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IN VITRO ADHERENCE OF COAGULASE-NEGATIVE *Staphylococcus* STRAINS IN CATHETERS OF DIFFERENT MATERIAL COMPOSITION

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Abstract- Coagulase-negative staphylococci (CoNS) are a major cause of infections associated with indwelling medical devices. Strains of CoNS produce slime and form biofilms on polymer surfaces, which is associated with their pathogenicity. This study evaluated the correlation between adherence of CoNS and composition of vascular catheters composed of polyurethane and silicone, by the phenotypic analysis of slime production, the presence of *ica*AD and atl*E* genes, and by comparing the relationship between oxacillin-resistant (MRS) and oxacillin-susceptible (MSS) CoNS and biofilm formation. All MRS isolates included in this study demonstrated the ability to form biofilms but on the other hand, only 50% of MSS were capable of forming biofilms. All CoNS demonstrated degrees of adherence using different materials (polyurethane and silicone). The results of this study suggest a strong correlation between slime production and the level of resistance to oxacillin, since 100% of MSS strains did not produce slime and 90% of MRS strains were slime producers. Two particular hospital samples were notable due to their high MICs (≥ 128 mg/mL), with both demonstrating the ability to produce slime and the presence of the icaAD gene. These findings emphasize the choice of material of the catheter and the required care to be taken at the time of insertion and maintenance to avoid contamination with CoNS. Our results highlight that continuous training for correct handling measures is necessary to prevent intravascular as well as peripheral catheter infections on the hospital ward.

Keywords- Coagulase-negative Staphylococci, Vascular catheter, Bacterial adherence

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Introduction

Coagulase-negative staphylococci (CoNS) are the most frequently isolated bacteria in clinical microbiology laboratories. They colonize the human skin, throat, nose and intestine and represent a major part of the normal bacterial flora of healthy individuals [1]. However, in the last years, CoNS have been recognized as relevant opportunistic pathogens [2-4].

Most of all CoNS related infections are hospital-acquired. Since many nosocomial isolates are capable of forming biofilms on indwelling devices, such as intravascular catheters, the eradication of CoNS is difficult and the removal of the catheter or device may be the only effective intervention. They are also the most common contaminants of blood cultures [5]. Bloodstream infections (BSI) caused by these organisms are frequently related to the use of medical devices. They account for significant morbidity and mortality, especially in immunocompromised individuals and neonates [6]. Mortality has been associated with severity and the inadequacy of empirical treatment but the relevance of the latter is not clearly recognized. Furthermore, pathogenic markers associated with biofilm formation and antimicrobial resistance, among others, have been described for CoNS [7].

Biofilms are bacterial agglomerations with increased resistance to antibiotic agents and mechanisms of host defense [8, 9]. Biofilm production have been described as a two-step process with an initial attachment process mediated by a number of factors like cell wall-anchored surface proteins (i.e., Fbe and Bhp) and the cell wall lytic enzyme autolysin E (AtlE) [10]. Fbe and AtlE have adhesive properties and bind to the host factors fibrinogen and vitronectin, respectively [11], while it has been suggested that Bhp contributes to primary attachment as well as intercellular adhesion [12]. The accumulative phase is linked to the production of polysaccharide intercellular adhesion (PIA), which is synthesized by *ica*ADBC-encoded proteins [13,14].

Furthermore, it has been shown that CoNS may be transmitted by hospitalized patients and by healthcare workers [15]. Isolates involved in transmission are often characterized by their resistance to many commonly used antimicrobial drugs, including methicillin [16].

Methicillin resistance in CoNS is mainly due to the expression of an altered penicillin-binding protein 2a (PBP2a), encoded by *mec*A [2]. Sometimes the control of these multi-resistant isolates is overlooked [17].

The aims of this study were to evaluate the adherence of CoNS to vascular catheters composed of two different materials (silicone and polyurethane); to determine their slime production and to detect the presence of specific genes associated with biofilm production in oxacillin-resistant (MRS) and oxacillin-susceptible CoNS (MSS) isolates.

Materials and Methods

Bacterial Isolates

Twenty CoNS isolates were included in this study and all samples used were part of a culture collection from the Department of Microbiology, Immunology and Parasitology. Ten isolates were obtained from hospital environment, such as, thermometers, stethoscopes and sphygmomanometers, in use at Pedro Ernesto University Hospital-HUPE (Rio de Janeiro, Brazil) during the period from January to December 2006 and all these isolates were resistant to oxacillin (MIC ≥ 32 µg/mL (MRS). In the same period, ten strains were obtained in the community, collected from the forearms of teenagers living in the western area of Rio de Janeiro city. All the latter isolates were sensitive to oxacillin with a MIC value of 0.25 µg/mL (MSS). The MIC for oxacillin was previously determined by a microdilution method according to CLSI 2009.

Identification

CoNS bacterial species were identified by a panel of phenotypic tests as previously described [18].

Catheters used for Adhesion Tests

Inserted central venous catheters (PICC) composed of silicone or polyurethane, respectively, were selected, both with 1.9 French (Biomedical, Rio de Janeiro, Brazil).

Quantitative Adherence Assay

The adherence assay was conducted according to [19] with some modifications. In brief, isolates were suspended in phosphate buffered saline (PBS) at concentrations ranging from 10^6 to 10^7 organisms per milliliter by visual comparison with a 0.5 McFarland standard. Aliquots of the final suspension were plated to obtain accurate counts of inoculum.

Initial Adherence Studies

From each segment of the sterile catheters, 4 to 5 cm were cut and placed into individual bacterial suspensions for 2 mins. These segments were then removed from these suspensions with sterile forceps, shaken to remove excess fluid from the surface and inner lumen, and immersed in individual large sterile tubes containing 1 mL of sterile water. All the tubes were then vortexed for 1 min.

Quantitative Culture

Liquid, previously obtained, was diluted 1:10 in sterile water and 0.1 mL was plated onto Muller-Hinton Agar (MHA) (Becton, Dickinson and Company; Sparks, USA) [19]. The plates were incubated aerobically for 18-24 hrs At 35°C ± 2 °C. The procedure was performed in triplicate. The average growth of bacterial cultures, obtained by CFU count was calculated thus obtaining the rate of adhesion.

Slime Assay

The slime producing isolates of MRS and MSS were detected using Congo Red Agar (CRA) (E. Merck, Darmstadt, Germany), following the method as described by [20]. The plates were incubated for 24 hrs at 35°C ± 2°C and subsequently overnight at room temperature. The experiment was performed in triplicate. The slime-positive strains were identified as black colonies and slime-negative strains as red colonies. *S. aureus* ATCC 35984 and *S. epidermidis* ATCC 12228 were used as positive and negative controls, respectively.

Biofilm Phenotypic Assay

Biofilm production was determined by microtiter plate assay (PlastBio, São Paulo, Brazil), and optical density results were scored and interpreted as described elsewhere [21].

CoNS strains were cultivated overnight in Tryptic soy broth (TSB) (Oxoid, Basingstone, England). The culture was adjusted to 0.5 McFarland and diluted 1:200 in TSB. From the previous dilution, 200 µL were transferred to a 96-well polystyrene microtiter plates (Greiner, Frickenhausen, Germany). After incubation for 24 hrs at 35°C ± 2°C, the wells were gently washed twice with 200 µL of sterile PBS and the plates were air dried under static conditions. The remaining surface-adsorbed cells of the individual wells were stained with 0.1% crystal violet (Serva) for 30 sec. Absorbance was measured with a Micro-ELISA Autoreader (Titertek Multiscan) (Biorad, Japan) at 490 nm. A well containing sterile TSB but lacking bacterial cells was used as a negative control and the value for this well was subtracted from the experimental readings. As a positive control we used the strain *S. aureus* ATCC 35984. Each assay was performed in triplicate.

The samples were classified into four categories based on optical density (OD): non-producer - OD obtained less than the OD of the negative control (ODc); weak producer - ODc < OD ≤ (2xODc), moderate producer (2x) ODc < OD ≤ (4xODc) and heavy producer - OD > (4xODc). All assays were performed in triplicate and the results considered were the mean of three assays.

Detection of the *ica*AD and atl*E* genes

Chromosomal DNA was extracted by thermal lysis. PCR for the detection of the *ica*AD gene was performed according to [22] and detection of atl*E* gene according to [22].

Statistical Analysis

As adhesion tests were conducted for two different materials (silicone or polyurethane) and in two different categories of microorganisms (resistant and susceptible to oxacillin), the statistical test used was the Student's t test (Prism® version 3.0) for mean comparison, with a significance level of $p < 0.05$. To compare biofilm production a chi-square test was applied (OpenEpi® version 2), defining the significance level at $p < 0.05$. All assays were also performed in triplicate and the results considered for each strain were the mean of the three assays.

Results and Discussion

Bacterial Identification

The MSS isolates (community isolates) were identified as *S. epidermidis* (n=7), *S. cohnii cohnii* (n=1), *S. saprophyticus* (n=1) and *S. cohnii urealyticum* (n=1). Among MRS isolates, *S. epidermidis* (n=3), *S. xylosus* (n=3), *S. cohnii cohnii* (n=4) and *S. simulans* (n=1) were identified. *S. xylosus* and *S. simulans* appeared exclusively in

the hospital samples.

Quantitative Analysis of Adhesion

Comparing the levels of adherence in the catheters composed from different materials (polyurethane and silicone), CoNS isolates showed significantly higher adherence rates in the polyurethane catheters (t test, p = 0.0040) [Fig-1](A).

The adherence to polyurethane catheters ranged from 1.3 x 10^4 to 2.34 x 10^6 CFU, with an average of 6.04 x 10^5 CFU and SD 34.68. The adherence to silicone catheters ranged from 2.6 x 10^4 to 1.14 x 10^6 CFU, with an average of 1.93 x 10^5 CFU and SD 34.71. It should be highlighted here the fact that both materials showed adherence even after cleaning. These results are of major importance to the clinicians at the moment of choice when purchasing equipment such as catheters, since the amount of microorganisms attached is directly correlated to the potential development of infection: the greater the number of microorganisms attached the greater the chance of infection [23].

The rate of adherence levels between MRS and MSS were similar, with no statistically significant differences (t test, p = 0.9155) [Fig-1] (B).

Samples that demonstrated a higher rate of adherence independent of catheter material were from the community (MIC 0.25 µg/mL) and were identified as *S. epidermidis*. One hospital strain also identified as *S. epidermidis* had greater rates of adherence to silicone when compared to polyurethane (1.14 x 10^6 and 5.66 x 10^5 CFU, respectively).

In a study by [4], the mean initial bacterial adherence of *S.epidermidis* in a group of polyurethane catheters was 2.04 x 10^5 CFU per catheter fragment at day 0. The number of microorganisms adhering to the untreated polyurethane catheter fragments varied between 1.41 x 10^5 to 2.09 x 10^6 CFU per fragment at various time durations. This is in agreement with the data found in the present study.

Slime Production

The majority of MRS strains were slime producers (90%) and all MSS were non-slime-producing strains. In 1972, Bayston and Penny published the first paper relating the production of the mucoid material by potentially pathogenic strains of *S. epidermidis* as a possible factor in the colonization of prosthetic materials installed for medical-surgical intervention in hospitalized patients. This mucoid material of polysaccharide composition has been termed slime [24]. Many strains of CoNS form slime, being the production of which regarded as a virulence factor. The results of this study suggest a strong correlation between slime production and the level of resistance to oxacillin, since 100% of MSS strains did not produce slime and 90% of MRS strains were slime producers. These results are consistent with the ones found in [25], study in which it was demonstrated that most of the slime-producing strains of coagulase -negative staphylococci were also resistant to oxacillin. Some authors [26] suggested the existence of a possible implication of the *mec*A gene in pathogenicity, since there was a decrease in the transcription of the *mec*A gene in oxacillin-resistant *S. epidermidis*, a non-slime producing phase variant. Furthermore, antimicrobial resistance can also be associated with the slime production as the exopolysaccharide matrix, contained in the slime, may create a physical barrier to the antimicrobial drugs.

Biofilm Production

All MRS (10/10) and 50% (5/10) MSS isolates were biofilm producers (p < 0.02). Among the MSS 60% were moderate producers and 40% were weak producers with all MRS being moderate producers. The adherence of staphylococcus is the first step in biofilm formation [12], the biofilm responsible for a better chance of survival, protecting the organism against the action of antimicrobials, opsonization and phagocytosis.

This data highlights the importance of care and good hygiene practices when inserting and maintaining catheters so that it may prevent contamination by CoNS, which are mostly oxacillin resistant in the hospital environment, especially the bacterial isolates from the ICU. Additionally, continuing education and training for all healthcare team members are essential to prevent equipment contamination [28].

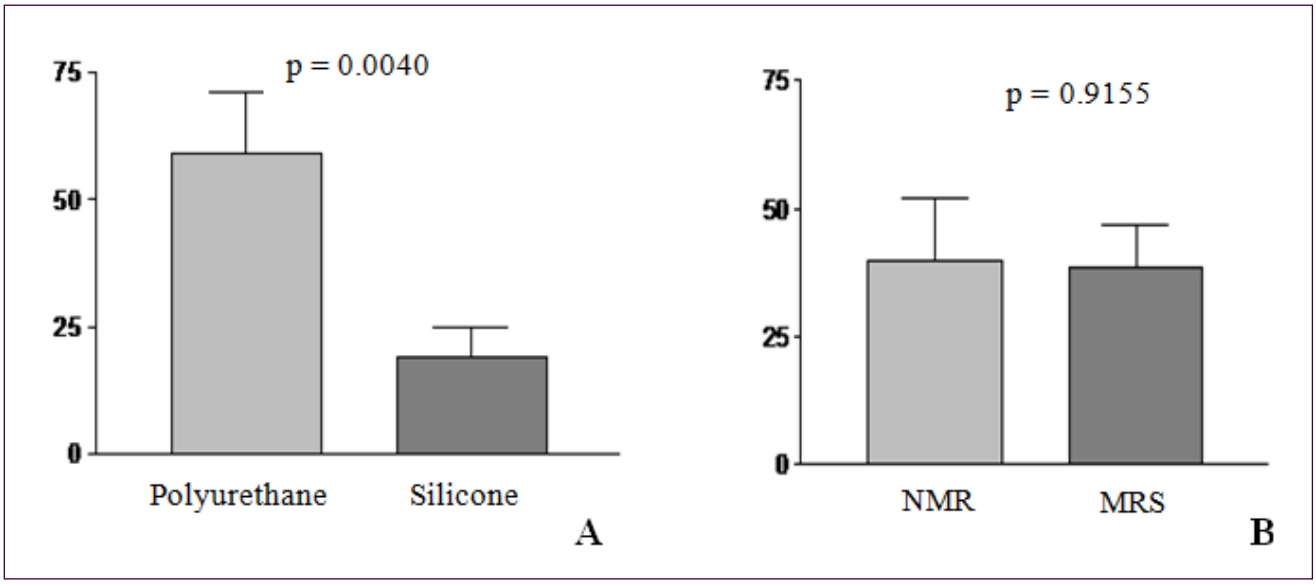

Fig. 1- Histograms representing the distribution of mean adherence rates of CNS: A – Susceptible to oxacillin (MSS) and resistant to oxacillin (MRS); B - Catheters composed of and silicone polyurethane , regardless of resistance phenotype.

PCR of the *ica*AD and atl*E* genes

A large number of *S. epidermidis* strains and other CoNS involved in central venous catheter infections produce extracellular slime in which cells are embedded and covered [29]. In particular, polysaccharide intercellular adhesion (PIA) is important in the pathogenesis of intravascular catheter-associated infection, and has an essential role in cellular aggregation and biofilm formation [30].

Detection of biofilm genes showed that 60% (6/10) of MRS and 10% (1/10) of MSS isolates harbored the *ica*AD gene. The slime may be related to actual biofilm production since the production of exopolysaccharides is necessary for biofilm formation at this stage [31]. In this study, our findings provide evidence of a relationship between the presence of the *ica*AD gene and the production of slime. Among the 9 slime producing samples (MRS), 6 samples also had the *ica*AD gene and from 10 non-slime producing samples (MSS) only 1 sample presented the *ica*AD gene.

The atl*E* gene was not found in any MSS samples, however, it was found in 10% (1/10) of the MRS samples.

The adherence was highest in catheters composed of polyurethane, in a community strain (4C) identified as *S. epidermidis*. This strain did not produce slime, was susceptible to oxacillin (MIC 0.25 µg/mL) and harbored the *ica*AD gene. These findings reinforce the indication that adherence is a characteristic of CoNS, regardless of its antimicrobial resistance profiles, and that other genes such as, atl*E*, *aap* and *ica*, may be involved in the adhesion of these microorganisms. Another strain identified as *S. epidermidis* (5H) gain our attention because it had higher rate of adherence to the silicone catheters rather than to the polyurethane ones. The 5H had the *ica*AD and atl*E* genes, was slime-producer and resistant to oxacillin (MIC 32 µg/mL) thus may be considered potentially more pathogenic. It should also be considered the expression of various bacterial cell mechanisms by 5H strain and its possible interaction with the biomaterials depending on the environmental conditions and surface characteristics of the silicone and/or the polyurethane materials.

Two particular hospital samples were notable due to their high MICs (≥ 128 mg/mL), with both demonstrating the ability to produce slime and the presence of the *ica*AD gene. One identified as *S. cohnii subsp. cohnii* with MIC of 512 mg/mL for oxacillin and was isolated from a thermometer used in the coronary ICU. This species is usually resistant to oxacillin and is common in opportunistic infections, but can also cause sepsis, especially in patients with cancer [32]. The other strain, identified as *S. xylosus*, showed a MIC of 128 mg/mL for oxacillin and was isolated from a thermometer in the neonatal ICU. There are reports of *S. xylosus* causing infection and sepsis in catheterized patients, especially in immunocompromised ones [33].

Our overall results raises important questions about the pathogenic profile of CoNS, since these microorganisms are colonizers in-healthy skin and mucosa of humans and animals and are considered to be carrying fewer virulence factors when compared to *S. aureus*, for example.

However, these microorganisms are resistant to oxacillin predominantly in the hospital environment [34]. The high levels of oxacillin resistance found among CoNS isolates from the hospital environment must be taken into consideration, since it is not clear, in the literature, if there is a raise of CoNS resistant strains over the sensitive strains. It may be the fact that resistant strains are better adapted to the hospital environment and therefore selected, or that there is transference of resistance genes to the sensitive strains.

This study corroborates the fact that coagulase-negative Staphylococcus (CoNS) has a high capacity of adherence to polymers, regardless of the strains resistance profiles. It also reinforces the idea that adherence is a characteristic of CoNS, regardless of antimicrobial resistance profiles and that other genes such as atlE and aap, as well as *ica*AD may be involved in the adhesion of these microorganisms. These findings emphasize the importance of choosing the catheter's material and the required care to be taken at the time of insertion and maintenance to avoid contamination with CoNS. Our results support the concept that measures, such as continuous training for correct handling, are paramount to prevent intravascular and peripheral catheter infections in hospital wards.

Conflicts of Interest: None declared.

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