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Original Article

Phenotypic and molecular characterization of resistance to macrolides, lincosamides and type B streptogramin of clinical isolates of *Staphylococcus* spp. of a university hospital in Recife, Pernambuco, Brazil

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ABSTRACT

Introduction: There is a mechanism of macrolide resistance in *Staphylococcus* spp. which also affects the lincosamides and type B streptogramins characterizing the so-called MLS_B resistance, whose expression can be constitutive ($cMLS_B$) or inducible ($iMLS_B$) and is encoded mainly by *ermA* and *ermC* genes. The $cMLS_B$ resistance is easily detected by susceptibility testing used in the laboratory routine, but $iMLS_B$ resistance is not. Therapy with clindamycin in cases of infection with isolated $iMLS_B$ resistance may fail.

Objective: To characterize the phenotypic (occurrence of $cMLS_B$ and $iMLS_B$ phenotypes) and molecular (occurrence of *ermA* and *ermC* genes) profiles of MLS_B resistance of clinical isolates of susceptible and methicillin-resistant *Staphylococcus aureus* and CNS (coagulase-negative *Staphylococcus*) from patients of a university hospital, in Pernambuco.

Methods: The antimicrobial susceptibility of 103 isolates was determined by the disk diffusion technique in Mueller–Hinton agar followed by oxacillin *screening*. The $iMLS_B$ phenotype was detected by D test. Isolates with $cMLS_B$ and $iMLS_B$ phenotypes were subjected to polymerase chain reaction (PCR) for the detection of *ermA* and *ermC* genes.

Results: The $cMLS_B$ and $iMLS_B$ phenotypes were respectively identified in 39 (37.9%) and five (4.9%) isolates. The $iMLS_B$ phenotype was found only in four (10.8%) methicillin-susceptible *S. aureus* and one (4.5%) methicillin-resistant *S. aureus*. In the 44 isolates subjected to PCR, four (9.1%) only *ermA* gene was detected, a lower frequency when compared to only *ermC* 17 (38.6%) gene and to one (2.3%) isolate presenting both genes.

Conclusion: In the *Staphylococcus* spp. analyzed, the *ermC* gene was found more often than the *ermA*, although the $iMLS_B$ phenotype had been less frequent than the $cMLS_B$. It was important to perform the D test for its detection to guide therapeutic approaches.

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Introduction

The increasing prevalence of methicillin resistance in *Staphylococcus* spp. is a growing problem. This has renewed interest regarding the use of macrolide, lincosamides, and type B streptogramin antimicrobials for the treatment of staphylococcal infections. Clindamycin, a lincosamide, represents a common choice for some of these infections, particularly for infections of the skin and soft tissues, and an alternative in case of intolerance to penicillin or methicillin resistance.[1–3] In *Staphylococcus* spp., one of the resistance mechanisms consists of ribosomal target modification, affecting macrolides, lincosamides, and type B streptogramins characterizes the so-called MLS_B resistance. Its expression can be constitutive ($cMLS_B$) or inducible ($iMLS_B$) and is encoded by *ermA* (erythromycin ribosome methylase) and *ermC* genes, which are the main determinants of resistance in staphylococci.[2,4,5]

It is important to know the type of MLS_B resistance for establishing adequate therapy, since *Staphylococcus* spp. with constitutive resistance present *in vitro* resistance to all macrolides, lincosamides, and type B streptogramins. In addition, *in vivo* therapy with clindamycin for *Staphylococcus* spp. infection with inducible resistance can select constitutive *erm* mutants, resulting in treatment failure.[3,4,6,7] It is noteworthy that $cMLS_B$ resistance is easily detected by susceptibility testing used in the laboratory routine, while $iMLS_B$ resistance is not. Using these detection methods, *Staphylococcus* spp. with inducible resistance has *in vitro* resistance to erythromycin and susceptibility to clindamycin.[5,6,8,9]

To detect the inducible clindamycin resistance in *Staphylococcus* spp., one of the tests recommended by the CLSI (Clinical and Laboratory Standards Institute) is the double-disk diffusion test (D Test) and when the isolated present such resistance, the CLSI recommends reporting them as resistant to clindamycin.[10] Then, data on the antimicrobial susceptibility are important in the choice of therapy against infections, but false susceptibility results can be obtained if the isolates are not subjected to tests that detect inducible resistance to clindamycin.[11]

Studies carried out in two Brazilian states with clinical isolates of *Staphylococcus* spp. reported the $cMLS_B$ phenotype as the most frequent.[12,13] Coutinho et al.[13] have also evaluated the occurrence of the *erm* genes among the isolates analyzed. However, the frequency of $cMLS_B$ and $iMLS_B$ resistance varies among different hospitals and there are other resistance mechanisms that confer resistance to only one or two classes of the MLS_B complex.[14,15]

The objective of this study was to characterize the phenotypic (occurrence of $cMLS_B$ and $iMLS_B$ phenotypes) and molecular (occurrence of *ermA* and *ermC* genes) profiles of MLS_B resistance of clinical isolates of susceptible and methicillin-resistant *Staphylococcus aureus* and CNS (coagulase-negative *Staphylococcus*) from patients of a university hospital in Pernambuco, Brazil. Obtaining local data relating to resistance, may be helpful in guiding therapeutic approaches.

Materials and methods

Clinical isolates

A total of 103 clinical isolates were gathered from various samples from patients infected with *S. aureus* or SCN of a university hospital of Pernambuco, Brazil, during the year 2012 and were stored in glycerol (25%) at −20 °C. To verify the purity, the colonies were inoculated into Brain Heart Infusion broth (BHI) and after incubation at 37 °C for 48 h were plated on blood agar.

Antimicrobial susceptibility profile

The antibiogram was performed by disk diffusion technique in Mueller–Hinton agar, using antibiotic clindamycin 2 μg, erythromycin 15 μg, cefoxitin 30 μg, and oxacillin 1 μg. The results were interpreted according to the standards determined by CLSI.[10]

Screening for oxacillin resistance

Isolates with resistance or intermediate resistance to oxacillin and/or cefoxitin were submitted to oxacillin *screening*, as proposed by Rabelo et al.[16]

D test

S. aureus and SCN isolates with resistance to erythromycin and susceptibility or intermediate resistance to clindamycin in the antibiogram were selected. For the execution of this test a disk of 2 μg of clindamycin was placed at a distance of 15 mm–26 mm from the edge of a disk of 15 μg of erythromycin in a plate containing Mueller–Hinton agar sown in the same way as it was for the antibiogram. After incubation at 35 °C for 16–18 h, isolates that showed no flattening of the inhibition zone around the clindamycin disk were reported as susceptible to clindamycin (negative D test) and isolates that showed flattening of the inhibition zone around the clindamycin disk adjacent to erythromycin disk ("D" zone) indicated inducible clindamycin resistance (positive D test).[10]

Extraction of total DNA

To examine the presence of *ermA* and *emrC* genes, the rapid extraction of the total DNA of isolates that showed phenotypes MLS_Bc and MLS_Bi was performed by a modified technique of thermal lysis directly from the colony, according to Hu et al.,[17] after inoculation of a colony of each isolate into 5 mL of BHI and incubated at 37 °C for 24 h.

Polymerase chain reaction (PCR) conditions

PCR was performed using the primers described by Lina et al.[18] for *ermA* and *ermC* genes. For the detection of *ermA* gene, each amplification reaction was prepared in a final volume of 25 μL for each tube and includes: 1 μL (40 ng) of total DNA, 1 μL (20 pmol) of each primer, 0.6 μL of deoxyribonucleotide triphosphate (dNTP) (8 mM), 5.0 μL of buffer (5×), 1.5 μL of

Table 1 – Susceptibility profile to erythromycin and clindamycin of susceptible and methicillin-resistant *S. aureus* and CNS.

Phenotypes	MSSA *n* (%)	MRSA *n* (%)	MSCNS *n* (%)	MRCNS *n* (%)	Total *n* (%)
ERY-S, CLI-S (susceptible)	25 (67.6)	5 (22.7)	4 (28.6)	7 (23.3)	41 (39.8)
ERY-S, CLI-I	1 (2.7)	0 (0)	1 (7.1)	0 (0)	2 (1.9)
ERY-S, CLI-R	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ERY-I, CLI-S	1 (2.7)	0 (0)	2 (14.3)	0 (0)	3 (2.9)
ERY-I, CLI-I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ERY-I, CLI-R	0 (0)	0 (0)	0 (0)	1 (3.3)	1 (1)
ERY-R, CLI-R (cMLS$_B$)	4 (10.8)	15 (68.2)	3 (21.4)	17 (56.7)	39 (37.9)
ERY-R, CLI-S/I, + D Test (iMLS$_B$)	4 (10.8)	1 (4.5)	0 (0)	0 (0)	5 (4.9)
ERY-R, CLI-S/I, – Test D (MS$_B$)	2 (5.4)	1 (4.5)	4 (28.6)	5 (16.7)	12 (11.6)
Total	37 (35.9)	22 (21.4)	14 (13.6)	30 (29.1)	103 (100)

ERY, erythromycin; CLI, clindamycin; S, susceptible; I, intermediary; R, resistant; MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; MSCNS, methicillin-susceptible coagulase-negative *Staphylococcus*; MRCNS, methicillin-resistant coagulase-negative *Staphylococcus*; MLS$_B$, macrolides, lincosamides and type B streptogramins; cMLS$_B$, phenotype of constitutive MLS$_B$ resistance; iMLS$_B$, phenotype of inducible MLS$_B$ resistance; MS$_B$, phenotype of resistance to macrolides and type B streptogramins.

$MgCl_2$ (25 mM), 0.4 μL of *Taq* DNA polymerase (5U). For the detection of *ermC* gene, each amplification reaction was prepared in the same manner as for *ermA* gene, except in relation to the quantity of *Taq* DNA polymerase (5U), which in this reaction was 0.3 μL. Amplification reactions were performed in the thermocycler at the following conditions: 30 cycles of 1 min at 94 °C in the denaturing step, 30 s at 49 °C in the annealing step and 30 s at 72 °C in the extension step, according to the conditions described by França et al.[19] with modification. In all PCR a negative control was included, corresponding to a tube containing all components of the mixture to which template DNA was not added. Identified positive isolates for the *ermA* and *ermC* genes were used as positive controls. The PCR products were subjected to electrophoresis on 1.5% agarose gel in 0.5× TBE buffer. These products were stained with blue, visualized on an ultraviolet transilluminator and photodocumented.

Sequencing of ermA and ermC genes

A PCR product positive for the *ermA* and *ermC* genes were purified through the kit Wizard® SV Gel and PCR Clean-Up System (Promega), according to manufacturer's protocol and then, quantified by spectrophotometry. Thereafter they were submitted to sequencing and analysis of the results was performed through the softwares Chromas Lite 2.1.1, Basic Local Alignment Search Tool (BLAST), and Expert Protein Analysis System (ExPASy). The analyzed sequences of *ermA* and *ermC* genes were deposited in the GenBank with the following access numbers, respectively: KT599443 and KU232395.

Results

Fifty-nine (57.3%) clinical isolates of *S. aureus* and 44 (42.7%) of SCN were analyzed, totaling 103 isolates. Of these, 37 (35.9%), 22 (21.4%), 14 (13.6%), and 30 (29.1%) were classified as methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), methicillin-susceptible coagulase-negative *Staphylococcus* (MSCNS) and methicillin-resistant coagulase-negative *Staphylococcus* (MRCNS), respectively (Table 1).

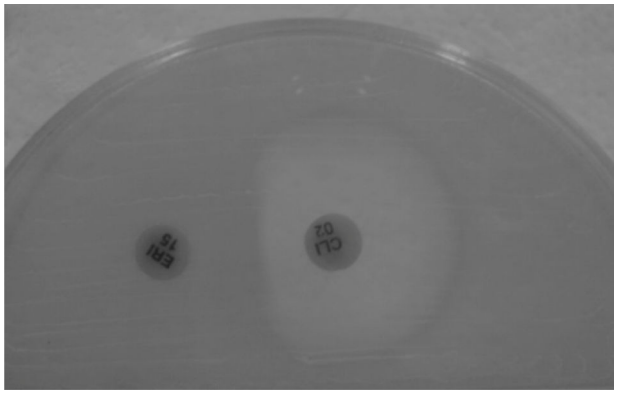

Fig. 1 – Positive D test, showing the flattening of the inhibition zone around the clindamycin disk adjacent to erythromycin disk (called "D" zone).

The susceptible phenotype (ERY-S, CLI-S) was detected in 25 (67.6%), five (22.7%), four (28.6%), and seven (23.3%) of MSSA, MRSA, MSCNS and MRCNS, respectively (Table 1). The phenotype cMLS$_B$ (ERY-R, CLI-R) was detected respectively, in four (10.8%) 15 (68.2%), three (21.4%), and 17 (56.7%) of MSSA, MRSA, MSCNS and MRCNS. The iMLS$_B$ phenotype (ERY-R, CLI-S, positive D test) (Fig. 1) was found in only four (10.8%) of the MSSA and in one (4.5%) of the MRSA. The MS$_B$ phenotype (ERY-R, CLI-S, negative D test) was detected in two (5.4%), one (4.5%), four (28.6%), and five (16.7%) of MSSA, MRSA, MSCNS, and MRCNS, respectively (Table 1).

For the identification of the *ermA* and *ermC* genes, 44 isolates with cMLS$_B$ and iMLS$_B$ phenotypes underwent PCR. Four (9.1%) isolates showed only *ermA* gene, 17 (38.6%) had only *ermC* gene, one (2.3%) had both genes, and 22 (50%) did not have the referred genes (Table 2).

Discussion

In this study, the frequency of MSSA (25; 67.6%) was greater than MRSA (five; 22.7%) and of MSCNS (four; 28.6%) was lower

Table 2 – Distribution of *ermA* and *ermC* genes among *Staphylococcus* spp. isolates with MLS_B resistance.

Genes	Isolates (%)
ermA	4 (9.1)
ermC	17 (38.6)
ermA + *ermC*	1 (2.3)
None	22 (50.0)
Total	44 (100.0)

than MRCNS (seven; 23.3%). In a study conducted in India, 313 isolates of *Staphylococcus* spp. there were 83 (64.84%) MSSA and 45 (35.15%) MRSA, in line to the present study; in contrast, there were 124 (67.02%) MSCNS and 61 (32.97%) MRCNS.[5] A total of 1687 isolates of *Staphylococcus* spp. investigated in Turkey consisted of 419 (24.8%) MSSA and 464 (27.5%) MRSA, unlike this study, and the rest of the isolates, similar to this study, consisted of 196 (11.6%) MSCNS and 608 (36.1%) MRCNS.[11]

The susceptible phenotype (ERY-S, CLI-S) found in 41 (39.8%) isolates in this study prevailed among the isolates tested. This phenotype was also prevalent in studies carried out in India, Libya, and Turkey where the referred phenotype was detected in 192 (51.5%), 87 (54.7%), and 688 (40.8%) isolates, respectively.[11,20,21]

In this study, the 39 (37.9%) $cMLS_B$ phenotype isolates prevailed over the five (4.9%) $iMLS_B$ phenotype and over the 12 (11.6%) MS_B phenotype resistant to macrolides and type B streptogramins, which is consistent with other studies conducted in Brazil.[12,13,22] One of the studies was performed in São Paulo revealed 37 (25.17%) and nine (6.12%) isolates with $cMLS_B$ and $iMLS_B$ phenotypes, respectively, while the MS_B was not detected.[12] Other studies were conducted in the state of Rio Grande do Sul; one of them identified 71 (46.7%), five (3.3%), and five (3.3%) isolates with $cMLS_B$, $iMLS_B$, and MS_B phenotypes, respectively.[13] The other study detected 25 (17.9%), 11 (7.9%), and two (1.4%) isolates with $cMLS_B$, $iMLS_B$, and MS_B phenotypes, respectively.[22]

In this study the $cMLS_B$ phenotype prevailed among the isolates resistant to methicillin, which is consistent with studies conducted in other countries[3,5,11,15,21] as well in Brazil.[12,22]

The MS_B phenotype was more frequent (12; 11.6%) than the $iMLS_B$ (five; 4.9%) in this study. Diverging from this finding, the study done by Kumar et al.[8] in India found the same frequency (33; 16.9%) of MS_B and $iMLS_B$ phenotypes among the *S. aureus* isolates analyzed. Merino-Díaz et al.[23] in Spain identified the *msrA* gene in all *Staphylococcus* spp. isolates with MS_B phenotype. In *Staphylococcus* spp. this gene is responsible for an efflux mechanism which confers resistance to macrolides and type B streptogramins, but not to clindamycin.[5,24]

Only *S. aureus* showed the $iMLS_B$ phenotype and its frequency was higher among the MSSA (four; 10.8%) than among the MRSA (one; 4.5%) in this study. Astudy conducted in Chicago detected the $iMLS_B$ phenotype in 59 (20%) MSSA isolates and in 14 (7%) MRSA isolates in one hospital, and the $iMLS_B$ phenotype in 94 (19%) and in 30 (12%) MSSA and MRSA isolates, respectively, in another hospital.[25] In a study conducted in Turkey, eight (5.8%) MSSA isolates and two (1.7%) MRSA isolates presented the $iMLS_B$ phenotype.[26] In the study conducted in São Paulo, the $iMLS_B$ phenotype was observed in seven (6.73%) MSSA and in two (4.65%) MRSA isolates.[12] These data from previously reported studies are similar to the findings of the present study. However, there are other studies carried out abroad, in which the $iMLS_B$ phenotype was prevalent among MRSA isolates.[3,8,15,27,28]

Relevant data for antimicrobial susceptibility are significant in establishing an appropriate therapy; therefore, the availability of the D test results are important.[20,25] Reporting an isolated *Staphylococcus* spp. as susceptible to clindamycin without verifying if it presents inducible resistance, may lead to inadequate therapy with this drug. In contrast, a negative result for inducible resistance to clindamycin, confirms the susceptibility of this antimicrobial, providing a very good therapeutic option.[3,8]

In this study, the *ermA* and *ermC* genes were detected individually in four (9.1%) and 17 (38.6%) isolates with MLS_B resistance phenotypes, respectively. In some studies, contrary to this, the *ermA* gene was identified more frequently than the *ermC* gene, as in a study conducted in Iran, in which the *ermA* and *ermC* genes were found in 76 (60.3%) and 69 (54.8%) *S. aureus* isolates, respectively.[29] Schmitz et al.,[30] when analyzing *S. aureus* isolates from 24 European university hospitals detected the *ermA* gene in 571 (67%) isolates and the *ermC* in 192 (23%). In Korea, Jung et al.[31] identified among 280 *S. aureus* isolates, the *ermA* gene in 250 and the *ermC* in 14 isolates. In Iran, Moosavian et al.[32] detected the *ermA* and *ermC* genes, respectively in 41.1% and 17.7% of the *S. aureus* isolates studied.

In other studies, consistent with this, the *ermC* gene was predominant relatively to the *ermA* gene, as in a study from Spain, in which the *ermC* gene was more frequently detected, in both *S. aureus* and SCN isolates with constitutive and inducible phenotypes.[23] In Greece, Spiliopoulou et al.[33] identified the *ermA* and *ermC* genes in 22% and 70% of *S. aureus* isolates, respectively. Vallianou et al.[34] have reported that the *ermC* gene was found more frequently among *S. aureus* and SCN isolates of a university hospital also in Greece.

In this study, one (2.3%) isolate presented both *ermA* and *ermC* genes. The association of these genes in *Staphylococcus* spp. isolates,[29,35,36] was also observed in other studies.

The *ermB* gene was not investigated, because it is usually detected in *Staphylococcus* spp. isolates of animal origin[13,23,30] and it is spread mainly between streptococci and enterococci.[4] In Rio Grande do Sul, Coutinho et al.[13] reported low frequency of the *ermB* gene. They found that among 152 *Staphylococcus* spp. isolates analyzed, 77 had one or more *erm* gene, the *ermA*, *ermC*, and *ermB* genes were found respectively at 49, 29, and three isolates and, the combination of these genes was found in four isolates,[13] data which differed from this study. However, in a work developed in Texas, with *S. aureus* isolates of pediatric origin, among the 67 isolates evaluated, the *ermB* gene was identified in 31 of these, and the *ermA* and the *ermC* genes were detected in 12 and 24 isolates, respectively.[37]

Then it was possible to observe the frequency variation of the phenotypes of the MLS_B resistance and of the *erm* genes amongst hospitals and geographic regions, already reported by other authors.[13,15,21,29] Therefore, the importance of determining these frequencies in specific location is highlighted.[13,21]

The good correlation between the phenotypic and genotypic methods allows us to infer the mechanism of resistance to erythromycin and clindamycin, to establish the most appropriate antimicrobial treatment and to appreciate the epidemiological differences in their distribution.[23]

Conclusion

The *ermA* gene was less frequent than the *ermC* gene among *Staphylococcus* spp. isolates with $cMLS_B$ and $iMLS_B$ phenotypes. Despite the lower frequency of iMLSB than cMLSB and MSB phenotypes, it is important to perform the D test in order to identify it and guide therapeutic procedures. As the phenotypic and molecular data about a particular mechanism of antimicrobial resistance vary between hospitals and geographic regions, obtaining local data is useful, since it can be helpful to emphasize the importance of the implementation of procedures that aim at controlling the spread of this mechanism, in the hospital where the study was conducted.

Conflicts of interest

The authors declare no conflicts of interest.

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