	0	0
Total	31	7	22.6

effectiveness of these substances within specified conditions. These results are summarized in Table 3. Sodium hypochlorite (0.5 to 2.0%), 2% chlorhexidine gluconate, quaternary ammonium, peracetic acid and formaldehyde were effective against the isolates tested. The strains showed higher resistance to vinegar (4% acetic acid) (62/95-65.3%), alcohol (61/95-64.2%) and glutaraldehyde (23/95-24.2%). No differences were observed in the disinfectant resistance frequency of MRSA and MSSA isolates (p-value <2.2e-16).

TABLE 2 - *Oxacillin resistance profile of Staphylococcus aureus isolated in hospital 2 from Vitoria da Conquista, Bahia.*

Sites	*Isolates*	*Resistance*	
		N.	*%*
Faucet	12	05	41.66
Infusion pump	01	0	0
Handles	06	03	50.0
Refrigerator	03	02	66.6
Prescription records	02	02	100.0
Blood-gas analyzer control panel	02	02	100.0
Floor	04	03	75.0
Infusion pump control panel	04	0	0
Hospital countertops	06	03	50.0
Hospital ventilator control panel	07	02	28.6
Telephone	03	01	33.3
Heart monitor	05	01	20.0
Cabinets	03	01	0
Hospital bed	02	02	100.0
Air conditioner	01	0	0
Computer	02	0	0
Table	02	0	0
Door	01	0	0
Emergency cart	01	01	100.0
Total	67	28	41.8

DISCUSSION

The present study disclosed staphylococci in various sites within the hospital environment. Similar results were reported by Masaki *et al*. (2003) who identified 19 clinical MRSA isolates from the hospital (floor). Two types and two subtypes were found both in patients and the environment. In another study, Brady *et al.* (2007) obtained 25 MRSA isolates from hospital beds, reporting the presence of this bacterium in different environments. These results may suggest ubiquity and relevance in environmental contamination.

The *S. aureus* isolated from certain points in the ICU environment in this study suggests that the main vector for the unit contamination may be the hands of health professionals. The bacteria are transmitted when these professionals are in contact with devices such as phones, control panels and electrical switches as they simultaneously care for patients. A study conducted by Lam *et al.* (2004) reported that health professionals recontaminate their hands during interruptions of patient care by touching objects such as a computer mouse, alarm buttons and pens before caring for patients. Moreover, many other microorganisms are transmitted to health professionals' hands through inanimate objects such as soap dispensers, tables, telephones, sphygmomanometers, monitors and bedside bars (Kerabey *et al.*, 2002). This transmission may render the health professionals' hands permanently colonized with pathogenic microorganisms acquired in a hospital environment. The two hospitals in the present study were colonized by the same type of epidemic clone of MRSA. Many health professionals work in both hospitals and they may have been vehicles of microorganism transmission between the two hospitals.

Approximately 36% of the isolates were resistant to oxacillin in the present study. Of these, 88.6% were the *mecA* gene in PCR reaction. According to Ito *et al.* (2003) the acquired drug resistance mechanisms in *S. aureus* are classified into two categories: bacterial chromosomal gene mutation and resistant gene acquisition from other mi-

croorganisms by genetic exchange. The ability of MRSA clonal spread contributes to increased morbidity and mortality and hospital costs in the world (Deurenberg and Stobbring, 2008). In this study, all MRSA isolates from two hospitals were SCCmec type III. This finding confirms the results found by Sousa-Junior *et al.* (2009), where the prevalent SCCmec found in Bahia was also type III, with 34 of the 45 isolates. Only one showed type II and six showed type IV. In Brazil,

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>dbj|AB539727.1| Staphylococcus aureus DNA, cassette chromosome mec (SCCmec) structure
(IIIR), strain: 16K
Length=118679

 Score =  291 bits (157),  Expect = 1e-75
 Identities = 157/157 (100%), Gaps = 0/157 (0%)
 Strand=Plus/Minus

Query  1      CGtaaaataaaaaaagtatctaaaaataaaaaacgagtagatgctcaatataaaattaaa  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  91770  CGTAAAATAAAAAAAGTATCTAAAAATAAAAAACGAGTAGATGCTCAATATAAAATTAAA  91711

Query  61     acaaaCTACGGTAACATTGATCGCAACGTTCAATTTAATTTTGTTAAAGAAGATGGTATG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  91710  ACAAACTACGGTAACATTGATCGCAACGTTCAATTTAATTTTGTTAAAGAAGATGGTATG  91651

Query  121    TGGAAGTTAGATTGGGATCATAGCGTCATTATTCCAG  157
              |||||||||||||||||||||||||||||||||||||
Sbjct  91650  TGGAAGTTAGATTGGGATCATAGCGTCATTATTCCAG  91614
```

FIGURE 1 - *Nucleotide sequence of the amplified product of mecA genes from MRSA isolated in two public hospitals from Vitoria da Conquista, Bahia. Alignment of the cassette chromosome mec (type III) sequence (Genbank AB539727.1) with the amplified sequence of an MRSA isolate.*

TABLE 3 - *Analysis of efficacy of different disinfectant solutions against Staphylococcus aureus isolated in two public hospitals in Vitoria da Conquista, Bahia.*

Disinfectant	*MRSA Resistance*		*MSSA Resistance*	
	N. total	*%*	*N. total*	*%*
Sodium hypochlorite 0.5%	03/35	8.57	3/60	5.00
Sodium hypochlorite 1.0%;	2/35	5.71	3/60	5.00
Sodium hypochlorite 2.0%	2/35	5.71	0/60	0
2.0% Glutaraldehyde	10/35	28.57	13/60	21.66
10.0% Formaldehyde	2/35	5.71	3/60	5.00
Ethanol at 70%	21/35	60.00	40/60	66.66
2.0% Chlorhexidine gluconate	2/35	5.71	5/60	8.33
2.0% Peracetic acid	2/35	5.71	0/60	0
Quaternary ammonium	6/35	17.14	5/60	8.33
100% White vinegar (4% acetic acid)	22/35	62.85	40/60	66.66

No differences were observed in desinfectant resistance frequency of MRSA and MSSA isolates (p-value <2.2e-16, R Project, Vienna, Austria).

a single clone is responsible for the majority of nosocomial infections, called the Brazilian epidemic clone (BEC) with an SCCmec IIIA (Teixeira *et al.*, 1995; Oliveira *et al.*, 2001). Oliveira *et al.* (2001) isolated 83 MRSA strains from 27 public and private hospitals located in 14 Brazilian states. DNA analysis by PFGE indicated that 78.3% showed the same pattern, suggesting an endemic MRSA clone widely disseminated in Brazilian hospitals.

The predominance of this clone was also observed in other studies, reflecting its wide dissemination in Brazilian hospitals (Soares *et al.*, 2000). There are reports of BEC isolation in other Latin American countries such as Argentina and Uruguay. In Europe, Souza *et al.* (1998) described the spread of BEC in hospitals in Portugal. These data demonstrate the BEC spread, even in hospitals where another MRSA clone was prevalent.

Although the BEC dominates most Brazilian hospitals, other epidemic clones are also frequently isolated in hospitals in Brazil, such as the New York/Japan clone, reported in Japan and the United States (Melo *et al.*, 2004). From 2003 to 2005, a study conducted in the United States demonstrated a very low prevalence of SCCmec type III, whereas a high prevalence of type IV and type II was shown (Davis *et al.*, 2006). In Sweden, Ender *et al.* (2009) observed a high prevalence of SCCmec type IV (34.3%). The remaining MRSA were distributed into 14.1% for type II, 9.1% for type III and 7% for type I. In Italy, Valaperta *et al.* (2010) observed an absolute prevalence of types I (74%) and II (23%) in 96 isolates obtained. The authors reported that the MRSA type identification (antibiotype-genotype correlation) led to the implementation of practices to interrupt cross-contamination between healthcare workers and patients.

Due to the high resistance of bacteria to antibiotics, the use of disinfectants with a broad spectrum of action is of great importance, since the elimination of these bacteria would prevent further spreading. In this study, sodium hypochlorite (0.5 to 2.0%), 2% chlorhexidine gluconate, quaternary ammonium, peracetic acid and formaldehyde were effective against the isolates tested. These results are consistent with findings in the literature (Bamabace *et al.*, 2003; Kuich *et al.*, 2004).

In this study, glutaraldehyde was not effective against all isolates, with 24.2% of *S. aureus* strains being resistant to this disinfectant. It is notable that these results are not in agreement with those obtained by Guimaraes *et al.* (2000) who reported the efficiency of glutaraldehyde in 100% of MRSA tested. Souza *et al.* (1998) also reported 100% effectiveness against these microorganisms after one hour at 37 °C, demonstrating that the incubation temperature is not a determining factor for reducing the effectiveness of the agent. The non-efficiency in this study may be related to its action time. A study by Vizcaino-Alcaide *et al.* (2003) showed that glutaraldehyde is effective when it acts over an extended period of time.

Although it is almost universally recognized as an effective agent, alcohol use is fraught with controversy and conflicting findings (Ali, 1991). In this study, alcohol was not effective against all isolates, and 64.2% of *S. aureus* strains were resistant to this disinfectant. These results are not in agreement with other studies (Dos Santos *et al.*, 2007). This result may have occurred due to volatilization so that alcohol requires time to act efficiently in bacterial proteins denaturation (Kalil and Da Costa, 1994). Dos Santos *et al.* (2007) observed that the action time of alcohol influenced its low efficiency. The authors report that alcohol lost its effectiveness when used for less than a minute. Pontual *et al.* (2003) did not observe the same result. However, the test consisted of immersing the material, and did not offer conditions of evaporation of alcohol.

Because it contains acetic acid in its formulation, vinegar (4% acetic acid) was included in the study to test its efficacy against clinical isolates of *S. aureus*. The results showed a low effectiveness, with only 34.8% of the strains being sensitive. Rutala *et al.* (1999) and Silva *et al.* (2008) also observed low efficacy against *S. aureus* strains. However, the authors found activity against gram-negative strains. On the other hand, Utyama (2003) observed disinfectant efficacy against all strains using white vinegar at a concentration of 3% of acetic acid. These contradictory results suggest that more research is needed to determine the best recommended uses of vinegar in the hospital routine.

To date, no epidemiological study involving genotypic typing of MRSA had been carried out in Vitoria da Conquista - BA. Further studies could

provide valuable information on the presence of this epidemic clone causing outbreaks. The determination of subtypes of SCCmec and their correlation with multidrug resistance in addition to the distribution pattern of the isolates as potential causes of infection make their detection and differentiation an important tool for epidemiological analysis of MRSA in hospital settings. In particular, this would serve to design a strategy aiming to stop the evolution of resistance. Moreover, despite some controversial results, perhaps because of the methodology used, the results of this study suggest that alternatives to infection control can be considered, such as chlorhexidine and quaternary ammonium compounds. Sodium hypochlorite and peracetic acid showed similar results, proving their effectiveness in disinfection. The findings of this study should assist in reducing the occurrence of nosocomial infections and, therefore, the morbidity, mortality and socio-economic burden caused by prolonged hospitalization.

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