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Evaluation of *Staphylococcus aureus* resistance profile isolated from nursing students in an institution of higher education

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ABSTRACT. *Staphylococcus aureus* causes a large variety of infections, where many of them are acquired in the hospital environment. A significant part of the population is a nasal carrier of this type of microorganism. The present study evaluated the nasal colonization by *S. aureus*, identifying its resistance profile in nursing students from a private educational institute of higher education. Nasal *swab* samples were collected and identified for *S. aureus*. Moreover, an antibiogram assay was performed, followed by the search for *ermA* and *ermC* genes using PCR. Sixty-two students were included and we isolated 20 positive samples (32,5%) for *S. aureus*. For the phenotypic profile, 30% were found to be resistant to Erythromycin and 10% to Oxacillin and Cefoxitin. For the D-test in the genotypic profile, 25% presented *mecA* gene (MRSA), 5% of *ermA* gene, 35% of *ermC* gene and 10% with *ermC* and *mecA* genes. These data reinforce the necessity of monitoring bacterial colonization in hospital environment, which are potentially resistant in health professionals.

Keywords: colonization, betalactam, macrolides, MRSA, MLSb.

Avaliação do perfil de resistência de *staphylococcus aureus* isolados de estudantes de enfermagem de uma instituição de ensino superior

RESUMO. *Staphylococcus aureus* causa uma grande variedade de infecções, muitas delas adquiridas no ambiente hospitalar. Uma parcela significativa da população é carreadora nasal desses micro-organismos. O presente trabalho avaliou a colonização nasal por *S. aureus* identificando seu perfil de resistência em estudantes de enfermagem de uma instituição privada de ensino superior. Foram coletadas e identificadas amostras de swab nasal para *S. aureus* e realizado o antibiograma e a detecção por PCR dos genes *mecA, ermA* e *ermC*. Foram incluídos 62 alunos e isoladas 20 amostras (32,3%) positivas para *S. aureus*, no perfil fenotípico, 30% apresentaram resistência à Eritromicina e 10% para Oxacilina, Cefoxitina e para o teste D, no perfil genotípico 25% apresentaram gene *mecA* (MRSA), 5% do gene *ermA* e 35% do gene *ermC*, e 10% com genes *ermC* e *mecA*. Esses dados reforçam a necessidade de monitoramento de colonização por bactérias potencialmente resistente em profissionais da saúde.

Palavras-chave: colonização, betalactâmicos, macrolídeos, MRSA, MLSb.

Introduction

Staphylococcus aureus can cause a wide variety of infections, where many of them are acquired in the hospital environment. The high transmissibility, the elevated pathogenic potential and the possibility of resistance to multiple antibiotics are items that contribute to staphylococcal infections in hospitals and other health-care services. The majority of the population are carriers of these bacteria, however they do not present any infection symptoms. The main niches are the nasal mucosa and perineum. This carrier pattern, where bacterial reproduction is observed without immunological interaction or

clinical disease is called 'colonization'. Either the colonized individual or those who present infection are able to transmit *S. aureus* through direct or indirect contact. This phenomenon, called 'cross transmission' presents increased dynamics in the hospital environment and this fact has a significant contribution to the inherent gravity of hospitalized individuals, to the invasive procedures executed in the hospital, to the use of antibiotics and the occurrence of 'understaffing' (in other words, a low number of professionals providing assistance to a large quantity of patients) (Muto et al., 2003).

Several virulence factors are responsible for the symptoms and the gravity of infections caused by *S*.

aureus. The cells of S. aureus are composed by certain components, including superficial adhesion molecules, peptidoglycan and protein A, which allow these microrganisms to evade the host immune system (Ladhani, Joannou, Lochrie, Evans, & Poston, 1999; Dinges, Orwin, & Schlievert, 2000; Foster, 2005). They also produce several extracellular enzymes such as catalase, nuclease, hyaluronidase, lipase, and fibrinolysin that hydrolyze proteins and other molecules, generating useful nutrients for S. aureus and at the same time facilitating the spread of these bacteria to the tissues (Archer & Climo,1998). Some strains are even capable of producing one or more toxins, which can be generally classified into two groups, the active agents in membranes, comprising toxins alpha, beta, delta, gamma and Panton-Valentine leukocidin (pvl, LukE-lukD genes), designated as PVL, and the toxins with superatingen activity (SAgs), including the family of pyrogenic toxins (PTs), which are the staphylococcal-entero-toxins (SEs), toxic shock syndrome toxin (TSST-1) and the family of exfoliative toxins (ETs) (Bohach, Fast, Nelson, & Schlievert, 1990). Some of the genes linked to these toxins can often be transported by mobile genetic elements, such as phages and pathogenicity islands (SaPIs), which are potentially mobile parts of DNA with different sizes, gene encoders associated with virulence, that are transferred horizontally among the strains, (for example the genes responsible for codifying enterotoxins B and C, and the toxic shock syndrome toxin)(Hanssen & Sollid, 2006). The importance of S. aureus as a pathogen is found in the combination of virulence mediated by its toxins, its invasive characteristic, its resistance profile to antibiotics (Le Loir, Baron, & Gautier, 2003) and the dissemination of isolates resistant to antibiotics utilized in clinical practice, which is a limiting factor to the treatment of staphylococcal infections (Fitzgerald, Sturdevant, Mackie, Gill, & Musser, 2001). An important mark in staphylococcal therapy was the appearance of Methicillin Resistant Staphylococcus aureus (MRSA), being noticed in the last decades a gram-positive pathogen predominant in hospital infections. It is estimated that this bacteria is involved in more than 50% of staphylococcal infections acquired in health-care services (Barrett, 2005; Deresinski, 2005; Finch, 2006). The MRSA are resistant to all beta-lactam antibiotics (Penicillin, Cephalosporin, Carbapenems and Monobactams), because they express a receptor with low affinity to these antibiotics. These antibiotics link themselves to constitutive bacterial enzymes, which participate in the synthesis of the cell wall and the Penicillin Binding Proteins (PBPs),

avoiding its correct function. MRSA has the capacity of synthesizing a variant of PBP2 called PBP2A, keeping its physiological function, but with low affinity to beta-lactams (Ito, Okuma, Ma, Yuzawa, & Hiramatsu, 2003; McCulloch, 2006). The gene that codifies the protein PBP2a, called mecA, along with its regulator gene is found in a mobile genetic element, sthaphylococcal cassette chromosome mec (SCCmec) (Enright et al., 2002). To date, eleven (I-XI) SCCmec types have been fully identified by the determination of mec (A, B, C1, C2 and D) and ccr (i.e. ccrAB1 to ccrAB5 and ccrC) complexes (Hiramatsu, Cui, Kuroda, & Ito, 2001; McCulloch, 2006; Mkrtchyan, Xu, & Cutler, 2015). Some of these genes are carriers of determinant genes to multiple antibiotics, besides the Macrolides, Lincosamides, Streptogramins, Aminoglycosides and Tetracyclines. Thus when a bacterial cell acquires these SCCmec genes at once, it acquires a phenotype of multi-resistance (Ito et al., 2003; McCulloch, 2006).

Nevertheless, the prevalence in the isolation of MRSA strain varies between 40 to 80% in Brazil, generally presenting resistance to Aminoglycosides, Chloramphenicol, Lincosamides, Macrolides, Quinolones, Sulfamethoxazole - Trimethoprim and Tetracyclines with a high susceptibility only to Rifampicin and Glycopeptides, therefore the demand for a glycopeptide in this type of infection is high (Oliveira, Faria, Levyand, & Mamizuka, 2001; Gardella et al., 2005). Studies regarding the geographic dissemination of multiresistant epidemic clones of S. aureus in Brazil demonstrated that tested isolates presented the same phenotype of resistance to Methicillin, and the majority of these isolates (> 70%) were multiresistant to at least 9 antibiotics (Vivoni et al., 2006). Furthermore the clones were largely disseminated in several areas of Brazil. The presence of a Brazilian endemic clone of MRSA (BEC) in many hospitals in South America and Europe has also been described (Sader, Pignatari, Hollis, Leme, & Jones, 1993; Pannuti & Grinbaum, 1995). The infections caused by MRSA are associated with considerable morbidity and mortality, being also one of the most expensive treatments in comparison to other infections. This considerable increase in the expenses related to the management of these infections is due to the prolonged hospitalization, the increase of isolation procedures, besides the additional medical care and the financial overload in secondary therapy. The high impact of MRSA upon the morbidity, mortality and expenses has been largely reported in the United States and among European countries (Barrett, 2005; Finch, 2006). Until 90's, MRSA used

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to be isolated only in hospital environments. However, the transmission of MRSA in the community has been reported in recent years (Rajana, Schoenfelderb, Ziebuhrb, & Gopal, 2015). In this context, isolates were identified with specific characteristics, genetic called CA-MRSA (Community-acquired Methicillin - Resistant Sthaphylococcus aureus). A large dissemination of CA-MRSA in the community is feared, which would turn innocuous any measurement control for MRSA in hospitals (Vivoni et al., 2006). In general, the epidemic occurrences of MRSA receive a higher attention. This fact is explained by the abrupt increase of morbidity and mortality by hospital infections in the presence of outbreaks. Outbreaks and epidemics can have devastating consequences, but they are more easily controlled, once the causative factors have been identified. On the other hand, endemic profiles are hard to be managed. Effective interventions to reduce the endemic rates of MRSA require a large review of work process and normalization of procedures for the identification and isolation of carriers (Muto et al., 2003). It is necessary to emphasize that colonization by MRSA is hard to eradicate. Furthermore, studies have pointed that individuals previously colonized are more susceptible to subsequent colonization during hospitalization and even after hospital discharge (Corso, Sanches, Sousa, Rossi, & Lencastre, 1998; Seas et al., 2006). The phenotypic test method of double disc diffusion (D test) provides an inducible resistance for the gene erm, and it is demonstrated by the narrowing of the inhibition zone around the Clindamycin disc and by the side of the Erythromycin disc, featuring the resistance to Erythromycin inducible to Clidamycin (iMLSb) (Teixeira et al., 1995). Clindamycin is considered an alternative drug for the treatment of infections caused by S. aureus in the cases of Penicillin intolerance or resistance to Methicillin, inhibiting the production of toxins and virulence factors through the inhibition of protein synthesis. There are two mechanisms, which can result in the resistance to this antibiotic, firstly the macrolide flux controlled by msrA and the second is the modification of the binding site in the ribosome, controlled by the erm gene (Erythromycin Ribosomal Methylase). The ribosomal methylation is responsible for cross-resistance to Macrolides, Lincosamides and Streptogramins B, called MLSb phenotype in staphylococci ermA and ermC. These are genes related to the expression of this phenotype, making the modification in the binding target due to the methylation of residue A2058, locatedin the V domain of 23s (Naimi et al., 2001; Pereira & Cunha,

2009). S. aureus synthesizes ribosomal methylase, which is encoded by the erythromycin resistance gene or another methylases (ERM). With methylation, the binding site of Macrolide -Lincosamide Streptogramin B is changed. The main methylases genes encoded by S. aureus are ermA, ermB and ermC (Heshiki, Quesada, Heshiki, Joaquim, & Brandão, 2002). The resistance of MLSb can be expressed in a constitutive and inductive form, the first presents cross-resistance to the antibiotics MLSb, and the second (MLSbi) presents in vitro resistance to macrolides (Naimi et al., 2001).

Some clinical failures related to mutation of Clindamycin resistance have been reported, however there is no chance of resistance appearing during treatment with Clindamycin. The causative factors for resistance are the frequency of resistance mutation, size of bacterial inoculum and type of infection. The understanding regarding the epidemiology of colonization/infection by S. aureus has important implications to control measures. In this way, it is necessary to document the dissemination of isolates and identify the resistance factors related to these isolates. These are the central aims of this project. The present study also aims to identify the prevalence of molecular factors linked to the resistance to macrolides and beta-lactams in S. aureus strains isolated from the nasal mucosa in nursing students from a private institution of higher education in the State of São Paulo.

Material and methods

Characterization and recruitment of sample

The present study was registered and approved protocol the of Brazil platform by nº 14274613.6.0000.5515.

The study included students that started the nursing course in 2012 and in the first period of 2013 and who agreed to participate in the project by signing the Informed Consent Form. Students who presented any sign of infection of upper airways and that used antibiotics up to seven days prior to the collection date were not included in the study. All the positive cultures for Staphylococcus aureus from nasal mucosa in students regularly enrolled in the nursing course were analyzed. Strains of S. aureus ATCC 25923 were used as a reference, while for quality control strains of S. aureus ATCC 33591, S. aureus 29213 and S. aureus 19095 were used. All the samples were identified and tested for the sensitivity to Erythromycin, Clindamycin, Oxacillin and Cefoxitin. All the samples were classified into 2 groups: Group I, new students in the course or

those enrolled in the course up to 1 year of study, and group II: =, students enrolled in the course over 1 year of study.

Statistical analysis

The percentage of the resistance frequencies related to the data was analyzed in a descriptive form. The G test was used for the comparison of the proportions among the samples of resistant *S. aureus* and the others sensitive to antibiotics and the duration of the study. The Fisher's exact test was used for the comparison of the proportions among the samples of resistant *S. aureus* sensitive to antibiotics and the fact of working or not in hospitals. The significance level for all the tests was defined as p < 0.05. The analysis was made using the BioStat 5.3 program.

Procedures and research tools

Samples from nasal mucosa were collected from nursing students. Sterile 'swabs' wet with previously sterilized saline were used during the collection of the material. The 'swab' was introduced in the anterior nasal mucosa during the collection procedure, executing delicate circle movements 3 times. After collection, all the materials were placed inside of dry sterile tubes and immediately sent to the Microbiology Laboratory for inoculation in Mannitol-Salt Agar plates, a selective media for *Staphylococcus*. After incubating in 37°C for 24 hours, the samples were submitted to the identification procedure of the isolates.

The microorganisms that grew in the culture plates were submitted to Gram staining, in order to observe morphology and specific coloration. After the confirmation of these characteristics, catalase and coagulase tests were carried out in tubes as recommended. Additional tests such as Maltose, Trehalose, Mannitol and Polymyxin B were performed for the differentiation from other species of coagulase positive *Staphylococcus*.

Antimicrobial susceptibility tests were performed for all isolates obtained through the drug diffusion in agar technique with impregnated discs according to the criteria recommended by Clinical Laboratory Standards Institute (CLSI, 2011). For the preparation of the inocula, cultures were used in BHI broth preincubated for 4 to 6 hours and then adjusted to 0.5 MacFarland scale. The drugs used were: Oxacillin, Cefoxitin, Clindamycin and Erythromycin. Once the density of the inoculums was adjusted, the inoculation was carried out through the use of sterile 'swab' on the surface of Mueller Hinton Agar, followed by the application of discs impregnated with the drugs. The plates were incubated at 35°C for 24 hours and the antimicrobial activity measured by the inhibition zone diameter through interpretation based on the rules established by the CLSI (2011). For genotypic determination, the polymerase chain reaction (PCR) was performed, which amplified *mecA*, *ermA* and *ermC* genes as described by Coutinho et al. (2010). The presence of the fragments was assessed by carrying out gel electrophoresis in 1.7% agarose in 1X TBE Buffer (90 mM Tris, 90 mM Boric acid, 2 mM EDTA pH 8.0) stained with Ethidium Bromide 0.5 mg mL⁻¹. The amplified products were compared with a 100 bp marker (Amersham Pharmacia Biosciences Inc.) and then visualized and photographed under ultraviolet light.

Results and discussion

Among the students from the first to the third period, 25, 20 and 26 samples were obtained respectively (Figure 1A). From a total of 71 students, 35.2% work in hospitals, where 20, 30 and 53.8% of students are from the first, second and third period, respectively. In addition, 78.9% of students were female and 21.1% male. From the total of students, 6 were presenting flu or cold symptoms on the day of collection and 3 were taking antibiotics, thus they were not included in the study, leaving 62 students in the sample.

The 62 samples were submitted to the identification process, and 20 samples (32.3%) were found to be positive for *Staphylococcus aureus*. In the assessment of positive samples for *S. aureus*, it was observed that 30% of the samples presented resistance to Erythromycin, no positive sample resistant to Clindamycin, 1 sample with intermediate profile, 10% of samples resistant to cefoxitin and 10% resistant to oxacillin.

In addition, 10% of the samples were positive for the D test (double-disk diffusion test) (Figure 2). In the genotypic determination provided by PCR, it was observed that 25% of the samples presented the mecA gene, which characterizes the Methicillin Resistant S. aureus- MRSA. It is important to highlight that only two samples presented a phenotype resistant to Cefoxitin, although 3 samples (14.3%) presented the mecA, however it was not expressed because all the samples were sensitive. In the genotypic assessment of macrolides resistance profile for the amplification of the ermC gene and ermA, 5% of the samples were positive for ermA and 35% of the samples were positive for ermC. It was observed that in the genotypic test 2 samples (10%) presented the ermC gene, despite the positive

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sensitivity profile to Erythromycin.10% of the samples were positive for the presence of *mecA* and *ermC* genes (Figure 3).



Figure 1. A) Percentage frequency of sampled students according to the period; and B) percentage frequency of students who work in hospitals according to the period.



Figure 2. Positive D - test. It is possible to observe the halo flattening forming latter 'D' aspect due to the induction by erythromycin.

Table 1 shows that there was no significant difference (p > 0.05) between the proportions of resistant strains to antibiotics and the evaluated samples from different periods.

Furthermore, there was no significant difference (p > 0.05) between students working in hospitals and proportions of antibiotic resistant samples (Table 2).



Figure 3. Electrophoresis in 1.7% agarose gel with amplification of the gene: *erm*C (572 bp) bands 1 to 7, *mec*A (533 bp) band 1 to 5 and *erm*A gene (421 bp) band 1 and L as a marker.

Table 1. Proportions of resistant samples to Erythromycin (ERI), Clindamycin (CLI), Oxacillin (OXA) e Cefoxitin (CFO) in the nursing students from the first (n = 8), s (n = 3) and third periods (n = 9).

Antibiotics	1° period (%)	2º period (%)	3° period (%)	p value
ERI	25.00	33.33	33.33	0.922
CLI	0.00	0.00	0.00	1
OXA	12.50	0.00	11.11	0.706
CFO	0.00	0.00	11.11	0.435

Table 2. Proportions of samples resistant to the antibiotics Erythromycin, Clindamycin and Cefoxitin in students that work (n = 9) or not in hospitals (n = 11).

Antibiotics	Work (%)	Does notwork (%)	p Value
ERI	33.33	27.27	0.5743
CLI	0.00	0.00	1
OXA	11.11	9.09	0.7105
CFO	11.11	9.09	0.7105

Table 3 shows there were no significant differences (p > 0.05) between the period (period) of students positive for the *mecA* gene, *ermA*, and *ermC*. There was also no significant difference (p > 0.05) between the positive results for the *mecA* gene, *ermA*, *ermC* and the fact of working in hospitals (Table 4).

Table 3. Proportions of positive samples to *mec*A, *erm*A and *erm*C in nursing students from the first (n = 8), s (n = 3) and third periods (n = 9).

Genes	1º period (%)	2° period (%)	3° period (%)	p Value
mecA	12.50	33.33	33.33	0.6062
ermA	0.00	33.33	0.00	0.3318
ermC	25.00	44.44	33.33	0.7319

Table 4. Proportions of resistant samples to the antibiotics Erythromycin, Clindamycin, Oxacillin and Cefoxitin in nursing students that work (n = 9) or not in hospitals (n = 11).

Genes	Works (%)	Does notwork (%)	p Value
mecA	33.33	18.18	0.3955
ermA	11.11	0.00	0.4500
ermC	33.33	36.36	0.6300

From 62 samples taken from students, 32.3% of the samples were positive for *S. aureus*. These data can be corroborated with the findings described by (Goldmann & Sands, 1992) who obtained an incidence rate of carriers of this microorganism between 30 to 40%, according to the author. Pereira and Cunha (2009) in their study of nasal colonization by *Staphylococcus spp.* resistant to Oxacillin in nursing students demonstrated a high rate of student carriers who had no prior contact to the hospital, which is consistent with the results obtained in this study, which observed 38% of positive samples for *S. aureus* in students from the first period, revealing that *S. aureus* is very present in the microbiota of people.

In the assessment of the phenotypic resistance, it was found that 32.3 and 6% (30%) samples were resistant to Erythromycin. These data differ from the findings of (Heshiki et al., 2002) who carried out the analysis of nasal colonization in resident doctors of the University hospital of Londrina (Paraná State), and which described that 19% of isolated samples showed high resistance to Erythromycin.

Among the 20 samples, 2 samples (10%) were resistant to Cefoxitin and 2 samples (10%) resistant to Oxacillin. The small number of samples found resistant to Cefoxitin and Oxacillin are consistent with the results observed by Pereira and Cunha (2009), where all the strains of *S. aureus* isolated from nursing students were susceptible to Oxacillin and Cefoxitin, suggesting that these bacteria are found in the community.

In the present study, all samples were sensitive to Clindamycin and only 1 sample (5%) presented an intermediate resistance profile. In the study of (Silva et al., 2012) regarding the colonization by S. aureus in the nursing staff from a teaching hospital in Pernambuco, it was reported that 11.9% of isolates were resistant to Clindamycin, differing from the results presented in this current paper. On the other hand, a study by Santos et al. (2007), which evaluated Staphylococcus aureus hospital importance, showed a sensitivity profile of 85-100% for Clindamycin, being similar to the results described in this study. Regarding to the evaluation of genotypic profile, 2 samples (10%) were positive for the mecA and ermC gene, characterizing a multidrug resistance profile. Coelho et al. (2007), conducted a mapping of resistance and detection of mecA gene profile of Staphylococcus aureus isolated from humans, which resulted in 12 (43%) of the samples with the presence of the mecA resistance gene out of 28 samples evaluated, thus indicating a higher potential.

According to these authors the increased strains of *S. aureus* resistant to methicillin (MRSA) is possibly favored due to the transfer of the *mecA* gene among the strains of *Staphylococcus spp.* in conjunction with the selective pressure derived from the indiscriminate use of antimicrobials. According to Coutinho et al. (2010) study on the evaluation of the prevalence of *erm* genes inducible to Clindamycin in *Staphylococcus aureus* isolates from patients treated at the Clinical Hospital of Porto Alegre, it was shown that from 94 positive samples for *S. aureus*, 46.9% presented *erm*A, results that disagree with the results obtained in this study, where it was observed 5% of positive samples for *ermA* was observed. Moreover, according to Coutinho et al. (2010), 29.8% of the positive samples for *erm*C gene were observed, corroborating the findings of this present study, where 33.3% of *S. aureus* samples were positive for the gene.

Conclusion

Considering the results, it can be concluded that students related to any health-care, such as nursing students, may be colonized with *S. aureus* with multidrug resistance potential. The monitoring of students and professionals linked to health-care, which attend the hospital environment, along with hygiene measures are essential to prevent and control the spread of these infectious agents, especially the more resistant strains.

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