

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 **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 is the Neonatal Intensive Care Unit (NICU), **for the initial post partum period serving as a home for** ~~of~~ newborns of ~~with~~ 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 immature 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, **originating from** ~~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 at the NICU. The samples were identified ~~y~~ and strains of *Staphylococcus* spp. were subjected to sensitivity, and to verify erythromycin-induced resistance, ~~it was used~~ 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 ~~se~~ ~~bacteria~~ belong to the genus *Staphylococcus* spp., representing 36 bacterial strains, of which 31 were **subspecies** *Staphylococcus aureus* and 5 were coagulase-negative **staphylococci (CoNS)**. There was **found** ~~ere a fee~~ 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 ~~(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.

12 **Keywords:** *Staphylococcus* spp, contaminated surfaces, bacterial resistance, MLSB.

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16 1. INTRODUCTION

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18 In recent decades, ~~the with~~ advances in staphylococcus classification and the development

19 of new gender identification techniques, **of** species and subspecies, ~~there~~ have allowed

20 researchers and clinicians **to** become more aware of the wide variety of coagulase-negative

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

28 So even though the exact function in the inanimate environment that plays staphylococcus
29 transmission is not yet determined, it can act as 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 is the neonatal ICU, home newborns of with low birth
32 weight, and immunologically immature, that require invasive procedures for administration of
33 nutritional and medicinal substances [1]. Thus, due to the immaturity of the immune system
34 of the newborn and the use of broad spectrum antibiotics, species commonly found in the air
35 can become pathogens, making the units of pediatric and neonatal intensive care and
36 neonatal critical areas [6,7,8].

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

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

53 The strains that express the MLS_B phenotype can be classified as induced (MLS_{Bi}) or
54 constitutive (MLS_{Bc}). When an MLS_B 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 to MLS class of antimicrobials (such as clindamycin). Even if the
57 strains with an inducible *erm* gene are resistant to inducers and remain susceptible to
58 macrolides do not inducers and lincosamides, in general, different inducible MLS_B
59 resistance patterns can be observed according to the type of *erm* gene or its expression
60 level. In this case, it is necessary to perform disk-approximation induction test (D-test). In
61 constitutive resistance methylase mRNA produced is active even in inducer default, which
62 gives a high level of 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, which gene pumps 14 or 15 macrolides members and
65 streptogramin type B [17, 13]. Therefore, bacteria are erythromycin resistant but remained
66 clindamycin susceptible, because it isn't an inducer or a substrate for the pump. When the
67 strains, previously sensitive to streptogramin B, become resistant after a macrolide
68 exposure, they are classified as efflux phenotype type M or MSB and can be differentiated
69 from MLS_{Bi} phenotype by double-disk testing, in this case no interactions between
70 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 [19,20,21,22]. 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 presents a is very serious problem, as the antimicrobials of this group are therapeutic options for treatment of staphylococcal infections of in skin and soft tissue, and are alternatives for to 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. MATERIAL AND METHODS

2.1 Study Design

This is a cross-sectional 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 within until two hours after collection.

2.3 Isolation and Identification

A 50 µl aliquot was removed from of 5.0 mL saline solution 0.9% containing the samples to be analyzed, and were seeded by ~~Set~~ spreading plate method on the surface of the blood agar and Mannitol Salt agar culture medium, ~~made~~ uniform spread ~~made~~ with a sterile glass rod (Drigalski spatula). Plates were incubated in-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

119 látex®), deoxyribonuclease search using the DNase agar and determining the enzyme
120 activity - pyrrolidonil arylamidase - PYR.

121 2.4 Resistotyping and Resistencia Induced to Macrolides

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

137 3. RESULTS AND DISCUSSION

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

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

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

165 Of the 36 sample isolates, only 26 were **with** resistance profile to all antibiotics mentioned
166 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. In Table 1 shows the results of antibiotic resistance of in the 22 tested strains of *Staphylococcus aureus* and the 4 strains of the CoNS. It was considered is 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 this result possible to be was extended to cephalothin, cefuroxime and oxacillin, where, in the first nominated latter 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 strength of the 9.0% to azithromycin, antibiotic belonging to the same group, our results and may have been extended our results 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 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, by virtue of which, at present, the use of these chemotherapeutics is limited [30]. Such resistance had been 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 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].

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		36,4	0,0
	Cephalothin		
	Cefoxitin	36,4	50

	Cefuroxime	36,4	50
Carbapenems	Imipenem	4,5	0,0
	Meropenem	9,0	0,0
		18,2	0,0
Aminoglycosides	Gentamicin		
	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 pathway inhibitors			
	Trimethoprim	72,7	75
Chloramphenicol	Chloramphenicol	0,0	0,0
Ansamycins	Rifampicin	54,5	75
Oxazolidinones	Linezolid	0,0	0,0

193

194 After analyzing the results of the **D** test Φ , it was observed that **of** the 07 bacterial strains
195 that had erythromycin resistance, 04 strains were resistant to erythromycin and sensitive to
196 clindamycin ~~and~~ **with** negative induction test, which is interpreted as a mechanism of
197 resistance for efflux (MSB). A strain showed resistance to erythromycin and clindamycin
198 sensitivity to false positive induction test, which is interpreted as an amendment to ribosomal
199 inductive resistance mechanism (MLS_{Bi}) and 02 strains were resistant to both erythromycin
200 **and** as clindamycin, and interpreted as modifying ribosomal with constitutive type resistance
201 mechanism (MLS_{Bc}). Table 2 shows the results of resistance testing after induction.

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

209 The macrolide resistance can be determined by mrsA gene, which ~~mediates~~ **ing**es an efflux
210 mechanism and **confer**ing resistance to macrolides and type B streptogramins, however,
211 ~~does not~~ **confer**ing 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 was 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 as our study found a value of more than twice [36].

In this study the frequency of the for 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 was assessed colonization by *Staphylococcus* spp. on surfaces of medical articles, and nostrils, and hands of professionals, finding a the 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 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].

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

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.

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250 **COMPETING INTERESTS**

251

252 Authors have declared that no competing interests exist

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254 **CONSENT (WHERE EVER APPLICABLE)**

255

256 All the authors declare that **the required** ~~no~~ consent was obtained for this study.

257 **ETHICAL APPROVAL (WHERE EVER APPLICABLE)**

258

259 Not applicable.

260

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