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ORIGINAL ARTICLE

Identification and characterization of bacterial biofilm production in clinical isolates of *Staphylococcus spp.* against antimicrobials

Identificação e caracterização da produção de biofilme bacteriano em isolados clínicos de Staphylococcus spp. frente aos antimicrobianos

Identificación y caracterización de la producción de biopelículas bacterianas en aislamientos clínicos de Staphylococcus spp. contra los antimicrobianos

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ABSTRACT

Justification and Objectives: Circulating blood is sterile and the presence of microorganisms can be of clinical interest, especially in the hospital environment, being able to cause infectious processes and substantially increase morbidity and mortality. The objective of this work was to characterize the isolates of the genus *Staphylococcus* spp. from bloodstream infections as to the production of bacterial biofilm and resistance to the main antimicrobials used in clinical practice. **Methods:** Blood cultures were collected with an indication of positivity for bacterial growth from multiple sectors of the study hospital, which were subsequently processed to identify the bacterial genus through the use of phenotypic tests for Gram positive bacteria. The verification of the resistance profile was performed following the Kirby-Bauer disk diffusion. The identification of the production and quantification of the bacterial biofilm occurred following the protocol described by O'toole (2010). **Results:** The most frequent clinical isolate was Coagulase negative *Staphylococci* 38 (54.29%), followed by *Staphylococcus aureus* 32 (45.71%). Resistance to erythromycin, norfloxacin, levofloxacin and azithromycin was observed in most isolates (70%). Regarding methicillin, more MRSA (59.38%) than MR-CONS (47.37%) were isolated. The ICU was the place where the formation of the biofilm showed indicative data of greater adherence, which was associated with MRSA strains. **Conclusion:** The bacterial isolates associated with bloodstream infections showed high resistance to antimicrobials. The presence of MRSA and MR-CONS with strong and/or moderate biofilm production capacity represents a greater risk to the health of patients affected by infections caused by these agents.

Keywords: *Biofilms. Blood culture. Staphylococcus.*

RESUMO

Justificativa e Objetivos: O sangue circulante é estéril e a presença de microrganismos pode ter interesse clínico, especialmente no ambiente hospitalar, sendo capaz de causar processos infecciosos e aumentar substancialmente a morbimortalidade. O objetivo deste trabalho foi caracterizar os isolados do gênero *Staphylococcus* spp. oriundos de infecções de corrente sanguínea quanto à produção de biofilme bacteriano e resistência aos principais antimicrobianos utilizados na prática clínica. **Métodos:** Foram coletadas hemoculturas com indicação de positividade para o crescimento bacteriano de múltiplos setores do hospital de estudo, as quais posteriormente foram processadas para identificação do gênero bacteriano através da utilização de testes fenotípicos para bactérias Gram positivas. A verificação do perfil de resistência foi realizada seguindo a metodologia de disco difusão de Kirby-Bauer. A identificação da produção e quantificação do biofilme bacteriano ocorreu seguindo o protocolo descrito por *O'toole* (2010). **Resultados:** O isolado clínico mais frequente foi o *Staphylococcus coagulase* negativo 38 (54,29%), seguido pelo *Staphylococcus aureus* 32 (45,71%). A resistência à eritromicina, norfloxacina, levofloxacina e azitromicina foi observada na maioria dos isolados (70%). Em relação à meticilina, foram isolados mais *Staphylococcus aureus* resistente à meticilina (MRSA) (59,38%) que *Staphylococcus* coagulase negativa resistente à meticilina (MR-CONS) (47,37%). A UTI foi o local onde a formação do biofilme apresentou dados indicativos de maior aderência, sendo essa associada às cepas MRSA. **Conclusão:** Os isolados bacterianos associados às infecções da corrente sanguínea apresentaram elevada resistência aos antimicrobianos. A presença de MRSA e MR-CONS com forte e/ou moderada capacidade de produção de biofilme representa maior risco à saúde dos pacientes acometidos por infecções causadas por estes agentes.

Descritores: *Biofilmes. Hemocultura. Staphylococcus.*

RESUMEN

Justificación y objetivos: la sangre circulante es estéril y la presencia de microorganismos puede ser de interés clínico, especialmente en el entorno hospitalario, ya que puede causar procesos infecciosos y aumentar sustancialmente la morbilidad y la mortalidad. El objetivo de este trabajo fue caracterizar los aislamientos del género *Staphylococcus* spp. de infecciones del torrente sanguíneo en cuanto a la producción de biopelículas bacterianas y la resistencia a los principales antimicrobianos utilizados en la práctica clínica. **Métodos:** Se recogieron hemocultivos con una indicación de positividad para el crecimiento bacteriano de múltiples sectores del hospital de estudio, que posteriormente se procesaron para identificar el género bacteriano mediante el uso de pruebas fenotípicas para bacterias Gram positivas. La verificación del perfil de resistencia se realizó siguiendo la metodología de difusión de disco de Kirby-Bauer. La identificación de la producción y cuantificación de la biopelícula bacteriana se produjo siguiendo el protocolo descrito por O'toole (2010). **Resultados:** El aislado clínico más frecuente fue *Staphylococcus* coagulasa negativo 38 (54.29%), seguido de *Staphylococcus aureus* 32 (45.71%). Se observó resistencia a la eritromicina, norfloxacina, levofloxacina y azitromicina en la mayoría de los aislamientos (70%). Con respecto a la meticilina, se aislaron más MRSA (59,38%) que MR-CONS (47,37%). La UCI fue el lugar donde la formación de la biopelícula mostró datos indicativos de una mayor adherencia, que se asoció con las cepas de MRSA. **Conclusión:** los aislamientos bacterianos asociados con infecciones del torrente sanguíneo mostraron una alta resistencia a los antimicrobianos. La presencia de MRSA y MR-CONS con una capacidad de producción de biopelículas fuerte y / o moderada representa un mayor riesgo para la salud de los pacientes afectados por infecciones causadas por estos agentes.

Palabras Clave: *Biopelículas. Cultivo de Sangre. Staphylococcus.*

INTRODUCTION

Health Care Related Infections (HAI) have *Staphylococcus* spp. as the main genus involved[1,2], and are observed at worrying levels mainly within the Intensive Care Unit (ICU), which patients are sometimes psychologically, physically and immunologically more compromised, thus being able to increase morbidity and mortality.[3] Blood, as a sterile tissue and with several functions in the body (such as the transport of nutrients, respiratory gases and defense against pathogens), has high relevance and the presence of any microorganism that has not been destroyed by the immune system may have clinical interest. Some species of *Staphylococcus* spp. are part of the resident skin microbiota, such as *Staphylococcus epidermidis* and *Staphylococcus aureus*, which can be easily inoculated into the bloodstream, through invasive procedures such as venous pulsation, use of central catheters or even probes, when performed without proper asepsis.[4,5]

The presence of microorganisms in the bloodstream can be aggravated by certain situations such as the type of etiological agent, the species, resistance levels and resistance factors. In this context, we highlight Methicillin-Resistant *Staphylococcus aureus* (MRSA), which may have mecA resistance gene and produce the beta-lactamase enzyme that degrades the ring of beta-lactam antibiotics, inactivating them during treatment, thus reducing

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the possibility of improvement of the infectious conditions of the patients. According to the Brazilian Health Regulatory Agency - ANVISA[6] (2016), more than 65% of *Staphylococcus* spp. identified in the ICU are MRSA, a factor that contributes towards increasing the length of stay of patients, who have an infectious condition resulting from this bacteria. The problem is exacerbated when such isolates are producers of bacterial biofilm that promotes the adhesion of bacteria to biotic surfaces. This adhesion is ideal for community life, as it helps in the nutrition process of microorganisms and contributes to their resistance to the microenvironment in which they are found. Considering the colonization of the circulatory system, the bacterial biofilm promotes greater resistance to leukocytes and antibiotic therapy, acting as a barrier to the diffusion of the antibiotic between the deeper bacterial cells. When the bacterial biofilm reaches a high level of maturation, it breaks down and free bacteria can easily reach other parts of the body, making it difficult to treat patients with antimicrobial resistant and biofilm--producing isolates.[4,7]

The most common technique for detecting microorganisms in the patient's circulatory system is blood culture, as this allows the isolation of most bacterial species present in the blood, in addition to being cost-effective and helping to determine the best line of treatment for the patient.[7-9]

This work aimed to characterize the isolates of the *Staphylococcus* spp. from bloodstream infections regarding the production of bacterial biofilm and resistance to the main antimicrobials used in clinical practice.

METHODS

This is an observational, analytical, quantitative and cross-sectional study, with a census sample, developed in 2018 from January to October, in a Brazilian hospital, including the following sectors: emergency, triage room, low-complexity room, high complexity room and ICU. Statistical tools and inferential calculations such as the chi-square test were used in order to assign statistical significance. The study was developed after approval by the Research Ethics Committee of Asces Unita, approval number CAAE 77393617.0.0000.5203.

Isolation and bacterial identification

Blood culture bottles with an indication of positivity for microbial growth were selected according to the automation system of the study hospital. A total of 500 uL of the blood culture content was removed from the original flask with the aid of sterile 3 mL syringes and seeded on the surfasse of Agar Blood and Agar MacConkey medium, and subsequently incubated in a bacteriological incubator at 37 °C for 18 to 24 hours for evaluating the growth and isolation of bacterial colonies. The isolates were stained by Gram's methodology and evaluated for macro and microscopic characteristics of the colonies to confirm the bacterial genus. Tests[10] to detect the production of catalase and DNase enzymes, as well as to evaluate the fermentation capacity of mannitol by bacterial isolates were developed for the differentiation of *Staphylococcus aureus* from other speies. Additionally, the novobiocin resistance test was performed to differentiate the specimens between S. coagulase negative and S. saprophyticus.

Antimicrobial tests

Antimicrobial susceptibility testing was performed following the Kirby-Bauer disk-diffusion methodology for 13 antimicrobials: penicillin (PEN - 10 und), cefoxitin (CFO - 30µg) (used for screening for Methicillin Resistant *Staphylococcus* - MRS), gentamicin (GEN - 10µg), azithromycin (AZI - 15µg), erythromycin (ERI - 15µg), tetracycline (TET – 30µg), levofloxacin (LEV – 5µg), lomefloxacin (LMX – 10µg), norfloxacin (NOR - 10µg), nitrofurantoin (NIT – 300µg), trimethoprim + sulfamethoxazole (TRS – 1.25 /23.75µg), chloramphenicol (CLO – 30µg) and linezolid (LIN – 30µg), according to CLSI 2018.[11]

Evaluation and quantification of bacterial biofilm

The evaluation of bacterial biofilm production by the isolates was performed according to the technique described by O'toole (2010).[12] Briefly, after microbial growth, the isolates were resuspended in saline at the 0.5 McFarland and seeded in the wells of the microdilution plate. As negative controls of the test, three wells with only Tryptic Soy Broth (TSB) culture medium were used and, as positive control, a strain of a known *S. aureus* was tested also in triplicate. Initially, 150µl of TSB and 100µl of the dilution containing the microbial cells were applied in microdilution plates, in duplicate, and incubated at 37°C for 18 to 24h. After growth, the cell surplus was removed by inversion and the fixed cells were stained with 0.4% crystal violet, later observed by optical microscopy. To quantify the biofilm, the isolates adhered to the wells of the microdilution plate were solubilized with an aqueous solution of acetic acid (30% v/v), subjected to rotation at 180 rpm for 10 minutes in a mechanical shaker, and subsequently evaluated under spectrophotometry UV light (570nm) for identification of remaining bacterial cells. For further quantification, the cut-off point of negative control was calculated at 0.088nm. Biofilm adherence was classificated as follows:[13] non-adherent if abs ≤ 0.088nm; weakly adherent if abs > 0.088 and ≤ 0.176nm; moderately adherent if abs > 0.176nm and ≤ 0.264nm; and strongly adherent if abs > 0.264nm.

Data analysis

Data analyzes were performed using Excel® 2018 (Microsoft Office) software, performing descriptive statistics for the present study, in addition to preparing the database and graphs. The *IBM SPSS® 20/2011* software was used to make graphs and perform the chi-square test.

RESULTS

Seventy samples were obtained from patients of both genders from all sectors of the hospital, 44 (62.86%)

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Table 1. Antimicrobial susceptibility tests of *Staphylococcus sp.*

Antimicrobial	*Coagulase negative Staphylococci* R n (%)	**p-value	*Staphylococcus aureus* R n (%)	**p-value
Gentamicin	15 (39,47)	0,225040	10 (31,25)	0,197156
Norfloxacin	34 (89,47)	0,177153	29 (90,63)	0,087439
Tetracycline	7 (18,42)	0,061524	7 (21,87)	0,809550
Erythromycin	34 (89,47)	0,177153	29 (90,63)	0,087439
Chloramphenicol	10 (26,32)	0,018703	11 (34,38)	0,907226
Cefoxitin*	18 (47,37)	0,000601	19 (59,38)	0,000239
Levofloxacin	27 (71,05)	0,004549	25 (78,13)	0,022216
Trimethopim	29 (76,32)	0,176827	11 (34,38)	0,050603
Linezolid	0 (0)	--	0 (0)	--
Penicillin	38 (100)	--	32 (100)	--
Azithromycin	35 (92,11)	0,249342	30 (93,75)	0,170067
Lomefloxacin	35 (92,11)	0,249342	24 (75)	0,170067
Nitrofurantoin	10 (26,32)	0,931859	8 (25)	0,809550

Cefoxitin - used for phenotypic identification of MRS strains. **P-value with respect to biofilm in association with MRS. R - resistant. -- No statistics are calculated because the values are a constant. The MDR p-value for CNS was 0.051889 and for S. aureus 0.014100.*

Table 2. Percentage of biofilm formation classification by evaluated species.

Biofilm classification	*Coagulase negative Staphylococci* MR-CONS n (%)	*Staphylococcus aureus* MRSA n (%)
Non-adherent	9 (23,68)	5 (15,63)
Weakly adherent	4 (10,33)	2 (6,25)
Moderately adherent	18 (47,37)	24 (75)
Strongly adherent	7 (18,42)	1 (3,12)

For quantification, the cut-off point of negative control of 0.088nm was calculated. Biofilm adherence classification was calculated as follows13: non-adherent if abs ≤ 0.088nm; weakly adherent if abs > 0.088 and ≤ 0.176nm; moderately adherent if abs > 0.176nm and ≤ 0.264nm; and strongly adherent if abs > 0.264nm.

male and 26 (37.14%) female.

Coagulase negative *Staphylococci* (CNS) were the most frequently microorganism isolated, representing 38 (54.29%) of the total, followed by *S. aureus*, which represented 32 (45.71%) of samples. The results of antimicrobial susceptibility tests are described in table 1. MRSA were identified as 19 (59.38%) of the isolates, while 18 (47.37%) of Methicillin Resistant *Coagulase negative Staphylococci* (MR-CONS) isolates were identified.

Multidrug resistance data related to biofilm production showed a p-value that for some antibiotics demonstrated that biofilm production may be associated with resistance.

A total of 31 (44.29%) of the isolates were retrieved from the ICU, followed by triage room (17 isolates / 24.29%) and high-complexity room (14 isolates / 20%). The low-complexity and emergence room corresponded to 2 (2.16%) and 6 (8.57%) of the total, respectively. The ICU had the highest number of MR-CONS and MRSA isolates, and it was also the hospital sector with the highest percentage (62.5%) of strains that strongly form bacterial biofilm.

Data of biofilm formation are described in table 2. The strains of S. *aureus* were the main representatives of the strong production (adherence) of biofilm (18.42%), allowing the realization of inferential statistics.

For quantification, the cut-off point of negative control of 0.088nm was calculated. Biofilm adherence classification was calculated as follows13: non-adherent if abs ≤ 0.088nm; weakly adherent if abs > 0.088 and ≤ 0.176nm; moderately adherent if abs > 0.176nm and ≤ 0.264nm; and strongly adherent if abs > 0.264nm.

Considering the different hospital isolation sectors, it was observed that the bacterial isolates that showed greater capacity for biofilm formation came from the ICU (41.93% of the total 31 isolates in the sector), as shown in table 3. In addition, 10 isolates (14.24%) were still associated with the profile of multi-resistance to antimicrobials

Table 3. Classification of biofilm formation by species and hospital section.

Aderence Hospital sector	*Coagulase negative Staphylococci* MR-CONS MODERATE n (%)	Strong n (%)	*Staphylococcus aureus* MRSA MODERATE n (%)	Strong n (%)
ICU	6 (33,33)	5 (71,43)	7 (29,17)	0 (0)
Emergency	1 (5,55)	2 (28,57)	2 (8,33)	0 (0)
High comp. room	6 (33,33)	0 (0)	5 (20,83)	1(100)
Triage room	5 (27,78)	0 (0)	7 (29,17)	0 (0)
Low comp. room	0 (0)	0 (0)	2 (8,33)	0 (0)

Sectors which isolates showed strong and moderate biofilm production.

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tested in the ICU.

Sectors which isolates showed strong and moderate biofilm production.

DISCUSSION

Although the presence of *Staphylococcus* spp. in the patient's microbiota, this group can be the causative agent of infections in the most diverse body sites; such as the skin, airways, soft tissue, conjunctiva and bloodstream,[14-17] affecting patients of different ages and of both sexes.[16]

In this work, it was observed that most of the isolates were from CNS, given that these diverge from the findings described by other studies[17,19] which aimed to evaluate the resistance profile of S. *aureus* and CNS in blood culture samples. This result may be associated with the wide dissemination of strains carrying specific genes (like mecA) that were identified as CNS, since the species that compose them also have this gene widely disseminated, in various species of clinical interest.[17-20]

The resistance patterns found for the antibiotics chloramphenicol, penicillin and linezolid corroborate the studies carried out with *S. aureus,*[14,15-18,20] emphasizing that penicillin is not a viable therapeutic option for most cases. The resistance pattern for tetracycline diverged from the previous study[17] which demonstrated a resistance around 30%, unlike this one, in which the levels were around 20%. In that study, the profile of resistance in blood cultures in a tertiary hospital was evaluated. The resistance observed in the antibiotics erythromycin, trimethoprim, norfloxacin, cefoxitin, azithromycin and gentamicin were lower than those identified in our study.[14-18, 20, 23] Nevertheless, the studies mentioned were carried out in multiple anatomical sites, such as those of conjunctival origin, keratitis and blood cultures[14-18, 20] of patients who occupied different hospital environments. The main isolation site for strains with strong and moderate biofilm production in association with methicillin resistance described in this study was the ICU and the high-complexity room, sectors of high and low turnover, respectively. The first is widely used for recovery after performing invasive surgical procedures, while in the high-complexity room, the procedures are emergency and the use of invasive devices (such as catheter) without proper asepsis is frequent due to the urgent need to perform it, as observed in other studies[21, 23] that evaluated the resistance of biofilm-producing S. *aureus* and CNS in hospital environments. The main sex affected by the occurrence of *Staphylococcus* spp. it was the male, as well as also described in studies[22, 25] developed in specific care hospitals. One of the possible causes for this is that the main users are young adults (18-25 years old) or adults (26-49 years old) and tend to use less primary health services. In addition, this group is also the main victim of car accidents, especially the male population. Another factor that can contribute is that in this age group, health care is sought later, leading to an overload of medium and high complexity services, in addition to underutilizing primary care. Multidrug resistance data (resistant to more than 9 (70%) antibiotics tested from 3 different groups) related to strong and moderate biofilm production in conjunction with the MRSA factor showed a p-value <0.05 (p-value 0, 004791 and 0.000030). These finds agree with previous studies,[24,25] which described that the isolates that produced biofilm and MRSA were more resistant to antimicrobials. Bacteria of the *Staphylococcus* spp. Resistant biofilm-producing multidrugs are involved in HAI processes at considerable levels, with MRSA and MR-CONS standing out in most cases. The ability of these to show moderately or strongly biofilm producers predisposes a greater risk to the health of patients affected by such infections. These finds reinforces the constant need for asepsis before any procedure performed in the hospital environment in order to minimize the occurrence of cross-infection by such agents.

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AUTHORS' CONTRIBUTIONS

Lamartine Rodrigues Martins contributed entirely to the conception of this manuscript, its design, data collection, analysis and interpretation of data.

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Igor Vasconcelos Rocha contributed significantly to data interpretation and drafting and critical review of this manuscript.

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All authors have approved the final version to be published and are responsible for all aspects of the work, including ensuring its accuracy.