ORIGINAL ARTICLE

Distribution of *erm* genes and low prevalence of inducible resistance to clindamycin among *staphylococci* isolates

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ABSTRACT

Introduction: Resistance to macrolides, lincosamides and streptogramins B (MLS_B antibiotics) in *staphylococci* may be due to modification in ribosomal target methylase encoded by *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 *erm* genes among 152 clinical isolates of *Staphylococcus aureus* and coagulase-negative *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 *staphylococci* were positive for $cMLS_B$; 3.3% for $iMLS_B$ and 3.3% for MS_B. One or more *erm* genes were present in 50.1% of isolates. The gene *erm*A was detected in 49 isolates, *erm*C in 29 and *erm*B in 3. **Conclusion**: The prevalence of the *erm*A, *erm*B and *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: *Staphylococcus*; resistance; *erm* genes; macrolides.

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INTRODUCTION

Staphylococcus aureus and coagulase negative *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.[1,2,3] Although β-lactam antibiotics are the main compounds used to treat infections due to *staphylococci*, macrolides, lincosamides e streptogramins type B (MLS_B) antibiotics are also widely used to treat staphylococcal infections. These antibiotics exert similar inhibitory effects on bacterial protein synthesis, but they are chemically distinct.[4,5] MLS_B resistance can be caused by several mechanisms, but the predominant form is target modification mediated by *erm*A, *erm*B e *erm*C (erythromycin ribosome methylase) genes.[4,5] The *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.[6,7] 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 *in vitro* cross resistance among MLS_B drugs. However, *staphylococci* isolates with inducible MLS_B resistance demonstrate clear *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 *in vitro* erythromycin resistance and false clindamycin susceptibility, because the conventional disk-diffusion may fail to detect inducible MLS_B resistance.[4,9,10] 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

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reaction (PCR) with specific primers is a genotypic method used to confirm the presence of the MLS_B genes, *erm*A, *erm*B e *erm*C.[12] The risk for therapeutic failure is increased as constitutive resistance may raise from $iMLS_B$ during the course of clindamycin therapy in patients with severe *staphylococci* infections.[11]

The objective of this study was to determine the prevalence of the MLS_B genes in *Staphylococcus aureus* and coagulase negative *staphylococci* from patients attending the *Hospital de Clínicas de Porto Alegre* (HCPA).

MATERIALS AND METHODS

Bacterial isolates

Isolates of *S. aureus* and of CNS were collected from consecutive clinical specimens sent to the of 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). *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.[11] 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 ($iMLS_B$). The phenotype $cMLS_B$ was characterized by erythromycin and clindamycin resistance. Finally, the phenotype (MS_B) was characterized by clindamycin susceptibility and erythromycin resistance, with negative D test.

*erm*A, *erm*B and *erm*C 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 µlL reaction mixture.[13]

PCR assays and primers specific from the *erm*A, *erm*B and *erm*C resistance genes used in this study have been previously described by Gerard, Lina *et al.* (Table 1).[14] Each reaction was carried out in a final volume of 25 µL and included 10 x PCR buffer (pht®); 3 mM of $MgCl_2$ (pht®); 5 µM of each *erm*A, *erm*B and *erm*C forward and reverse primers (Invitrogen®); RNAse and DNAse free water; 1.25 U of *Taq* DNA polymerase (pht®); 2.5 mM of each dATP, dTTP, dCTP, and dGTP (ABgene®); and 5 µL of DNA. The PCR mixture was subjected to thermal cycle (30 cycles of 30 s at 94°C as the denaturation step, 30 s at 57°C as the annealing step, and of 5 min at 72°C as the extension step) with a JMR® PTC-100. The PCR-amplified reaction mixture was resolved by electrophoresis through a 2% agarose gel containing ethidium bromide in Tris-borate-EDTA buffer at 12 V/cm for 30 min. The gel was visualized under UV light and the sizes of the amplification products were estimated by comparison with 100 bp molecular size standard ladder.

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.

Table 1. Correlation between* erm *genes and MLSB resistance phenotypes

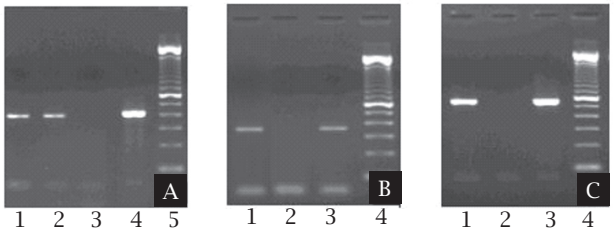
Isolate	Phenotype	Genotype					
		*erm*A	*erm*B	*erm*C	*erm*A/*erm*C	*erm*A/*erm*B	*erm*A/*erm*B/*erm*C
S. aureus	40 ($cMLS_B$)	36	1	3	0	0	0
	3 ($iMLS_B$)	2	0	1	0	0	0
	2 (MS_B)	0	0	0	0	0	0
CNS	24 ($cMLS_B$)	0	0	20	2	1	1
	2 ($iMLS_B$)	0	0	2	0	0	0
	3 (MS_B)	0	0	0	0	0	0

RESULTS

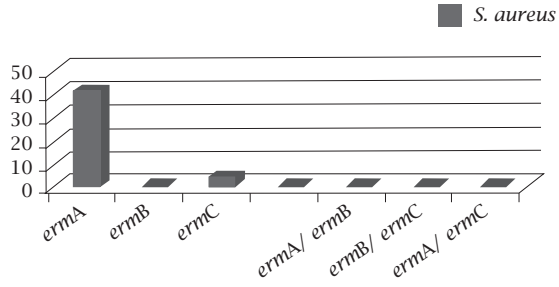
A total of 152 strains including 94 *S. aureus* and 58 CNS were included in this study. Eighty-one (53.3%) exhibited erythromycin resistance and were considered for evaluation of the three distinct MLS_B resistance phenotypes ($cMLS_B$, $iMLS_B$, MS_B). Among these 81 erythromycin-resistant strains, 10 showed clindamycin susceptibly and were tested by double-disk diffusion method. We found only five (6.2%) isolates with $iMLS_B$ resistance phenotype (three *S. aureus* and two CNS) and five (6.2%) with MS_B resistance phenotype (two *S. aureus* and three CNS). The remaining 71 (87.7%) isolates were considered as $cMLS_B$ resistance phenotype (46 *S. aureus* and 25 CNS).

All the 152 strains were tested for the presence of MLS_B resistance genes and 77 (50.1%) contained one or more *erm* genes (Figure 1). The *erm*A gene was detected in 44 isolates (41 *S. aureus* and three CNS), the *erm*B gene was found in only one isolate of *S. aureus* and the *erm*C 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 $cMLS_B$ resistance phenotype, 36 presented the *erm*A gene, only one exhibited the *erm*B gene and three had the *erm*C gene. Moreover, in three of the *S. aureus* isolates with $iMLS_B$ resistance phenotype, two isolates were *erm*A positive and one was *erm*C positive. The *erm*C gene was identified in 20 isolates of CNS with $cMLS_B$ resistance phenotype and in two isolates of CNS with $iMLS_B$ resistance phenotype. Seven (six *S. aureus* and one CNS) isolates with $cMLS_B$ resistance phenotype did not present any of the three *erm* genes (Table 1). Resistance to non-MLS_B 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 multiresistance among the *staphylococci* strains studied was 48.2%.

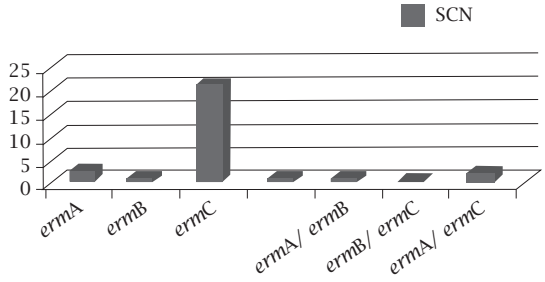
Figure 1: (A) Lanes 1 and 2 *erm*A positive in 421 bp; lane 3 negative control; lane 4 positive control; and lane 5 100 bp molecular size ladder. (B) Lane 1 *erm*B positive in 359 bp; lane 2 negative control; lane 3 positive control; and lane 4 100 bp molecular size ladder. (C) Lane 1 *erm*C positive in 572 bp; lane 2 negative control; lane 3 positive control; and lane 4 100 bp molecular size ladder.



Graphic 1: Frequency of *erm* genes in *S. aureus* isolates.



Graphic 2: Frequency of *erm* genes in SCN isolates.



DISCUSSION

The incidence of constitutive and inducible MLS_B 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.[15,16] In our study 53.3% of *staphylococci* presented one of three MLS_B resistance phenotypes. In fact, $cMLS_B$ resistance phenotype was the most common (46.7%) and $iMLS_B$ and MS_B phenotype were each detected in only 3.3% of the *staphylococci*.

In a study conduced in Texas by Fiebelkorn *et al.* the $cMLS_B$ resistance phenotype was also the most prevalent phenotype (41.7% of *staphylococci*) but the $iMLS_B$ was found in 25.2% of the isolates, indicating a difference in relation to $iMLS_B$ data of the present study.[10] In Europe where the MLS_B phenotype prevalence are somehow variable, in London Hamilton-Miller *et al.* detected *staphylococci* with $iMLS_B$ as the predominant phenotype (43% of isolates) and the $cMLS_B$ resistance phenotype was detected in only 24% of isolates.[17] The D test is critical, in this scenario, to avoid therapeutic failure. On the other hand, CNS isolates studied in Sevilla demonstrated that the MS_B resistance phenotype was more common (11.2%) in relation to the other phenotypes ($iMLS_B$

7.4% and $cMLS_B$ 3.2%).[16] 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 MLS_B resistance phenotypes, the prevalence of *erm* genes has been reported to be similar in various countries. According to our findings, the *erm*A gene was the most prevalent among the *S. aureus* isolates (43.6%) and the *erm*C gene was the most prevalent among the SCN isolates (37.9%). Only three isolates of *staphylococci* presented the *erm*B 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 e 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 *msr*A and *msr*B, 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 *lin*A and *lin*A´.[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*; *erm*A was the most prevalent gene in *S. aureus* strains, whereas *erm*C 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.

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