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Induced and Constitutive Clindamycin Resistance in *Staphylococcus* spp. Strains Isolated from a Neonatal Intensive Care Unit

Sávio Benvindo Ferreira[1*], José Ricardo Tomé Lopes Martins[2], Paula Benvindo Ferreira[1] and Zilka Nanes Lima[3]

[1]Program of Postgraduate in Natural Products and Synthetic Bioactive, Federal University of Paraiba, Paraiba, Brazil.
[2]Multiprofessional residence in Health, Federal University of Rio Grande do Norte, Rio Grande do Norte, Brazil.
[3]Department of Pharmacy, State University of Paraíba, Paraíba, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Authors SBF and ZNL did the study design, wrote the protocol and analyses of study. Author JRTLM did the processing samples while the literature searches were by author PBF. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The hospital environment can act as a reservoir for microorganisms, which in turn can contaminate a range of hospital equipment and survive for long periods of time. One of these environments the Neonatal Intensive Care Unit (NICU), for the initial post partum period serving as a home for newborns of low birth weight and needing invasive procedures for administration of nutritional and medicinal substances, which makes the NICU a critical area for housing individuals with immune system. Thus, the aim of this study was to evaluate the phenotypic appearance of resistance of the *Staphylococcus spp.* compared to erythromycin and clindamycin, originating from

**Corresponding author: Email: saviobenvindo@gmail.com;*

isolated areas of a NICU in the city of Campina Grande - PB.
Place and Duration of Study: Sample: Neonatal Intensive Care Unit of a public hospital in the city of Campina Grande – PB, Brazil. Processing and Analysis of Samples: Clinical Analysis Laboratory of the State University of Paraíba, between August and October 2012.
Methodology: Samples were collected from surfaces present at the NICU. The samples were identified and strains of *Staphylococcus* spp. were subjected to sensitivity, and to verify erythromycin-induced resistance the D-test was used, following the CLSI standards-M100-S22 (2012).
Results: Bacterial strains from all surfaces analyzed were isolated, 59.02% of isolates belong to the genus *Staphylococcus* spp., representing 36 bacterial strains, of which 31 were subspecies *Staphylococcus aureus* and 5 were coagulase-negative *staphylococcus* (CoNS). There was found more than 70% resistance to the group of penicillins and more than 30% to methicillin. Among the 36 strains of *Staphylococcus* spp. 19.45% were resistant to erythromycin. The rate found for constitutive resistance to macrolides (MLSBc) was 5.56% and was observed induced resistance to the macrolide type (MLSBi) in 2.78% the strains.
Conclusion: The resistotyping of isolated strains for inducible and constitutive resistance may be considered a test of substantial importance not only as an epidemiologic marker in view of analyzing possible dissemination of hospital strains, but with respect to adequate, and precise determination of the antibiotic treatment of neonates.

Keywords: Staphylococcus spp; contaminated surfaces; bacterial resistance; MLSB.

1. INTRODUCTION

In recent decades, the advances in *staphylococcus* classification and the development of new gender identification techniques of species and subspecies have allowed researchers and clinicians to become more aware of the wide variety of coagulase-negative *staphylococcus* (CoNS), which were previously described as non-pathogenic, are now recognized as opportunistic microorganisms that prevail in many different situations to produce serious infections [1]. The transmission of these microorganisms can occur by direct contact, where health professionals coming into contact with patients or objects colonized, can serve as transmitters of organisms to other patients. Not forgetting the environmental and airway transmissions, which are uncommon in certain cases, but in some circumstances may occur [2].

Even though the exact function in the inanimate environment that plays *staphylococcus* transmission is not yet determined, it can act as reservoir for microorganisms, which in turn can contaminate a range of hospital equipment and survive for long periods of time [3-5]. One of these environments is the neonatal ICU, homing newborns of low birth weight, and immunologically immature**,** that require invasive procedures for administration of nutritional and medicinal substances [1]. Thus, due to the immaturity of the immune system of the newborn and the use of broad spectrum antibiotics, species commonly found in the air can become pathogens, making the units of pediatric and neonatal intensive care critical areas [6-8].

In addition to that, a growing number of microorganisms develop resistance to drugs used to treat infections, as they are also impervious to new drugs [9]. Among them, has been isolated in recent year's coagulase negative *staphylococcus* (CoNS) resistant to multiple antimicrobials, which has leveraged the interest in studying their susceptibility to commercial drugs [10]. Given that studies have shown multiresistant microorganisms to antimicrobial surfaces of beds and equipment after cleaning and disinfection not appropriate, the interest in this monitoring is only going to increase further [11,12].

Recently, the detection of macrolide-lincosamide-streptogramin B resistance (MLSB) among *staphylococcus* has attracted the attention of clinical laboratories. Three mechanisms have been reported of the MLSB resistance to antibiotics: modification of the action site, efflux of antibiotics and modifying drugs [13]. MLSB carries the erm gene (erythromycin ribosome methylase) encoding rRNA methylase which modifies the binding site of the antimicrobial agent by mutation or methylation of 23S rRNA, resulting in resistance to macrolides, lincosamides, and streptogramin B. There are four main classes erm gene [*erm* (A), *erm* (B), *erm* (C) and *erm* (TR)], and the types *erm* (A)

and *erm* (C) are often responsible for resistance in *staphylococci* of the type MLSB [14,13].

The strains that express the MLSB phenotype can be classified as induced (MLSBi) or constitutive (MLSBc). When an MLSB inducible strain is exposed to an inducer (such as a low-erythromycin), increases the level of expression of rRNA methylase resulting in an increased resistance to MLS class of antimicrobials (such as clindamycin). Even if the strains with an inducible *erm* gene are resistant to inducers and remain susceptible to macrolides inductors and lincosamides, in general, different inducible MLSB resistance patterns can be observed according to the type of *erm* gene or its expression level. In this case, it is necessary to perform disk-approximation induction test (D-test). In constitutive resistance methylase mRNA produced is active even in inducer default, which gives a high level of cross-resistance to MLSB group drugs [13,15,16].

The msrA gene is responsible for efflux mechanism for *staphylococci*, activated after a macrolide exposure, which gene pumps 14 or 15 macrolides members and streptogramin type B [17,13]. Therefore, bacteria are erythromycin resistant but remain clindamycin susceptible, because it isn't an inducer or a substrate for the pump. When the strains, previously sensitive to streptogramin B, become resistant after a macrolide exposure, they are classified as efflux phenotype type M or MSB and can be differentiated from MLSBi phenotype by double-disk testing, in this case no interactions between erythromycin and clindamycin (no D-shaped zone). Resistance to macrolides mediated efflux is common among coagulase-negative *staphylococci* (CoNS) and is increasingly found in methicillin-sensitive *Staphylococcus aureus* (MSSA) [18].

Some researchers recommend that treatment with clindamycin is avoided in cases of infections caused by *S. aureus* strains with MLSBi phenotype, then there are reports of clinical failures in the treatment of patients with this resistance phenotype [19-22]. Similarly, the classification of all *S. aureus* resistant to erythromycin and clindamycin-resistant, can prevent the clindamycin use in cases where the drug would be an alternatively effective alternative treatment [23]. The presence of these drug-resistant strains presents a serious problem, as the antimicrobials of this group are therapeutic options for treatment of staphylococcal infections of skin and soft tissue, and are alternatives for individuals with hypersensitivity to penicillin [24]. Thus, both the characterization of the sensitivity profile, and the investigation of microorganisms with MLSBi resistance phenotype, are necessary.

Therefore, this study was aimed to isolate, identify and evaluate the sensitivity of *Staphylococcus* spp isolated in cultures of various sites of surfaces in a neonatal intensive care unit localized at Campina Grande city, and evaluates the phenotypic appearance of resistance in these microorganisms front to erythromycin and clindamycin, correlating the results with the presence of ermA and msrA genes.

2. MATERIALS AND METHODS

2.1 Study Design

This is a cross-sectional study in the sector of the Neonatal Intensive Care Unit (NICU) of a hospital localized at Campina Grande city, Paraíba State, Brazil. Samples were taken from surfaces in the environment: mattress incubators (n = 10), the stethoscope diaphragm (n = 09), door handles (n = 8), taps (n = 4), telephone (n = 1), computer keyboard (n = 1), mouse (n = 1) and monitor (n = 1). These locations were selected because are frequently handled by visitors and / or health professionals in the unit, making possible cross contamination sources with patients. The collection was during August and October 2012, with the knowledge and authorization of the hospital board and the head nursing sector under study.

2.2 Sampling

For sample collection, it was used a sterilized swab moistened with saline solution 0.9%, it was rubbed and rolled in random locations of the surfaces of the materials investigated. After collection, the swabs were placed in test tubes containing 5 mL of 0.9% saline solution and immediately transferred to a cooler and brought to the Clinical Analysis Laboratory of the State University of Paraíba for sample processing within two hours after collection.

2.3 Isolation and Identification

A 50 µl aliquot was removed from 5.0 mL saline solution 0.9% containing the samples to be analyzed, and were seeded by Spread plate method on the surface of the blood agar and

Mannitol Salt agar culture medium, uniform spread with a sterile glass rod (Drigalski spatula). Plates were incubated for 24 - 48 hours at 35°-37°C. The identification was carried out according to the macroscopic aspects (characteristics of the bacterial colony) and microscopic (Gram stain) of colonies isolated in the culture media used. The bacteria in the Gram staining were identified as Gram-positive cocci, catalase test passed through, where the obtained a result catalase positive, were subjected to the latex agglutination assay, based on the search of A protein and clumping factor (Staphclin látex®), deoxyribonuclease search using the DNAse agar and determining the enzyme activity **-** pyrrolidonil arylamidase - PYR.

2.4 Resistotyping and Resistencia Induced to Macrolides

The resistance profile was determined by the disk diffusion method. A bacterial suspension using 0.9% saline solution was taken and normalized to a concentration in the range of 0.5 McFarland (1.5 × 108 CFU / ml). Then, the disks containing ampicillin (10 mg), amoxicillin / clavulanic acid (20 / 10 mg), ampicillin / subactam (10 / 10 mg), cefepime (30 µg), 30 µg ceftazidime, cefuroxime (30 µg), cephalothin (30 µg), imipenem (10 mg), meropenem (10 mg), gentamicin (10 mg), amikacin (30 µg), tobramycin (10 mg), azithromycin (15 µg), erythromycin (15 µg), tetracycline (30 µg), cirpofloxacina (5 µg), nitrofurantoin (300 µg), clindamycin (2 mg), trimethoprim/ sulfamethoxazole (1.25 / 23.75 µg), trimethoprim (5 µg), chloramphenicol (30 µg), rifampicin (5 µg), linezolid (30 µg), penicillin G (10 mg), oxacillin (1 mg) and cefoxitin (30 µg) (Cecon Ltda. Sao Paulo, Brazil) were placed on plates containing Mueller Hinton Agar (Difco, Sparks, MD, USA) and then incubated at 37°C for 24 to 48 hours. Samples with phenotypic profile of sensitivity to clindamycin and erythromycin resistance exhibited by the agar diffusion test were submitted to the test "D" or the double disk diffusion test. The test "D" was performed according to CLSI methodology - M100-S22 (2012).

3. RESULTS AND DISCUSSION

After processing and analysis of samples, were isolated 36 strains of *Staphylococcus* spp., of which 31 were identified as *Staphylococcus aureus* and 5 identified as coagulase-negative *staphylococcus* (CoNS). According to the literature, a high contamination in the environment can be a mirror of a bad adherence to hygiene measures, both hands as well as the environment [25]. Research on the occurrence of multiresistant bacteria in an intensive care unit found that after identification, 55.7% of the identified bacterial strains were *Staphylococcus* spp., corroborating with the 59.02% found in this work [9].

The cultures of the areas analyzed the presence of *Staphylococcus* spp, namely 88.89% of the stethoscopes, 25% of taps, 25% of the handles and 50% of the mattresses. Also isolated the same bacterial genus in single samples analyzed from phone, mouse, keyboard and monitor. It is observed that the stethoscopes (30,5%) had greater relative frequency due to the higher number of strains, followed by telephone (19,4%) and mattresses (16,7%).

The surfaces of stethoscopes are a place of proven contamination, though given due importance [26]. The 88.89% infection rate found in this study correlates with values reported by other authors, which evaluated the bacterial contamination rate of stethoscopes and found the value of 86.8 and 97.9% respectively of contamination of this equipment [27,26]. According to a previous study, the mattress is one of the objects that have the most contact time with patients, which may serve as a reservoir for infection-causing microorganisms. The same paper cites a percentage of 72.2% of contaminated mattresses. This result proves higher than the 50% observed in this study [25]. Very touched surfaces such as doorknobs, telephones, computers, support the hypothesis that the more you handle such items, more contaminated they become. So when a professional touches these places, not clinging to the importance of hand hygiene, he/she can spread microorganisms to other locations or patients [28,25].

Of the 36 sample isolates, only 26 were with resistance profile to all antibiotics mentioned above, the remaining needed repeated testing to confirm the results, which was not possible. Thus, of the 26 samples 22 were *Staphylococcus aureus,* and 4 CoNS. Table 1 shows the results of antibiotic resistance of the 22 tested strains of *Staphylococcus aureus* and the 4 strains of CoNS. It was considered possible to observe a resistance of more than 70% to the class of penicillins. Obtained as a resistance percentage of more than 30% to cefoxitin, and therefore, the

result possible to be extended to cephalothin, cefuroxime and oxacillin, where, the first nominated drug, cefoxitin, functions as resistance marker. Strains of *S. aureus* showed a resistance percentage of 31.8% to erythromycin, in contrast to the resistance percentage of 9.0% to azithromycin, antibiotic belonging to the same group, our results may have been extended without the need for testing the two drugs. Comparing the strains of *S. aureus* and CoNS, one realizes that *S. aureus* showed a wider resistance profile than that of CoNS.

Research conducted in Brazil says that more than 70% of the bacterial strains having been isolated, both in community and hospital settings, are resistant to the class of penicillins [29]. This confirms the proportion of 77.3% to penicillin G and ampicillin. Such resistance is already expected, due to the fact that these antibiotics have their widespread use in the treatment of infections, by virtue of which, at present, the use of these chemotherapeutics is limited [30]. Such resistance had been conferred by the action of penicillinase enzyme, soon after the appearance of penicillins, appear already *staphylococci* capable of producing such an enzyme [31]. The constitutive resistance to oxacillin is determined by the presence of the mecA gene, which is located on a chromosome [32]. In a previous study investigating the prevalence of *Staphylococcus aureus* resistant to methicillin in an ICU, the value of these resistant microorganisms was 60.4%, which differs from the value found in this work that was 36.4% [5].

After analyzing the results of the D test, it was observed that of the 07 bacterial strains that had erythromycin resistance, 04 strains were resistant to erythromycin and sensitive to clindamycin with negative induction test, which is interpreted as a mechanism of resistance for efflux (MSB). A strain showed resistance to erythromycin and clindamycin sensitivity to false positive induction test, which is interpreted as an amendment to ribosomal inductive resistance mechanism (MLSBi) and 02 strains were resistant to both erythromycin and clindamycin, and interpreted as modifying ribosomal with constitutive type resistance mechanism (MLSBc). Table 2 shows the results of resistance testing after induction.

The interpretive criteria for erythromycin predicting the same outcome for clarithromycin and azithromycin, may be used for any of the three tested antibiotics [33]. However, in this study, the results between azithromycin and erythromycin proved to be discordant with 9.0 and 31.8% respectively, this fact may lead to treatment failure if these samples are isolated from infectious processes and suggest a further study. A previous study resistotyping community strains of *S. aureus* isolated in João Pessoa city have found partial resistance to 14- and 15-member macrolides [34].

The macrolide resistance can be determined by mrsA gene, mediating an efflux mechanism and conferring resistance to macrolides and type B streptogramins, however, not conferring resistance to lincosamides (clindamycin and clarithromycin), or can occur by a change in ribosomes, which affects the activity of macrolides, lincosamides and type B streptogramins [35]. The resistance phenotype most frequently found was MSB with 18.2% of *S.* aureus strains isolated. This result was also consistent with the frequency found by previous studies, where the MSB phenotype was the most present, differing only in the incidence value, since they showed a rate of 7.2% of *S.* aureus strains with type MSB resistance, and our study found a value of more than twice [36].

In this study the frequency of the constitutive resistance to clindamycin (MLSBc) was 28.6% among strains of *S. aureus* that showed erythromycin resistance. The resistance rate found in this research was close to the value found in the literature, eg, a survey which assessed colonization by *Staphylococcus* spp. on surfaces of medical articles, and nostrils, and hands of professionals, finding a value of 20.4% of strains resistant to erythromycin with type MLSBc profile [37]. The MLSBi samples exhibit a high rate of spontaneous mutations for constitutive resistance which can hinder treatment and aggravate the problem of infections caused by *S. aureus* in hospitals and in the community setting [38]. This study showed that the number of strains with MLSBi type resistance was lower compared to the one found for MLSBc, with a value of 14.3% among *S. aureus* strains resistant to erythromycin, i.e. 4.54% of *S. aureus* isolates in this case. This value was close to the frequency of 5.2% obtained in previous studies [36].

Table 1. Resistance percentage of strains of *S. aureus* and coagulase-negative staphylococcus

Class	Antibiotic	% Resistance to *Staphylococcus aureus* (n=22)	% Resistance to coagulase-negative *Staphylococcus* (n=04)
	Penicillin G	77,3	100
Penicillins	Ampicillin	77,3	100
	Oxacillin	36,4	50
Penicillin + Inhibitor	Amoxicillin/Ac. Clavul.	63,6	75
β-lactam	Ampicillin/ Sulbactam	0,0	0,0
	Cephalothin	36,4	0,0
Cephalosporins	Cefoxitin	36,4	50
	Cefuroxime	36,4	50
Carbapenems	Imipenem	4,5	0,0
	Meropenem	9,0	0,0
	Gentamicin	18,2	0,0
Aminoglycosides	Amikacin	18,2	0,0
	Tobramycin	13,6	0,0
Macrolides	Azithromycin	9,0	0,0
	Erythromycin	31,8	0,0
Tetracyclines	Tetracycline	0,0	0,0
Fluoroquinolones	Ciprofloxacin	13,6	0,0
Nitrofurans	Nitrofurantoin	31,8	75
Lincosamides	Clindamycin	13,6	0,0
Folate pathway	Sulfamet./Trimeth.	72,7	75
inhibitors	Trimethoprim	72,7	75
Chloramphenicol	Chloramphenicol	0,0	0,0
Ansamycins	Rifampicin	54,5	75
Oxazolidinones	Linezolid	0,0	0,0

Table 2. Values of resistance phenotypes observed after induction test

Erythromycin	Clindamycin	Induction test	Resistance phenotype	Determinant gene	Percentage resistance (%)
R	S	-	MSB	msrA	57,1
R	S	+	MLSBi	erm	14,3
R	R	-	MLSBc	erm	28,6

4. CONCLUSION

The results of the present study demonstrated that the hospital environment of a neonatal intensive care unit may be contaminated with multidrug-resistant bacteria. The resistotyping of isolated strains for inducible and constitutive resistance can be considered a test of substantial importance not only as an epidemiologic marker in view of analyzing possible dissemination of hospital strains, but with respect to adequate, and precise determination of the antibiotic treatment of neonates.

CONSENT

All the authors declare that the required consent was obtained for this study.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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