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Original Article

Microbiology Section

Biofilm Formation in Clinical Isolates of *S. aureus* is Associated with Presence of Device and Dissemination of Infection

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ABSTRACT

Introduction: Biofilms are complex microbial communities attached to abiotic or biotic surfaces. These communities produce their own extracellular matrix, where they interact with one another and with the environment.

Aim: To observe the biofilm formation isolates of Staphylococcus aureus from South Brazil.

Materials and Methods: A total of 126 consecutive *S. aureus* isolates were collected, causing a variety of infections at a tertiary hospital from 2011 to 2014. We investigated biofilm-forming ability by using a microtiter plate assay (crystal violet method) and compared the clinical characteristics and outcomes of infected patients with biofilm-forming ability. The following clinical characteristics were evaluated: presence of polymicrobial infection; presence of another micro-organism (in another clinical material at the same time); recurrence of infection; presence of device and site of infection.

Results: Biofilm forming bacteria were categorized as high producers (n=46, 36.5%), moderate producers (n=59, 46.8%) and weak producers or non-producers (n=21, 16.7%). The presence of another microorganism isolated in the same day in another clinical specimen was significantly associated with biofilm-formation ($p<0.006$) as well the presence of invasive devices ($p<0.02$).

Conclusion: This study allows planning medical conducts, e.g., the choice of appropriate antimicrobials, in patients with devices such as catheters and patients with infections at different sites to adequately adjust treatment of infections by biofilm-forming bacteria.

Keywords: Bacterial dissemination, Biofilm production, Hospital infection, Medical device

INTRODUCTION

The ability to form a biofilm is a characteristic associated with the persistence of a bacterium in an infection and it is believed that several chronic bacterial infections are related to the biofilm formation [1]. *Staphylococcus aureus* is a major cause of healthcare associated infections as well as community acquired infections and represents a significant cost in the health system. *Staphylococcus aureus* attachment to medical implants and host tissue and the establishment of a mature biofilm play an important role in the persistence of chronic infections [2].

Biofilms are defined as communities of bacteria submerged in an extracellular matrix produced by them that adheres to a biotic or abiotic surface. *Staphylococcus aureus* and *Staphylococcus epidermidis* are well known as major causes of chronic infection associated with biofilm formation [3].

The biofilm formation, especially on the surfaces of medical implants such as catheters, increases tolerance to antibiotic drugs and may lead to therapeutic failure [4,5].

One of the most important problems in implant infection is the general lack of epidemiological data due to the absence of studies that assess the capacity of a clinical isolate to produce biofilm and its epidemiological data. The objective of this study was to observe the biofilm-forming ability of *S. aureus* isolated from different clinical specimen by crystal violet staining and to analyse the relationship between biofilm and clinical features.

MATERIALS AND METHODS

This is a prospective study conducted with the permission of the hospital and since it did not use any data from the patient, it did not require the approval of an ethics committee.

Isolates and growth media: The strains used for biofilm formation in microtiter plates were *S. aureus* from a variety of infections collected from May 2011 to April 2015. A total of 126 isolates were collected and only the first strain of each patient was used in the study. Clinical isolates were collected at Mãe de Deus hospital (about 400-bed hospital) and stored in skim milk with glycerol. Clinically information of each patient was collected from hospital records and the second isolate from each patient was not processed (it was treated as recurrence of infection). Susceptibility to antimicrobial agents was determined by Minimal Inhibitory Concentrations (MICs) on the Micro Scan Walk Away system (Siemens Healthcare, USA) according to protocols of the Clinical and Laboratory Standards Institute 2011 [6].

Biofilm formation: Clinical isolates of *Staphylococcus aureus* were cultured on blood agar and from their cultures a sterile NaCl suspension of about $3,0x10^8$ CFU per well (reached by spectrophotometer reading at an optical density of 600 nanometers) were allowed to form biofilms overnight at 37°C in 96-well flat bottom microplates. Biofilm formation was monitored by crystal violet staining and classified according to Stepanović S as strong, moderate, weak and no biofilm formers [7]. To classify, the Optical Density (OD) of each well stained with crystal violet is measured at 570 nm and the cut-off value (ODc) should be established. It is defined as three Standard Deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3XSD of negative control). The isolates with OD below ODc were classified as no biofilm producer; the isolates with OD reading average between ODc and 2xODc were classified as weak biofilm formers; the isolates with mean OD reading between 2xODc and 4xODc were classified as moderate biofilm formers and the isolates with OD reading average above 4xODc were classified as strong biofilm formers.

STATISTICAL ANALYSIS

Data analysis was performed using GraphPad Prism Software version 6.0. Statistical significance was assessed via Chi-square and Fisher's-exact test for categorical variables and the Student's t-test or the Mann-Whitney U test for continuous variables. Statistical significance was considered for p-value of <0.05.

RESULTS

One hundred twenty-six clinical isolates of *Staphylococcus aureus* were collected. All clinical isolates classified as strong or moderate were considered biofilm formers, and all clinical isolates classified as weak were considered non-biofilm formers according to Stepanović S. Biofilm forming bacteria were categorized as high producers (n=46, 36.5%), moderate producers (n=59, 46.8%) and weak producers or non-producers (n=21, 16.7%). After that, the clinical characteristics were compared to biofilm formers and non-formers and it is possible to verify that there is presence of another micro-organism, other than *S. aureus* isolated on the same day in another clinical specimen, was significantly associated with biofilm-formation and this can be visualized in [Table/Fig-1] in the line "other micro-organism" (p<0.006). Another feature to be highlighted in [Table/Fig-1] is the line "presence of invasive devices" (p<0.02), even though, these devices were not the site of infection. An inverse association between biofilm and skin and soft tissue origin was observed (p<0.0003). No association was found with the presence of polymicrobial infection (isolation of micro-organism other than *S. aureus* at the same site of infection) recurrence of infection (same infection during hospitalization period). Also, when we evaluated the site of infection, no association was found between any site, except for skin and soft tissue infection, where there is a negative association with biofilm formers (p 0.0003).

	Biofilm formers 105 (83.3%)	Biofilm non-formers 21 (16.7%)	p
Characteristics			
Age, years			
Mean ± SD	57±24	49±28	
Median	53	65	
Male	62	13	
Female	43	8	
MRSA isolates	32 (25,4%)	9 (7,14%)	0.3
Polymicrobial infecion	9	2	1.0
Other microorganism*	27	0	0.006**
SCoN (blood)	4		
E. faecalis (urine)	4		
P. mirabilis (urine)	4		
S. aureus (catheter)	3		
S. aureus (blood)	2		
C. albicans (blood)	2		
P. mirabilis (blood)	1		
S. aureus (SSTI)	1		
S. aureus (tracheal aspirate)	1		
P. mirabilis (cateter)	1		
E. coli (urine)	1		
Acinetobacter spp. (blood)	1		
Acinetobacter spp. (urine)	1		
E. faecalis (cateter)	1		
Recurrence of infection	11	1	0.68
Presence of an invasive device	30	1	0.02*
Catheter	26	1	
Breast implant	3		
Endotracheal tube	1		
Site of infection			
Blood	55	10	0.81
Breast implant	3	0	1.0
Catheter tip	11	1	0.68
CSF	1	0	1.0
Nasopharyngeal	0	2	0.02*
Ocular	1	2	0.07
Sputum	2	0	1.0
Skin and Soft Tissue Infection	6	8	0.0003***
Tracheal aspirate	13	3	0.73
Urine	6	0	0.58
Wound (cirurgic)	2	0	1.0

[Table/Fig-1]: Summary of clinical characteristics.
*other than *S. aureus*

When we observe the antimicrobial profile of bacteria comparing biofilm formers and biofilm non-formers [Table/Fig-2], there is a tendency to higher resistance in biofilm-forming strains, 4.76% of isolates were resistant to all (except vancomycin) antibiotics tested. Besides this, we found a higher number of biofilm non-formers (47.62%) with susceptibility to all agents tested, compared to biofilm formers (38.10%).

	Antibiotic resistance profiles	No of isolates	%
Biofilm formers (105)	No one	40	38.10
	OX	3	2.86
	OX-CIP	2	1.90
	CIP	2	1.90
	ERI	9	8.57
	SUT	5	4.76
	SUT-ERI	2	1.90
	ERI-CLI	17	16.19
	CLI-ERI-SUT	2	1.90
	OX-CIP-ERI	4	3.81
	OX-CIP-CLI-ERI	14	13.33
	All (OX-CIP-CLI-ERI-SUT) except VA	5	4.76
Biofilm non-formers (21)	No one	10	47.62
	OX	3	14.29
	OX-CIP	1	4.76
	CIP	-	-
	ERI	-	-
	SUT	2	9.52
	SUT-ERI	-	-
	ERI-CLI	1	4.76
	CLI-ERI-SUT	-	-
	OX-CIP-ERI	1	4.76
	OX-CIP-CLI-ERI	3	14.29
	All (OX-CIP-CLI-ERI-SUT) except VA	-	-

[Table/Fig-2]: Comparison of antimicrobial susceptibilities between biofilm-forming and non-forming isolates.
CIP Z ciprofloxacin; CLI Z clindamycin; OX Z oxacillin; P Z penicillin; SXT Z trimethoprim-sulfamethoxazole; TET Z tetracycline

DISCUSSION

Our results showed that biofilm-formation was significantly associated with the presence of another micro-organism (p<0.006) in other sites of infection and the presence of invasive devices (p<0.02). Even though, our study has not found statistically significant differences regarding proportions of patients with polymicrobial infection or recurrence of infection being higher in biofilm-forming isolates than non-forming isolates, other authors were able to find this correlation [8].

Regarding our expectation to observe the association between biofilm and the presence of devices, it is possible to affirm that these devices perform essential life-saving functions on one hand, while conversely they are also the leading cause of blood stream infections [9]. It is widely known that biofilm might play a role in the pathogenesis of device-associated *S. aureus* infections. Particularly, the presence of biofilms on intravascular catheters and their role in catheter-related blood infection is widely accepted [10]. Biofilm-formation was significantly associated with the presence of invasive devices (such as catheter, breast implant and endotracheal tube) ($p<0.02$) in our study, suggesting that biofilm infection may be the hidden focus of blood infections.

Furthermore, following the establishment of a biofilm, individual cells can detach/disperse from the original biofilm and seed new sights of infection [11]. This phenomenon represents a reservoir of dissemination of bacterial infection to other sites in the human body, as already shown by other authors [12]. Our study showed that biofilm-forming bacteria was significantly associated with the presence of another micro-organism in another site of infection ($p<0.006$). This finding corroborates with an earlier study which reported that dispersal of bacteria may explain the high rate of infection in distant sites symptomatically observed with *S. aureus* [13]. When bacteria living in the form of biofilms disperse and return to a planktonic state, dissemination to other secondary sites is possible and worsens infection [2], and our work shows that association.

Staphylococcus aureus have become some of the most important pathogens in nosocomial infections associated with the use of catheters and other medical implants. However, because these species are part of the normal bacterial flora of human skin and mucosal surfaces, it is difficult to discern when a microbial isolate may be the cause of infection or is the result of sample contamination. It is well known that in the clinical laboratory, cut-offs are used for differentiating infection from contamination with normal bacterial flora [14-16]. It is also widely accepted that the detachment rate of all species within the biofilms is not necessarily the same. Since we are working with clinical isolates, our hypothesis is that these strains may be part of normal patient microbiota and this may have contributed to the absence of correlation between polymicrobial infection in biofilm-forming isolates and non-forming isolates [17].

The presence of Skin and Soft Tissue Infection (SSTI) was significantly associated with biofilm non-formers ($p<0.0003$) and, as far as we know, this is the first report of this association. Likewise, Qi R et al., showed that production of PSMs (Phenol-Soluble Modulins) was higher in *S. aureus* SSTIs than other sites of infection and Joo H-S et al. [18,19], demonstrate that low PSM production causes strongly increased biofilm formation, not to mention PSMs disperse biofilm. Usually SSTI are attributed to community-associated *S. aureus* (CA-MRSA) isolates and in the last decade the number of reports of CA-MRSA isolates increased [20,21] even in nosocomial isolates [22]. The different virulence phenotypes of these two MRSAs are suggested to be due to phenol-soluble modulin a (PSMa), which is encoded in the core genome. Moreover, the expression of PSMa is elevated in most prevalent CA-MRSA strains [23].

The global emergence of organisms with Multiple Drug Resistances (MDRs) is an important factor in acute and chronic infections that leads to increased mortality rates and increased healthcare costs [24]. In addition to antibiotic resistance, the other factor that causes treatment failure and chronic and recurrent staphylococcal infections in patients is biofilm formation in these strains [25,26]. In our work, as expected, we found a higher number of biofilm non-formers (47.62%) with antimicrobial sensitivity profile, compared to biofilm formers (38.10%).

LIMITATION

It is difficult to demonstrate the independent effects of biofilm, since biofilm is the product of various factors, including not only bacterial factors but also host factors and this is the reason why studies with clinical isolates of the relationship of specific epidemiological characteristics and biofilm formation require further investigation. Besides this, the isolates were collected from a single hospital. Future studies with an approach in more than one health center are needed. Despite such limitations, this work managed to associate the presence of device and dissemination of bacteria in other sites with the ability to form biofilm. The information on the capacity of a clinical isolate to produce biofilm would help to understand its virulence and provide better planning of future preventive actions.

CONCLUSION

This study allows planning medical conducts, e.g., the choice of appropriate antimicrobials, in patients with devices such as catheters and patients with infections at different sites to adequately adjust treatment of infections by biofilm-forming bacteria.

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