Distribution of \textit{erm} genes and low prevalence of inducible resistance to clindamycin among \textit{staphylococci} isolates

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ABSTRACT

Introduction: Resistance to macrolides, lincosamides and streptogramins B (MLS$_B$ antibiotics) in \textit{staphylococci} may be due to modification in ribosomal target methylase encoded by \textit{erm} genes. The expression of MLS$_B$ resistance lead to three phenotypes, namely constitutive resistance (cMLS$_B$), inducible resistance (iMLS$_B$), and resistance only to macrolides and streptogramins B (MS$_B$). The iMLS$_B$ resistance is the most difficult to detect in the clinical laboratory.

Objective: This study investigated the expression of MLS$_B$ resistance and the prevalence of the \textit{erm} genes among 152 clinical isolates of \textit{Staphylococcus aureus} and coagulase-negative \textit{Staphylococcus} (CNS) from Hospital de Clínicas de Porto Alegre.

Methods: Primary MLS$_B$ resistance was detected by the disk diffusion method. Isolates with iMLS$_B$ phenotype were tested by double-disk induction method. All isolates were tested by a genotypic assay, PCR with specific primers.

Results: A total of 46.7\% of \textit{staphylococci} were positive for cMLS$_B$; 3.3\% for iMLS$_B$ and 3.3\% for MS$_B$. One or more \textit{erm} genes were present in 50.1\% of isolates. The gene \textit{erm}A was detected in 49 isolates, \textit{erm}C in 29 and \textit{erm}B in 3.

Conclusion: The prevalence of the \textit{erm}A, \textit{erm}B and \textit{erm}C genes were 29.6\%, 17.1\% and 0.66\% respectively, and constitutive resistance was the most frequent as compared to the other two phenotypes.

Keywords: \textit{Staphylococcus}; resistance; \textit{erm} genes; macrolides.

INTRODUCTION

\textit{Staphylococcus aureus} and coagulase-negative \textit{staphylococci} (CNS) are recognized to be causing nosocomial and community-acquired infections worldwide. A great concern related to these microorganisms is their ability to develop resistance to antibiotics which originally were active against these species.¹²³ Although \textit{β}-lactam antibiotics are the main compounds used to treat infections due to \textit{staphylococci}, macrolides, lincosamides and streptogramins type B (MLS$_B$) antibiotics are also widely used to treat \textit{staphylococcal} infections. These antibiotics exert similar inhibitory effects on bacterial protein synthesis, but they are chemically distinct.⁴⁵ MLS$_B$ resistance can be caused by several mechanisms, but the predominant form is target modification mediated by \textit{erm}A, \textit{erm}B and \textit{erm}C (erythromycin ribosome methylase) genes.⁴⁵ The \textit{erm} genes encode enzymes that confer inducible or constitutive resistance to MLS$_B$ agents via methylation of the 23S rRNA, thereby reducing binding by MLS$_B$ agents to the ribosome.⁶⁷ Constitutive MLS$_B$ resistance can be detected by the disk diffusion test in laboratorial routine.⁸ Strains with constitutive MLS$_B$ resistance show high-level \textit{in vitro} cross resistance among MLS$_B$ drugs. However, \textit{staphylococci} isolates with inducible MLS$_B$ resistance demonstrate clear \textit{in vitro} resistance to 14 and 15-member macrolides (e.g., erythromycin), while they seem to be susceptible to 16-member macrolides, lincosamides and streptogramins type B. Therefore, strains can show \textit{in vitro} erythromycin resistance and false clindamycin susceptibility, because the conventional disk-diffusion may fail to detect inducible MLS$_B$ resistance.⁹¹⁰ The Clinical and Laboratory Standards Institute (CLSI) developed a phenotypic method (the double-disk diffusion test (D test)) to screen for inducible resistance.¹¹ However, the polymerase chain
reaction (PCR) with specific primers is a genotypic method used to confirm the presence of the MLSB genes, \textit{ermA}, \textit{ermB} e \textit{ermC}. The risk for therapeutic failure is increased as constitutive resistance may raise from iMLSB during the course of clindamycin therapy in patients with severe \textit{staphylococci} infections. The objective of this study was to determine the prevalence of the MLSB genes in \textit{Staphylococcus aureus} and coagulase negative \textit{staphylococci} from patients attending the \textit{Hospital de Clínicas de Porto Alegre (HCPA)}.

**MATERIALS AND METHODS**

**Bacterial isolates**

Isolates of \textit{S. aureus} and of CNS were collected from consecutive clinical specimens sent to the microbiology laboratory of the HCPA. The period of the study was between September and October 2007. The bacterial identification was performed through Gram's technique and catalase and coagulase tests. Isolates were stored in glycerol broth at -20°C until use.

**Susceptibility tests**

The antimicrobial susceptibility test was performed by the disk diffusion method on Mueller Hinton Agar (bioMérieux, Marcy L’Etoile, France), according to the Clinical and Laboratory Standards Institute (CLSI 2008), with the following antibiotic (Oxoid®): oxacillin (1 µg), cefoxitin (30 µg), vancomycin (30 µg), gentamicin (10 µg), clindamycin (2 µg), chloramphenicol (30 µg), doxycycline (30 µg), erythromycin (15 µg), levofloxacin (5 µg), rifampin (5 µg) and trimethoprim-sulfamethoxazole (25 µg). \textit{S. aureus} ATCC 25923 was used for quality control.

The standard CLSI double-disk diffusion (D test) test was performed using Mueller Hinton agar (bioMérieux, Marcy L’Etoile, France) with a 15 µg erythromycin disk and 2 µg clindamycin disk (Oxoid®) placed at distances of 15 and 26 mm and incubated for 24 h at 35°C. The inducible phenotype was characterized by a positive D test, a flattening of the inhibition zone around the clindamycin disk near to the erythromycin disk and indicates that erythromycin has induced clindamycin resistance (iMLSB). The phenotype cMLSB was characterized by erythromycin and clindamycin resistance. Finally, the phenotype (MSB) was characterized by clindamycin susceptibility and erythromycin resistance, with negative D test.

**\textit{ermA, ermB} and \textit{ermC} gene detection**

A direct colony suspension of the culture equivalent to a 1.0 McFarland standard was prepared in 500 µL of 10 mM Tris-1 mM EDTA (pH 8.0), vortexed, and boiled for 10 min an aliquot of 5 µL of the suspension was used for each 25 µL reaction mixture.

PCR assays and primers specific from the \textit{ermA, ermB} and \textit{ermC} resistance genes used in this study have been previously described by Gerard, Lina et al. (Table 1).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Isolate</th>
<th>Phenotype</th>
<th>\textit{ermA}</th>
<th>\textit{ermB}</th>
<th>\textit{ermC}</th>
<th>\textit{ermA/ermB}</th>
<th>\textit{ermA/ermC}</th>
<th>\textit{ermA/ermB/ermC}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>\textit{S. aureus}</td>
<td>40 (cMLSB)</td>
<td>36</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (iMLSB)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (MSB)</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>\textit{CNS}</td>
<td>24 (cMLSB)</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>2</td>
<td>1</td>
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<td>0</td>
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<td></td>
<td></td>
<td>3 (MSB)</td>
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Three clinical samples with positives results for each of the three genes were submitted to sequencing and analyzed by BLAST and Chromas and DDBJ/EMBL/GenBank. These isolates were used as positive control in all experiments.
RESULTS
A total of 152 strains including 94 S. aureus and 58 CNS were included in this study. Eighty-one (53.3%) exhibited erythromycin resistance and were considered for evaluation of the three different MLSB resistance phenotypes (cMLSB, iMLSB, MSB). Among these 81 erythromycin-resistant strains, 10 showed clindamycin susceptibility and were tested by double-disk diffusion method. We found only five (6.2%) isolates with iMLSB resistance phenotype (three S. aureus and two CNS) and five (6.2%) with MSB resistance phenotype (two S. aureus and three CNS). The remaining 71 (87.7%) isolates were considered as cMLSB resistance phenotype (46 S. aureus and 25 CNS).

All the 152 strains were tested for the presence of MLSB resistance genes and 77 (50.1%) contained one or more erm genes (Figure 1). The ermB gene was detected in 44 isolates (41 S. aureus and three CNS), the ermC gene was found in only one isolate of S. aureus and the ermA gene was detected in 28 isolates (four S. aureus and 24 CNS). Combination of erm genes was detected in 4 CNS isolates (Graphics 1 and 2). For S. aureus isolates with cMLSB resistance phenotype, 36 presented the ermA gene, only one exhibited the ermB gene and three had the ermC gene. Moreover, in three of the S. aureus isolates with iMLSB resistance phenotype, two isolates were ermB positive and one was ermC positive. The ermC gene was identified in 20 isolates of CNS with cMLSB resistance phenotype and in two isolates of CNS with iMLSB resistance phenotype. Seven (six S. aureus and one CNS) isolates with cMLSB resistance phenotype did not present any of the three erm genes (Table 1). Resistance to non-MLSB antibiotics in S. aureus and CNS isolates with erm genes was higher in relation to the isolates without the erm genes: chloramphenicol (p = 0.004), doxycycline (p < 0.001), gentamicin (p < 0.001), levofloxacin (p < 0.001), oxacillin (p < 0.001), rifampin (p < 0.001) and, trimethoprim-sulfamethoxazole (p < 0.001). Of the 77 isolates who harbored erm genes, 65 (40 S. aureus and 25 CNS) were multidrug resistant (resistant to five or more antimicrobial class). The overall range of multidrug-resistance among the staphylococci strains studied was 48.2%.

DISCUSSION
The incidence of constitutive and inducible MLSB resistance may vary according to different geographic region and even from hospital to hospital or patient group. This variability is usually associated with the inconsistent use of erythromycin in different institutions; the origin of the isolate (nosocomial versus community acquired); patient age and clinical samples. In our study 53.3% of staphylococci presented one of three MLSB resistance phenotypes. In fact, cMLSB resistance phenotype was the most common (46.7%) and iMLSB and MSB phenotype were each detected in only 3.3% of the staphylococci.

In a study conducted in Texas by Fiebelkorn et al. the cMLSB resistance phenotype was also the most prevalent phenotype (41.7% of staphylococci) but the iMLSB was found in 25.2% of the isolates, indicating a difference in relation to iMLSB data of the present study. In Europe where the MLSB phenotype prevalence are somehow variable, in London Hamilton-Miller et al. detected staphylococci with iMLSB as the predominant phenotype (43% of isolates) and the cMLSB resistance phenotype was detected in only 24% of isolates. The D test is critical, in this scenario, to avoid therapeutic failure. On the other hand, CNS isolates studied in Sevilla demonstrated that the MSB resistance phenotype was more common (11.2%) in relation to the other phenotypes (iMLSB
In contrast, the cMLS\(_{B}\) resistance phenotype was most frequent (46.9%) as compared to iMLS\(_{B}\) (30.2%) in France.\(^{14}\)

In Turkey it was demonstrated that the prevalence of the cMLS\(_{B}\) phenotype is higher than that of the iMLS\(_{B}\) phenotype and the MS\(_{B}\) phenotype is low, data similar to our study.\(^{15,18-20}\)

A previous study conducted in our city evaluated 200 CNS and showed that only 2.5% of isolates presented the iMLS\(_{B}\) resistance phenotype.\(^{21}\) Therefore, one could speculate that the prevalence of the inducible phenotype is low in our city.

Despite the fact that there is geographic variability among MS\(_{B}\) resistance phenotypes, the prevalence of \(erm\) genes has been reported to be similar in various countries. According to our findings, the \(ermA\) gene was the most prevalent among the \(S. aureus\) isolates (43.6%) and the \(ermC\) gene was the most prevalent among the SCN isolates (37.9%). Only three isolates of \(staphylococci\) presented the \(ermB\) gene (2.0%). The presence of more than one \(erm\) gene was not detected in \(S. aureus\) but it was observed in four SCN isolates. According to Martineau et al., in Canada, 20.9% of the \(S. aureus\) were positive for the \(erm\)A gene and 66% of CNS were positive for the \(erm\)C gene, demonstrating that the prevalence of the \(erm\)A gene in \(S. aureus\) is slightly lower in comparison to other studies.\(^{22}\) A multicenter study in 24 European university hospitals confirmed the high prevalence of \(erm\)A gene and the low prevalence of \(erm\)C and \(erm\)B genes among 851 \(S. aureus\).\(^{23}\) Lina et al. found 63.2% of \(S. aureus\) with \(erm\)A gene positive and 44% of CNS strains \(erm\)C gene positive, while the \(erm\)B gene was present in only 1% of \(staphylococci\).\(^{14}\) The results reported by Westh et al. in Denmark, also showed a high prevalence of the \(erm\)A gene in \(S. aureus\) isolates and the \(erm\)C gene in CNS strains, as well as a low prevalence for the \(erm\)B gene.\(^{24}\) In our study, the \(erm\)B gene was also detected in a small percentage of \(staphylococci\) isolates. This gene is generally found in animal \(staphylococci\) strains.\(^{6,14,17}\)

In the present study, eight isolates (three \(S. aureus\) and five SCN) susceptible to erythromycin proved to carry \(erm\) genes (seven \(erm\)A one \(erm\)C). The presence of \(erm\) genes among isolates of \(staphylococci\) susceptible to erythromycin had already been demonstrated in another study.\(^{22}\) This may be due to the lack of expression of \(erm\) genes due to factors which down regulate the expression of this gene.\(^{22,23}\)

In our study we found six \(S. aureus\) isolates and one CNS resistant to erythromycin and clindamycin but with negative genotypic test. These results were probably associated with the presence of other genes, such as \(msrA\) and \(msrB\), with low frequency in \(staphylococci\) species isolated form humans,\(^{25}\) which were not evaluated in this study.

We detected three \(S. aureus\) resistant to clindamycin and susceptible to erythromycin, which did not harbor \(erm\) genes. In a study conducted by Lina and et al., the only SCN sample that presented this susceptibility profile was positive for the genes \(linA\) and \(lina\).\(^{14}\) These genes confer lincosamides resistance only in \(S. heamolyticus\) and \(S. aureus\). Incidence of \(staphylococci\) with lincosamide resistance but without resistance to macrolides and streptogramins is usually very low.\(^{14,26}\)

**CONCLUSION**

The aim of this study was to determine the prevalence of the MLS\(_{B}\) phenotypes and genes in \(staphylococcus aureus\) and coagulase-negative \(staphylococci\) from patients receiving care at our hospital. We found that constitutive MLS\(_{B}\) resistance was the most prevalent phenotype in \(staphylococci\); \(ermA\) was the most prevalent gene in \(S. aureus\) strains, whereas \(ermC\) was the most frequent gene in CNS isolates. Therefore, \(staphylococci\) with resistance to MLS\(_{B}\) are usually detected directly in routine susceptibility test and the “D test” is not required to be performed in most of our isolates. However, other regions in our country may not present the same resistance profile as ours and, therefore, surveillance studies are warranted in different institutions.

**REFERENCES**


