

1 Original Research Article
2 **Induced and Constitutive Clindamycin**
3 **Resistance in *Staphylococcus* spp. Strains**
4 **Isolated from a Neonatal Intensive Care Unit**

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10 **ABSTRACT**
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Aims: The 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 is the Neonatal Intensive Care Unit (NICU), home of newborns with low 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 immature. Thus, the aim of this study was to evaluate the phenotypic appearance of resistance of the *Staphylococcus* spp. compared to erythromycin and clindamycin, in 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 in the NICU. The samples were identified and strains of *Staphylococcus* spp. were subjected to sensitivity, and to verify erythromycin-induced resistance, it was used the D-test, following the CLSI standards-M100-S22 (2012).

Results: Bacterial strains from all surfaces analyzed were isolated, 59.02% of isolated bacteria belong to the genus *Staphylococcus* spp., representing 36 bacterial strains, of which 31 were species *Staphylococcus aureus* and 5 were coagulase-negative staphylococci (CoNS). There was a fee more than 70% resistance to the group of penicillins and more than 30% 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 of inducible and constitutive resistance may be considered a test of substantial (not paramount) 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.

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13 **Keywords:** *Staphylococcus* spp, contaminated surfaces, bacterial resistance, MLSB.
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16 **1. INTRODUCTION**
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18 In recent decades, with advances in staphylococcus classification and the development of
19 new gender identification techniques, species and subspecies, there have allowed
20 researchers and clinicians to become more aware of the wide variety of coagulase-negative
21 staphylococcus (CNS), the which were previously described as non-pathogenic, are now
22 recognized as opportunistic microorganisms that prevail in many different situations to
23 produce serious infections [1]. The transmission of these micro-organisms can occur by

24 direct contact, where health professionals to come into contact with patients or objects
25 colonized, can serve transmitters of organisms to other patients. Not forgetting the
26 environmental and airway transmissions, which are uncommon in certain cases, but in some
27 circumstances may occur [2]).

28 So even though the exact function in the inanimate environment plays staphylococcus
29 transmission is not yet determined, it can act a reservoir for microorganisms, which in turn
30 can contaminate a range of hospital equipment and survive for long periods of time [3,4,5].
31 One of these environments it is the neonatal ICU, home newborns with low birth weight, and
32 immunologically immature that requires invasive procedures for administration of nutritional
33 and medicinal substances [1]. Thus, due to the immaturity of the immune system of the
34 newborn and the use of broad spectrum antibiotics, a species commonly found in the air can
35 become pathogens, making the units of pediatric intensive care and neonatal critical areas
36 [6,7,8].

37 In addition to that, a growing number of micro-organisms develop resistance to drugs used
38 to treat infections, as they are also impervious to new drugs [9]. Among them, has been
39 isolated in recent years coagulase negative staphylococcus (CNS) resistant to multiple
40 antimicrobials, which has leveraged the interest in studying their susceptibility commercial
41 drugs [10]. Given that studies have shown multiresistant microorganisms to antimicrobial
42 surfaces of beds and equipment after cleaning and disinfection inappropriate, interest in this
43 monitoring is only going to increase further [11,12].

44 Recently, the detection of macrolide-lincosamide-streptogramin resistance (MLS) between
45 staphylococcus has attracted the attention of clinical laboratories. Three mechanisms have
46 been reported to the MLS B resistance to antibiotics: modification of the active site, efflux of
47 antibiotics and modifying drugs [13]. MLSB carries the *erm* gene (erythromycin ribosome
48 methylase) encoding rRNA methylase which modifies the binding site of the antimicrobial
49 agent by mutation or methylation of 23S rRNA, resulting in resistance to macrolides,
50 lincosamides, and streptogramin B. There are four main classes *erm* gene [*erm* (A), *erm* (B),
51 *erm* (C) and *erm* (TR)], and the types *erm* (A) and *erm* (C) often responsible for resistance in
52 staphylococci MLSB [14,13].

53 The strains that express the MLSB phenotype can be classified as induced (MLSB_i) or
54 constitutive (MLSB_c). When an MLSB inducible strain is exposed to an inducer (such as a
55 low-erythromycin), increases the level of expression of rRNA methylase resulting in an
56 increased resistance MLS class of antimicrobials (such as clindamycin). Even if the strains
57 with an inducible *erm* gene are resistant to inducers and remain susceptible to macrolides do
58 not inductors and lincosamides, in general, different inducible MLSB resistance patterns can
59 be observed according to the type of *erm* gene or its expression level. In this case, it is
60 necessary to perform disk-approximation induction test (D-test). In constitutive resistance
61 methylase mRNA produced is active even in inducer default, which gives a high level of
62 cross-resistance to MLS B group drugs [13,15,16].

63 The *msrA* gene is responsible for efflux mechanism for staphylococci, activated after a
64 macrolide exposure, where pumps 14 or 15 macrolides members and streptogramin type B
65 [17, 13]. Therefore, bacteria are erythromycin resistant but remained clindamycin
66 susceptible, because it isn't an inducer or a substrate for the pump. When the strains,
67 previously sensitive to streptogramin B, become resistant after a macrolide exposure, they
68 are classified as efflux phenotype type M or MSB and can be differentiated from MLSB_i
69 phenotype by double-disk testing, in this case no interactions between erythromycin and
70 clindamycin (no D-shaped zone). Resistance to macrolides mediated efflux is common

among coagulase-negative staphylococci (CNS) 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 [19,20,21,22]. 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 is very serious, as the antimicrobial of this group are therapeutic options for treatment of staphylococcal infections in skin and soft tissue, and are alternatives to individuals with hypersensitivity to penicillin [24]. Thus, the characterization of the sensitivity profile and the investigation of microorganisms with MLSBi resistance phenotype are necessary.

Therefore, this study 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 evaluate the phenotypic appearance of resistance in these microorganisms front of erythromycin and clindamycin, correlating the results with the presence of *ermA* and *msrA* genes.

2. MATERIAL AND METHODS

2.1 Study Design

This is a cross-experimental 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 they are often manipulated, acting as potential sources of contamination by health professionals exposure. 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 until two hours after collection.

2.3 Isolation and Identification

A 50 µl aliquot was removed of 5.0 mL saline solution 0.9% containing the samples to be analyzed, and were seeded at spreading plate method on the surface of the blood agar and Mannitol Salt agar culture medium made uniform spread with a sterile glass rod (Drigalski spatula). Plates were incubated in for 24 - 48 hours at 35⁰-37⁰C. 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 A protein and clumping factor (Staphclin látex®), deoxyribonuclease

118 search using the DNase agar and determining the enzyme activity pyrrolidonil arylamidase -
119 PYR.

120 **2.4 Resistotyping and Resistencia Induced to Macrolides**

121 The resistance profile was determined by the diffusion with disks method. A bacterial
122 suspension using 0.9% saline solution was taken and normalized to a concentration in the
123 range of 0.5 McFarland (1.5×10^8 CFU / ml). Then, the disk containing ampicillin (10mg),
124 amoxicillin / clavulanic acid (20 / 10mg), ampicillin / subactam (10 / 10mg), cefepime (30µg),
125 30µg ceftazidime, cefuroxime (30µg), cephalothin (30µg), imipenem (10mg), meropenem
126 (10mg), gentamicin (10mg), amikacin (30µg), tobramycin (10mg), azithromycin (15µg),
127 erythromycin (15µg), tetracycline (30µg), cirpofloxacin (5µg), nitrofurantoin (300µg),
128 clindamycin (2mg), trimethoprim/sulfamethoxazole (1.25 / 23.75µg), trimethoprim (5µg),
129 chloramphenicol (30µg), rifampicin (5µg), linezolid (30µg), penicillin G (10mg), oxacillin
130 (1mg) and cefoxitin (30µg) (Cecon Ltda. Sao Paulo, Brazil) was placed on plates containing
131 Mueller Hinton Agar (Difco, Sparks, MD, USA) and then incubated at 37 °C for 24 to 48
132 hours. Samples with a phenotypic profile of sensitivity clindamycin and erythromycin
133 resistance exhibited by the agar diffusion test were submitted to the test "D" or the double
134 disk diffusion test. The test "D" was performed according to CLSI methodology - M100-S22
135 (2012).

136 **3. RESULTS AND DISCUSSION**

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

145
146 The cultures of the areas analyzed the presence of *Staphylococcus* spp. 88.89% of the
147 stethoscope 25% of taps, 25% of the handles and 50% of the mattresses. Also isolated the
148 same bacterial genus in single samples analyzed phone, mouse, keyboard and monitor. It is
149 observed that the stethoscopes (30,5%) had greater relevance in the number of strains,
150 followed by telephone (19,4%) and mattress (16,7%).

151 The surfaces of stethoscopes are a place of proven contamination, but without given due
152 importance. [26] The 88.89% infection rate found in this study correlates with values
153 reported by other authors, which evaluated the bacterial contamination rate of stethoscopes
154 and found the value of 86.8 and 97.9% respectively of contamination of equipment [27,26].
155 According to a previous study, the mattress is one of the objects that have the most contact
156 time with patients, which may serve as a reservoir for infection-causing microorganisms. The
157 same paper cites a percentage of 72.2% of contaminated mattresses. This result proves
158 higher than the 50% observed in this study [25]. Very touched surfaces such as doorknobs,
159 telephones, computers, support the hypothesis that the more you handle such fomites, more
160 contaminated they become. So when professional touch these places, not cling the
161 importance of hand hygiene and can spread microorganisms to other locations or patients
162 [28,25].

163 Of the 36 isolates sampled, only 26 were resistance profile to all antibiotics mentioned
164 above, the remaining need repeated to confirm the results, which was not possible. Thus,
165 the 26 samples 22 were *Staphylococcus aureus* and **4 CoNS**. In Table 1 shows the results

of antibiotic resistance in the 22 tested strains of *Staphylococcus aureus* and 4 strains of the CoNS. It is possible to observe a resistance of more than 70% to the class of penicillins. Obtained as a resistance percentage of more than 30% cefoxitin, therefore, this result was extended to cephalothin, cefuroxime and oxacillin, where, in the latter drug, cefoxitin functions as resistance marker. Strains of *S. aureus* showed a resistance percentage of 31.8% of erythromycin, in contrast to the percentage strength of the 9.0% azithromycin antibiotic belonging to the same group and may have extended our results without the need for test the two drugs. Comparing the strains of *S. aureus* and CoNS, one realizes that *S. aureus* showed a wider resistance profile than CoNS.

Research conducted in Brazil says that more than 70% of the bacterial strains are 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, which, at present, the use of these chemotherapy is limited [30]. Such resistance is 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 chromosome [32]. In a previous study investigated 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].

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

Class	Antibiotic	% resistance to <i>Staphylococcus aureus</i> (n=22)	% resistance to coagulase-negative staphylococci (n=04)
Penicillins	Penicillin G	77,3	100
	Ampicillin	77,3	100
	Oxacillin	36,4	50
Penicillin + Inhibitor β-lactam	Amoxicillin/Ac.	63,6	75
	Clavul.		
	Ampicillin/ Sulbactam	0,0	0,0
Cephalosporins	Cephalothin	36,4	0,0
	Cefoxitin	36,4	50
	Cefuroxime	36,4	50
Carbapenems	Imipenem	4,5	0,0
	Meropenem	9,0	0,0
Aminoglycosides	Gentamicin	18,2	0,0
	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
Inhibitors pathway	Sulfamet./Trimeth.	72,7	75
folate	Trimethoprim	72,7	75
Chloramphenicol	Chloramphenicol	0,0	0,0
Ansamycins	Rifampicin	54,5	75
Oxazolidinones	Linezulida	0,0	0,0

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

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

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

213 In this study the frequency of constitutive resistance to clindamycin (MLSBc) was 28.6%
214 among strains of *S. aureus* that showed erythromycin resistance. The resistance rate found
215 in this research was close to the value found in the literature, which was assessed
216 colonization by *Staphylococcus* spp. on the surfaces of medical articles and nostrils and
217 hands of professionals, finding the value of 20.4% of strains resistant to erythromycin with
218 type MLSBc profile [37]. The MLSBi samples exhibit a high rate of spontaneous mutations
219 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 values 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].

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Table 2. Values of resistance phenotypes observed after induction test

<i>Erythromycin</i>	<i>Clindamycin</i>	<i>Induction</i>	<i>Resistance</i>	<i>Determinant</i>	<i>Percentage</i>
		<i>Test</i>	<i>Phenotype</i>	<i>gene</i>	<i>Resistance (%)</i>
R	S	-	MSB	<i>msrA</i>	57,1
R	S	+	MLSBi	<i>erm</i>	14,3
R	R	-	MLSBc	<i>erm</i>	28,6

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229

4. CONCLUSION

230

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 of 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.

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COMPETING INTERESTS

245

Authors have declared that no competing interests exist

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CONSENT (WHERE EVER APPLICABLE)

249

All the authors declare that no consent was obtained for this study.

251

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

252

Not applicable.

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