Biofilm production by resistant non-fermenting gram-negative bacilli from intensive care units

Produção de biofilme por bacilos Gram-negativos não fermentadores resistentes de unidade de terapia intensiva

Producción de biopelículas por bacilos gramnegativos no resistentes a la fermentación en la unidad de cuidados intensivos

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ABSTRACT

Introduction: The Intensive Care Unit is one of the hospital departments in which the highest number of bacterial isolates multidrug-resistant to antimicrobials prevails. Infections caused by Non-Fermenting Gram-Negative Bacillus (NFGNB) are of great importance and clinical concern, especially when they are related to multidrug-resistant microorganisms. One of the main mechanisms associated with bacterial resistance is the production of biofilm. Objective: To verify the presence, resistance profile as well as bacterial biofilm production of NFGNB isolates from tracheal secretion, blood culture, and surfaces of an Intensive Care Unit. Outline: Samples were collected from February to September 2018. MacConkey Agar, Triple Sugar Iron, and oxidase test were used for bacterial identification. Antimicrobial resistance occurred by the Kirby-Bauer disk diffusion method, and the presence of bacterial biofilm was verified by Quantitative Microtiter Dish Biofilm Formation assay. Results: Of 30 gram-negative isolates, seven were NFGNB. Four of the genus Acinetobacter sp., two of the genus Pseudomonas sp., and one of the genus Burkholderia sp. were identified. The results showed moderately and strongly biofilm producing isolates. Implications: The bacteria found showed high resistance to the antibiotics tested associated with biofilm production. Identifying these pathogens can contribute greatly to more effective hospital antibiotic therapy.

DESCRIPTORS

Biofilms; Drug Resistance, Microbial; Intensive Care Units; Bacteria.

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INTRODUCTION

The Intensive Care Unit (ICU) is one of the places where the isolation of pathogenic microorganisms occurs most frequently since the patients present there are more susceptible to the development of infections, especially by resistant microorganisms, with rates of Healthcare-Associated Infections (HAI) ranging from 18 to 54%. HAI are responsible for increasing mortality and morbidity in addition to psychological causes and social consequences to patients. With increased HAI, cross-contamination emerges as an important factor for the patient’s condition, as it can happen not only from person to person but also between them and the surrounding ICU surfaces.

Studies developed to investigate the most contaminated surfaces inside an ICU reported greater contamination in the right and left bed rails, along with the heart rate monitor shelf, bed height adjusters, infusion pump buttons, among the surfaces near the patient. In general, when evaluating biological samples collected from the ICU for microbiological examinations, tracheal secretion, blood culture, and urine stand out, while other studies have associated a higher prevalence of Non-Fermenting Gram-Negative Bacilli (NFGNB) in tracheal samples. Regarding the blood cultures, considering the sterile physiological property of blood, the finding of microorganisms should be investigated, given the possibility of sepsis. The presence of bacteria in blood cultures suggests a clinical significance because the patient’s bacteremia may develop into septicemia.

Among the main isolated microorganisms in ICU, the NFGNB are often associated with a high mortality rate, and they can be found in tracheal secretion, blood cultures, and surfaces near the patient. These are non-sporulated aerobic bacteria that do not use carbohydrates as a source of energy through fermentation, degrading them by the oxidative pathway. More than 30 genera were classified as pathogenic, and the following stand out: Pseudomonas sp., Acinetobacter sp., Stenotrophomonas sp., and Burkholderia sp.

These microorganisms have been commonly associated with high rates of antimicrobial resistance and also with biofilm production, posing a threat to public health, and it hinders the treatment of infections caused by them. The biofilm production also potentiates the bacterial colonization and is responsible for the selection of resistant bacterial lineages, as the microorganisms present in the core of the biofilm are not affected by the antimicrobials used in patient treatment and remain viable.

Due to the increase in healthcare-associated infections, the identification and knowledge about the etiological agent and the mechanisms related to their resistance to antimicrobials constitutes a fundamental strategy in the control of HAI. Thus, the purpose of this study was to verify the presence, the biofilm formation ability, and the antimicrobial resistance profile of NFGNB isolated from tracheal secretion, blood culture, and hospital surfaces, since such contamination aggravates the clinical profile of patients.

METHOD

This is a descriptive cross-sectional study developed in an ICU of a hospital from Caruaru-PE, Brazil, from January to November 2018. Ethical approval was obtained from the ethical committee of the Centro Universitário Tabosa de Almeida (ASCES-UNITA) (code number 77393617.0.0000.5203/2.348.001 and 77418217.0.0000.5203/2.348.003). Tracheal secretion and blood culture specimens were obtained from the hospital laboratory routine and sent, under...
appropriate biosafety conditions, to the ASCES-UNITA Microbiology Laboratory.

Surface samples were collected during the patient's hospitalization, by convenience, from right and left rails of the beds with a sterile swab moistened in Tryptic Soy Broth (TSB) (KASVI® - São José do Pinhais-PR, Brazil).

The swabs were deposited again in TSB and incubated at 37°C for 18 to 24 hours. Bacterial growth was evaluated by visual inspection and then seeded on MacConkey Agar plates (KASVI® - São José do Pinhais-PR, Brazil).

Tracheal secretions were recovered from the original collection bottles by using a sterile swab, while for bacterial blood analysis, about 0.25mL of the blood culture was drawn from each vial using a 3 mL syringe. All tracheal secretion and blood culture samples were seeded on MacConkey Agar plates (KASVI® - São José do Pinhais-PR, Brazil) and incubated for 18 to 24 hours to verify bacterial growth.

The identification of the isolates was performed according to macro and microscopic characteristics of the colonies, Gram stain and biochemical test results. The carbohydrate fermentation test was performed in Triple Sugar Iron (TSI) (KASVI® - São José do Pinhais-PR, Brazil), as well as oxidase-based tests (LABTEST® - Lagoa Santa-MG, Brazil) for the identification of non-fermenting gram-negative bacteria and bacterial genus.

Microbial resistance profile analysis was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (KASVI® - São José do Pinhais-PR, Brazil) as proposed by the Clinical and Laboratory Standard Institute (CLSI).

The biofilm production and quantification were determined by O’Toole and Trentin’s methodology. Briefly, the isolates were diluted in saline solution until reaching OD625nm density of 0.08 - 0.13 AU (0.5 McFarland scale). 80μL of each bacterial suspension and 120μL of TSB were added in different wells of a microtiter dish. The micro dish was incubated at 37°C for 18 to 24 hours, and the bacterial suspensions were removed by water washing. Subsequently, 200μL of an aqueous solution of 0.4% crystal violet was added to each well of the microtiter dish, which was subsequently incubated for 15 minutes, followed by a new water washing. The production of bacterial biofilm was evaluated by optical microscopy, observing the colonies adhered to the wall and/or bottom of the microtiter dish, like a lumpy. Tests were performed in triplicate, and wells containing sterile TSB were used as negative control for biofilm production.

The biofilm quantification test was performed by adding 200μL of a 30% acetic acid solution in each well to solubilize the biofilm, when it was present. Optical Density (OD) reading from each well was conducted using a microplate reader (Thermo Plate® - China) at 570nm, and the biofilm producers were classified considering the highest OD reading for each isolate (ODi) and comparing it to the reading of the OD negative control (ODc) as follows: i) non-adherent (ODi ≤ ODc); ii) weakly adherent (ODi < ODc ≤ 2 × ODc); iii) moderately adherent (2 × ODc < ODi ≤ 4 × ODc); and iv) strongly adherent (4 × ODc < ODi).

## RESULTS

It was observed that from 68 clinical samples used in the study (10 from surface, 49 blood culture, and 9 from tracheal secretion), 64 samples (94%) showed bacterial growth, being 32.81% gram negative. Of these, 33% of NFGNB were identified, being Acinetobacter sp. (57%), Pseudomonas sp. (28%), and Burkholderia sp. (15%) the principal genera of NFGNB, as shown in Table 1. Just one patient had the samples from three different places from the hospital, with positivity for all the samples.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Tracheal Secretion No.(%)</th>
<th>Blood Culture No.(%)</th>
<th>Surface No.(%)</th>
<th>Total No.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter sp.</td>
<td>0(0)</td>
<td>2(100)</td>
<td>2(50)</td>
<td>4(57%)</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>1(100)</td>
<td>0(0)</td>
<td>1(25)</td>
<td>2(28%)</td>
</tr>
</tbody>
</table>
Biofilm production by resistant non-fermenting gram-negative bacilli from intensive care units

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sample</th>
<th>Bacterial genus NFGNB</th>
<th>Antimicrobial resistance</th>
<th>Production and quantification of bacterial biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tracheal secretion</td>
<td><em>Pseudomonas</em> sp.</td>
<td>Imipenem, aztreonam, ceftazidime, piperacillin-tazobactam, gentamicin</td>
<td>Moderately adherent</td>
</tr>
<tr>
<td>2</td>
<td>Left rail</td>
<td><em>Acinetobacter</em> sp.</td>
<td>Ceftriaxone, ciprofloxacin, levofloxacin, ampicillin-sulbactam, imipenem, cefotaxime, ceftazidime, piperacillin-tazobactam</td>
<td>Strongly adherent</td>
</tr>
<tr>
<td>3</td>
<td>Right rail</td>
<td><em>Pseudomonas</em> sp.</td>
<td>Imipenem, aztreonam, ceftazidime, piperacillin-tazobactam</td>
<td>Strongly adherent</td>
</tr>
<tr>
<td>4</td>
<td>Right rail</td>
<td><em>Acinetobacter</em> sp.</td>
<td>Ceftriaxone, ciprofloxacin, levofloxacin, ampicillin-sulbactam, imipenem, cefotaxime, piperacillin-tazobactam, gentamicin, ceftazidime</td>
<td>Moderately adherent</td>
</tr>
<tr>
<td>5</td>
<td>Left rail</td>
<td><em>Burkholderia</em> sp.</td>
<td>No resistance</td>
<td>Moderately adherent</td>
</tr>
<tr>
<td>6</td>
<td>Blood culture</td>
<td><em>Acinetobacter</em> sp.</td>
<td>Amikacin, ceftriaxone, ciprofloxacin, levofloxacin, ampicillin-sulbactam, imipenem, cefotaxime, ceftazidime, piperacillin-tazobactam, gentamicin</td>
<td>Moderately adherent</td>
</tr>
<tr>
<td>7</td>
<td>Blood culture</td>
<td><em>Acinetobacter</em> sp.</td>
<td>Amikacin, ceftriaxone, ciprofloxacin, ampicillin-sulbactam, imipenem, cefotaxime, gentamicin, ceftazidime</td>
<td>Moderately adherent</td>
</tr>
</tbody>
</table>

DISCUSSION

This study initially aimed to evaluate the hospital surface and tracheal secretion samples and their relationship with *Acinetobacter* sp.; however, due to the limited number of samples, blood cultures were also included, as well as all NFGNB isolates. Due to the study hospital’s policy and its Ethics Code restrictions, it was not possible to access the complete patient data, making it difficult to know whether the isolates found were related to their clinical condition.

There are numerous risk factors that lead a patient to be colonized by microorganisms, including prolonged hospitalization, invasive procedures, and underlying diseases such as diabetes mellitus, cardiovascular diseases, malnutrition cases, gastric achlorhydria, among others. Most hospitalizations are not caused by infectious diseases, but these may affect hospitalized patients, thus developing an infectious condition, leading to more extensive treatment and higher risk of acquiring new infections.17-18
The most isolated microorganisms in the ICU are gram-negative bacteria of the Enterobacteriaceae family.18-20 Bacteria that do not ferment glucose, most of the time, are not evidenced, mainly due to the difficulty of their phenotype laboratory identification. Nowadays, these microorganisms have become of greater clinical significance, given its resistance profile and bacterial biofilm production.

In the present study, the NFGNB represented 10.93% of growing samples, with prevalence in surface, blood culture and tracheal secretion samples, the latter with low occurrence of isolates (14.28%) when compared to another study in which of 326 NFGNB isolates from various samples, such as urine, tracheal secretion, sputum, 38.34% were isolated just from tracheal secretion, varying due to study time design and number of samples.11

In this study, there was a similar occurrence found in blood culture and surfaces, when compared to studies by Deliberali11, Oliveira8 and Rocha5.

The present work evidenced the genus Acinetobacter sp. as the most prevalent on surfaces, and this prevalence was probably related to its nutritional and metabolic versatility since it can survive in low-nutrient environments, using various substrates as carbon source, which increases its colonization time in the hospital environment.5,23 Because it is a pathogen of great clinical significance, being considered the major cause of infections in the ICU by NFGNB, it was also the most isolated of gram-negative bacteria in the bloodstream in similar studies by Cunha24 and Pailhoriès25, that during the period studied by the survey, 24 5,759 blood culture tests were analyzed, with 1,019 positive results, Acinetobacter baumannii was found in 31 samples, it was the sixth most isolated pathogen and corresponded to 3.04% of positive blood cultures. Bacterial studies conducted in 75 different countries showed that hospital ICU patients were infected with Acinetobacter sp. in about 9% of participating countries.26

Acinetobacter sp. is a pathogen of great importance because it is usually multidrug-resistant to numerous antimicrobials in clinical practice.24 In this study, isolates of this genus showed sensitivity to the antibiotic minocycline (100%), corroborating other study in this field,8,27 which presented similar results to this one. Regarding the bacterial resistance profile, other studies8 showed that Acinetobacter sp. isolates showed resistance to amikacin (100%), ciprofloxacin (100%), ceftriaxone (100%), ceftazidime (100%), imipenem (100%) and gentamicin (100%), similar to the present study which highlights what the literature portrays regarding its resistance.

According to a study28 that evaluated tracheobronchial secretion, in relation to Pseudomonas sp., regarding tracheal secretion isolates, there was a single isolate, differing from another similar study18 in which the prevalence of these bacteria represented 36.43% of the total of 129 tracheal secretion samples, which demonstrates the value of epidemiological analysis of bacterial isolates of clinical importance, as one of the ways to contribute to the control of hospital infection rates.

In this study, biofilm production was observed in all isolates of NFGNB analyzed, being quantified as strongly adherent (28.57%) and moderately adherent (71.43%), which was higher than that reported by similar biofilm production study,29 which showed 75% of isolates as biofilm producers, with 10% strongly adherent. The same authors also found that 46.7% of the bacteria were multidrug sensitive, and in the present study, it is observed that, in relation to the genus Pseudomonas sp., all isolates analyzed were multidrug resistant. This result is similar to those of other studies, in which this bacterial genus was strongly and moderately biofilm producer. The authors also reported the growth capacity of this bacterial genus even in environments with minimal supply of organic matter.29-30

NFGNB have acquired importance as causes of HAI, such as the respiratory tract, urinary tract, surgical wounds, among others.11,31 This has been owing to increased antimicrobial resistance, which is due to the presence of bacterial biofilm that is an
aggravation factor of these infections as it can be formed in some areas of the body and environments, especially the surfaces close to the patients. Bacterial multidrug resistance may persist even after treatment with high doses of antimicrobial drugs.\(^{11,32-33}\)

Pinheiro\(^{34}\) found that 70% of all hospital infections are linked to the presence of biofilm in medical devices, also showing that bacteria associated with biofilm production behave differently in their growth rate, resistance to antimicrobial agents, and increased resistance to the host immune response.

Moskowitz\(^{35}\) reports that the antibiogram may evidence an effective \textit{in vitro} antibiotic therapy, but this method is performed with the bacteria in its planktonic form, which limits the correct evaluation of the antibiotic tested. These bacteria may be protected by the biofilm in the patient, which suggests that the response is not the same as that obtained through the standard antimicrobial resistance test. In the present study, the genus \textit{Burkholderia} was moderately producer of bacterial biofilm, despite its total \textit{in vitro} sensitivity through antibiogram.

The production of bacterial biofilm in hospital samples is associated with public health problems as this mechanism contributes to the decrease in patients’ quality of life, increase in length of stay and hospital costs as well as increase in morbimortality.\(^{31,36}\)

Regarding the measures to contain bacterial biofilm colonization, it is realized that health professionals are among the main vehicles for microbiological dissemination among patients. Early identification of colonized patients can help to control the spread of this kind of infection,\(^{37}\) and hand hygiene, while simple and affordable, is one of the most effective proven methods to prevent the transmission of resistant isolates and should be constantly stimulated and performed whenever patient contact begins and ends.\(^{38}\)

**CONCLUSION**

The NFGNB found in this study proved to be quite resistant to most of the antibiotics tested, which hinders the treatment of infected patients. These microorganisms were moderately and strongly adherent in relation to biofilm production, further aggravating the action of possible antimicrobials used. Strongly adherent bacterial isolates in relation to biofilm production were found on surfaces close to the patient, suggesting greater care for local hygiene in order to prevent possible cross infections. Studies such as this one, in a hospital environment, can help in actions aimed at improving the reduction of resistance rates.
RESUMEN
Introducción: La Unidad de Cuidados Intensivos es uno de los sectores en el entorno hospitalario con altas tasas de aislamiento de bacterias multirresistentes a los antimicrobianos. Las infecciones causadas por bacilos gram-negativos no fermentadores (BGNNF) son de gran importancia y preocupación clínica, especialmente cuando están relacionadas con microorganismos multirresistentes. Uno de los principales mecanismos asociados con la resistencia bacteriana es la producción de biopelícula.

Objetivo: Para verificar la presencia, el perfil de resistencia, así como la producción de biopelícula bacteriana, de aislados de BGNNF de la sección traqueal, hemocultivo y superficies de una Unidad de Cuidados Intensivos. Delineación: Las muestras fueron recolectadas de febrero a septiembre de 2018. Se utilizaron el Agar MacConkey, Triple Sugar Iron y la prueba de oxidasa para la identificación de bacterias. La resistencia a los antimicrobianos se produjo utilizando el método de difusión en disco de Kirby-Bauer y la presencia de la biopelícula bacteriana se verificó por cuantificación evidenciada a través de un lector de placas de microaloración. Resultados: De los 30 aislamientos gram-negativos, siete eran BGNNF. Fueron identificados cuatro del género Acinetobacter sp., dos del género Pseudomonas sp., y uno del género Burkholderia sp. Los resultados mostraron moderadamente aislado y fuertemente productores de biopelícula. Implicaciones: La bacteria encontrada mostró una gran resistencia asociada con la identificación de estos patógenos puede contribuir en gran medida a una terapia antibiótica hospitalaria más efectiva.

DESCRIPTORES
Biopelículas; Farmacorresistencia Microbiana; Unidades de Cuidados Intensivos; Bacterias.

REFERENCES


COLLABORATIONS
MISP and CAMN have contributed entirely to the data collection, analysis, interpretation, and design of this manuscript of the project. LRM and MQMS have significantly contributed to the drafting, design, and critical review of this manuscript. IVR and SRO have supervised the final version of this manuscript. All the authors discussed the results, commented on the article, and approved the final manuscript.

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There are no conflicts of interest to declare.