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Biofilm Formation in Bovine Pericardium Biomaterial by Multi-Drug Resistant *Staphylococcus haemolyticus* Nosocomial Blood Isolates

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ABSTRACT

Bovine pericardium a cellular material (BPAM), composed of decellularized collagen fibers, has been used in varied surgical procedures. Biomaterial-associated infections by human pathogens, during clinical procedures, are of significant medical concern. Biofilm formation by human pathogens has been increasingly described as a multifactorial and multistep process associated with infection of implanted medical devices. This study aimed to investigate the ability of biofilm formation on bovine pericardium patches (BPP), and the influence of virulence features of multi-drug resistance– MDR *Staphylococcus haemolyticus* blood isolates from neonate and adult hospitalized patients. The influence of phenotypic and genotypic virulence features in the ability of biofilm formation on BPP and abiotic surfaces were analyzed. Collagen-binding properties and ability of biofilm formation favored colonization, viability, and persistence in BPAM by *S. haemolyticus* strains. A higher adhesive activity, autoaggregation microcolonies, and ability of biofilm formation on BPP, independent of *slime* production were expressed by the invasive strain from neonate patients. The genes *atl* and *fbp* were detected for both *S. haemolyticus* strains with adhesive properties to collagen type I fibers and biofilm formation on BPAM. BPAM-biofilm producer *S. haemolyticus* strains also expressed the ability of biofilm production in the presence of Oxacillin, Vancomycin, and Linezolid antimicrobial agents, as shown on glass and polystyrene surfaces. Data indicate the involvement of multiple selective activities of virulence mechanism of varied nosocomial pathogens in contamination, persistence, and dissemination from biomaterials, through the bloodstream and tissues during human infections, such as MDR *S. haemolyticus* strains.
Significance and Impact of Study: This work emphasized the possibility of contamination of BPP by human pathogens and the persistent infection following surgery of patients involving exposure to antimicrobial agents, including the ability of biofilm formation by *S. haemolyticus* on surfaces abiotic and biotic (BPAM).

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INTRODUCTION

Staphylococcus haemolyticus is the second most frequently isolated coagulase-negative staphylococcus (CoNS) from clinical nosocomial cases, notably from blood and infections related to implanted medical devices, including septicemia in adults and infants, well as bacteremia in neonatal intensive care units (NICUs). Moreover, *S. haemolyticus* nosocomial clinical isolates have been also increasingly reported to express higher levels of antimicrobial multidrug resistance (MDR) including heteroresistance to glycopeptides, thereby limiting therapeutic options (Jain *et al.* 2004; Klingenberg *et al.* 2007; Pereira *et al.* 2014; Czekaj *et al.* 2015; Panda & Singh 2018; Argemi *et al.* 2019; Pereira-Ribeiro *et al.* 2019).

Biofilm formation often occurs on medical devices which are in direct contact with human blood. Bacterial pathogens were found to exploit extracellular components and/or plasma elements to colonize human tissues or to evade immune mechanisms, including CoNS and *S. aureus*. Previous studies verified the influence of collagen-binding proteins (CBPs) on the interaction of human pathogens with collagenous tissues. CBPs were also found involved in biofilm formation and colonization of damaged heart tissues (Singh *et al.* 2010; Chen *et al.* 2020).

Bovine pericardium patch (BPP), is a biomaterial commonly used in vascular surgery, composed of decellularized collagen type I fibers (Sellaro *et al.* 2007; Li *et al.* 2011; Athar *et al.* 2014; Cuando-Espitia *et al.* 2018; McMillan *et al.* 2019; Zouhair *et al.* 2020). Infection is a serious complication that can result from any surgical procedure, especially when dealing with the implantation of artificial materials. Infection after patch angioplasty has been generally associated with additional surgery, patch explant, and increased risk of mortality and morbidity, including methicillin-resistant and –sensitive *S. aureus* and *Staphylococcus epidermidis* (Stone *et al.* 2011; Hualong *et al.* 2016). Little information is available in the literature concerned with cases of BPP-related infection. The possibility of contamination of BPP by human pathogens is of significant medical relevance. The aim of this study was to investigate the ability of biofilm formation on BPP and the influence of virulence features of MDR*S. Haemolyticus* isolated from neonate and adult hospitalized patients with bacteremia.

MATERIAL AND METHODS

Origin and identification of clinical isolates

In this study, MDR*S. Haemolyticus* (n=2) blood clinical isolates were obtained from the culture collection of our laboratory, previously obtained from patients presenting catheter-related bloodstream infections: one Neonatal Intensive Care Unit patient and General Nursery adult patient. Microorganisms were identified by conventional phenotypic and MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) methods and also submitted to antimicrobial susceptibility testing (Sued *et al.* 2017; Pereira-Ribeiro *et al.* 2019).

Bacterial adherence and biofilm formation on pericardium bovine patches.

Quantitative and semiquantitative analyses of biofilm formation by using *in vitro* model system with bovine pericardium patches were based on methods previously described (Pereira-Ribeiro *et al.* 2019). Briefly, sterile 5 mm segments of bovine pericardium patches, were washed according to the manufacturer, immersed for 24 h in 3 x 10^7 CFU ml suspended in TSB medium. After 24 h the bovine pericardium segments were removed, gently rinsed in PBS (0.01 M; pH 7.2) to eliminate loosely attached planktonic cells. This method was performed in triplicate.

Scanning electron microscopy (SEM)

Sections of 1 cm^2 bovine pericardium infected with CoNS strains were fixed with 2.5% glutaraldehyde, washed in 0.1 M cacodylate buffer, pH 7.2, post-fixed with 1% OsO_4 and 0.8% $K_3Fe(CN)_6$, washed in water, dehydrated in a graded ethanol series (30°-100° GL), critical point dried in CO_2, mounted on stubs, coated with gold (20–25 nm) and examined using the conventional scanning electron microscope (SEM) Jeol JSM-6510LV and field emission SEMJeol JSM-7100F.

Congo red agar (CRA) method

Slime producing ability analysis was conducted by culturing *S. haemolyticus* strains on CRA plates (CRA; Sigma Chemical Company, St Louis, MO, USA*)*, based on methods previously described, supplemented with 0.08% sucrose(Pereira *et al.* 2014; Sued *et al.* 2017).

Influence of antimicrobial agents on slime production and biofilm formation on abiotic surfaces.

Congo red agar tests and biofilm formation on hydrophilic (glass) and hydrophobic (polystyrene) surfaces were performed with and without antibiotics, based on previously described methods. Experiments were added of sub-MICs of OXA, VAN or LZD equivalent to 1/4 MIC (Sued *et al.* 2017; Pereira-Ribeiro *et al.* 2019).In biofilm formation on glass surface assays microorganisms were inoculated in glass tubes (15x100 mm) containing 5 mL of Trypticase Soy Broth (TSB) medium and incubated at 37ºC for 48 h. Fresh sterile TSB (5 mL) was added to the test tubes and re-incubated for 48 h. This procedure was repeated twice. Glass-adherent bacteria created a confluent coat of cells on the sides of the tube. Microorganisms were classified as the non-adherent (-: absence of adherence), weakly adherent (+: adherent bacteria appeared as a ring at the interface between the medium and the air), moderately adherent (++: bacteria attached on the side of the glass tubes), or strongly adherent (+++: bacteria attached on the side of the glass tubes and at the interface between the medium and the air (Moreira *et al.* 2003; Pereira *et al.* 2014).

Qualitative and semi-quantitative adherence and biofilm formation on polystyrene surfaces assays were performed by using sterile 96-well flat-bottomed plastic tissue culture plates (JET BIOFIL®). Based on the optical density (OD) of biofilm formation, tested strains were classified into the following categories: non-adherent (-: OD ≤ ODc), weakly adherent (+: ODc> OD ≤ 2x ODc), moderately adherent (++: 2x ODc> OD ≤ 4x ODc), or strongly adherent (+++:OD>4x ODc). The cut-off OD (ODc) was defined as the mean OD of the negative control (TSB only). Each assay was performed in triplicate and repeated three times (Sued *et al.* 2017; Pereira-Ribeiro *et al.* 2019).

PCR of genes coding for staphylococci adhesins

PCR assays were performed for detection of *icaA*, *aap*, *atl*, and *fbp* genes involved in staphylococci adhesive properties to biotic and abiotic surfaces and/or biofilm formation, in accordance with methods previously described (Santos *et al*.1999; Araujo *et al.* 2006; Potter *et al*. 2009; Barros *et al*. 2015). The primers and amplicons used in this study were listed in **Table 1**.

Table No. 1: Primers used in this study

Primers	**Sequence of forward and reverse primers 5'→ 3'**	**Product size (bp)**	**References**
*ica*A-F *ica*A-R	CGATGGGCTCAAGGTGG TTCTTTTCGTAGCGACTGTC	287 pb	Potter et al. 2009
aap-F *aap*-R	CAACGAAGGCAGAAGAAGGA CATCCCCATCTTTCTTGCTG	719 pb	Araujo et al. 2006
atl-F *atl*-R	TAACTCAACAATCGATGGCG GTACCCCAAGGTGCTACTTG	446 pb	Barros et al. 2015
fbp-F *fbp*-R	GGTGATACCATTTACCGCAC CGTGCATCGTAGTAGCGATC	512 pb	Barros et al. 2015

RESULTS

Biofilm formation and bacterial viability of sessile forms onbovine pericardium patches surface.

Results displayed in **Table 2** showed that both MDR *S. haemolyticus* 3754 and 9N strains were found capable of producing mature biofilm (24 h) on bovine pericardium patches, but at different levels, S. *haemolyticus* 9N strain isolated from neonate patient, presented 8.3 x 10^7 CFU while the 3754 clinical isolates obtained adult patient, showed 6.7 x 10^7 CFU viable cells. A significant ability of biofilm formation and a higher number of viable sessile bacterial cells was recovered from the MDR *S. haemolyticus* 9N strain (19.3%; $p<0.05$).

Morphological features of bacterial adherence and biofilm formation on bovine pericardium patch.

The presence of microcolonies, hollow voids, and autoaggregation sessile forms were indicative of mature biofilm formation on the surface of BPP during 24h incubation by both MDR *S. haemolyticus* isolated from blood samples of neonate 9N and 3754 adults in patients, as demonstrated with FESEM (**Figures 1** and **2**).

Figures **1A** and **B** showed images of commercially available BPPconfirming the presence of wavy bundles of collagen and lack of cells. **Figures C** and **D** demonstrated that *S. haemolyticus* 9N (neonate) strain expressed the ability to colonize and form a large amount of biofilm within BPP fibers. Bacterial autoaggregation microcolonies and hollow voids on the BPP were all indicative of mature biofilm structure. However, the presence of extracellular *slime* amorphous material on bacterial sessile forms adherent to BPP was not evident. **Figures E** and **F** showed *S. haemolyticus* 3754 (adult) strain ability of interaction with BPP biomaterial and biofilm formation at a lower intensity. **Figures 2 -B1/B2** showed maintained collagen fiber architecture (laminated) and variable invasion of bacterial aggregative sessile forms demonstrating that both MDR *S. haemolyticus* isolated from blood samples of neonate 9N and 3754 adult inpatients exhibit similar mechanisms during BPP interaction. Both strains remained viable on biofilm formation in the presence of 1/4 MIC of OXA, VAN, and LZD ($p<0.05$).

Slime production on Congo red agar medium.

Results of CRA phenotypic assays were shown in **Figures A1** and **A2** and **Table 2.** MDR *S haemolyticus* SH-3754 (adult) strain was verified as a *slime*-producing variant, exhibiting reddish-black colonies with a rough, dry, and crystalline consistency on CRA medium surface. MDR *S. haemolyticus* SH-9N (neonate) strain was found as *slime*-negative in CRA medium with sucrose, presenting pinkish-red smooth colonies with a darkening color at the center. Data showed that MDR *S. haemolyticus* SH-3754 (adult) and SH-9N (neonate) strains showed differences in the ability of *slime* production on Congo red agar medium, including in the presence of 1/4 MIC of OXA, VAN, and LZD.

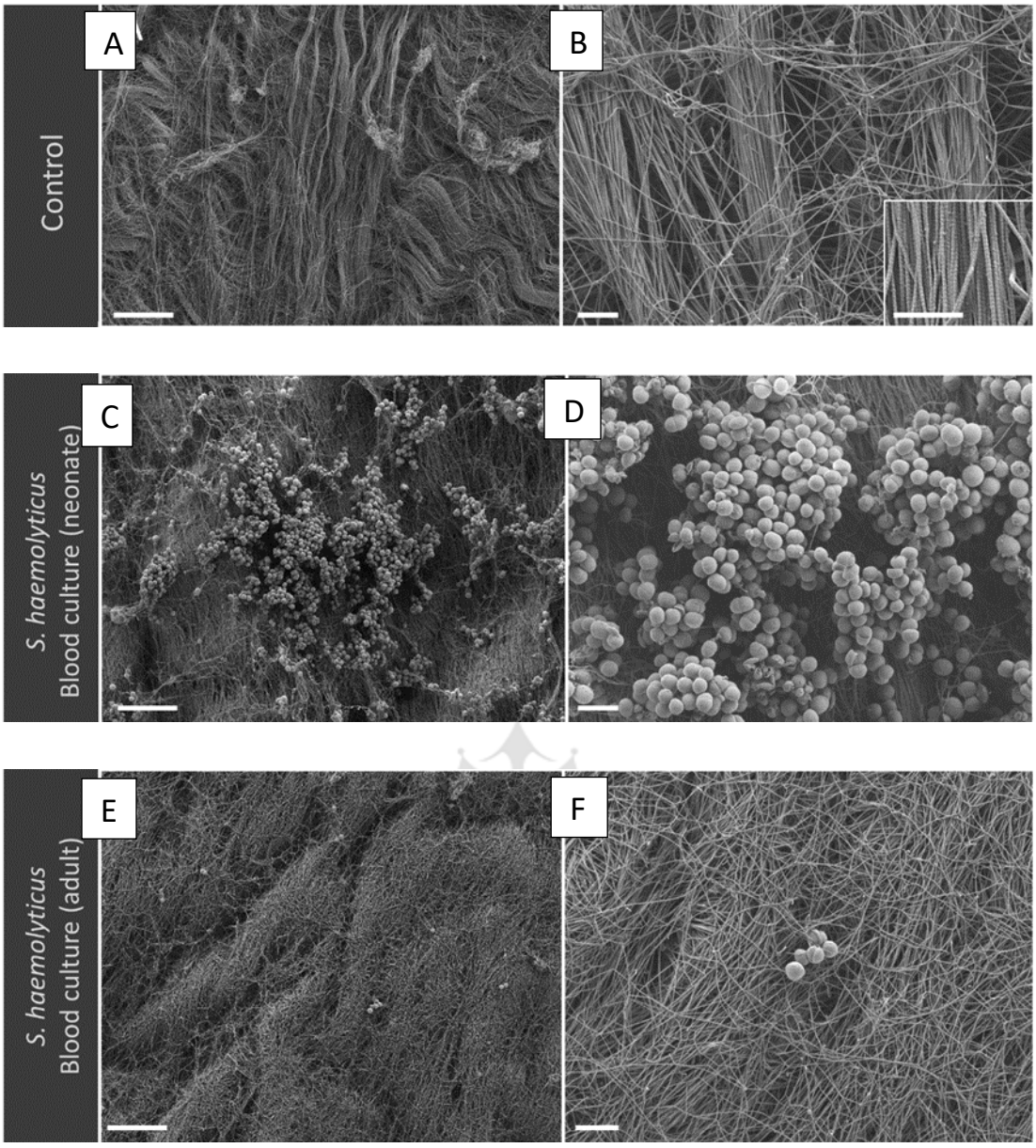

Figure No. 1: Field Emission Scanning electron microscopy (FESEM) of the internal surface on the bovine pericardium matrix in interaction with *Staphylococcus haemolyticus*: (A, B) surface of a sterile bovine pericardium (control group); (C, D) *Staphylococcus haemolyticus* (SH9N) strain isolated from blood culture of neonate forming a structured biofilm on the surface of the bovine pericardium; (E, F) *Staphylococcus haemolyticus* (SH3754) strain isolated from blood culture of adult forming separated colonies; Scale bars: 10µm and inset: 1µm.

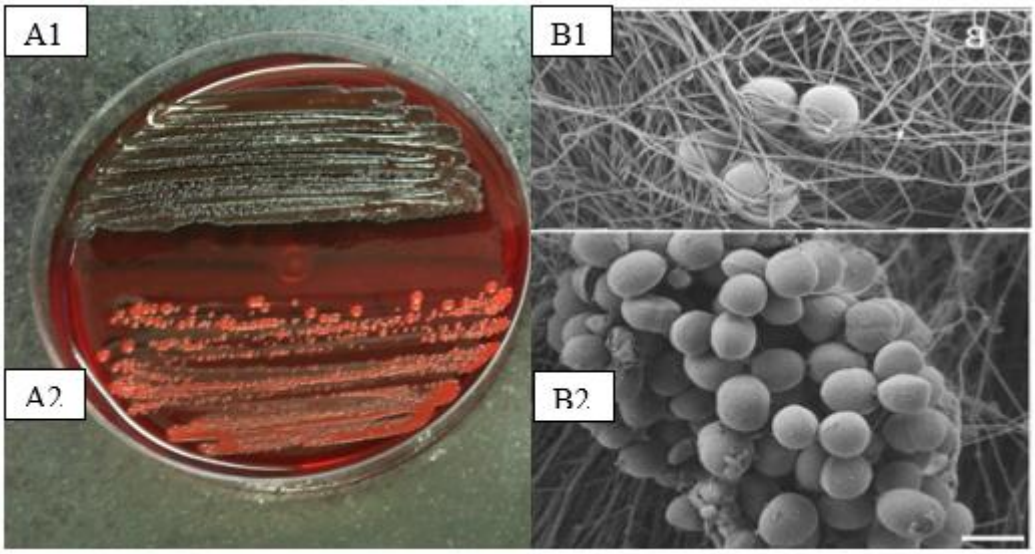

Figure No. 2. Field Emission Scanning electron microscopy (FESEM) showing a detailed interaction of the *Staphylococcus haemolyticus* with the PB matrix and *slime* production on CRA medium: (A1/B1) *Staphylococcus haemolyticus* (SH3754) strain showed *slime*-producing variant, exhibiting reddish-black colonies on CRA medium surface demonstrating bacterial aggregative sessile forms; (A2/B2) *Staphylococcus haemolyticus* (SH9N) strain was found as *slime*-negative in CRA medium, presenting pinkish-red smooth colonies, forming a biofilm structure on the pericardium matrix. Scale bars: 1µm.

Biofilm formation on hydrophobic and hydrophilic abiotic surfaces

Analysis of the influence of antibiotics by subinhibitory concentrations on pathogenicity of *S. haemolyticus* showed that multidrug-resistant MRSH strains grown in the presence of OXA, VAN, and LZD were able to produce biofilm on glass and polystyrene surfaces. OXA and/or VAN, LZD were unable to significantly inhibit biofilm formation on abiotic surfaces by MRSH strains (**Table 2**). Data of experiments without antibiotics showed that MDR *S. haemolyticus* SH-3754 (adult) and SH-9N (neonate) strains the ability of biofilm on hydrophobic (polystyrene) or hydrophilic (glass) abiotic surfaces but at different levels. Both strains expressed moderate adherence to polystyrene, while strong (+++) biofilm formation was detected on glass surfaces. Interestingly, both strains remained viable, although expressing moderate adherence on polystyrene surfaces in the presence of linezolid. SH-9N neonate strain expressed an increased ability of biofilm formation in the presence of oxacillin.

PCR detection of oxacillin-resistance, biofilm formation, and slime-producing genes.

Table No. 2 demonstrated that MDR *S. haemolyticus* strains presented the following tested genes: 9N (neonate) - *ica*A, *atl*, *fbp*; SH-3754 (adult) -*mec*A and *aap*, *atl*, *fbp*.

SH-9N neonate strain expressed an increased ability of biofilm formation independent of the presence of *mec*A gene coding for oxacillin-resistance.**Table No. 2:** Phenotypic and genotypic features of *slime* and biofilm formation on the bovine pericardium (BP) biomaterial and abiotic surfaces by MDR *Staphylococcus haemolyticus* strains.

Strains	*mec*A gene	*Slime* production				Biofilm formation												Biofilm genes
		Congo red agar medium				Polystyrene binding assays				Glass binding assays				BP patches				
		C	OXA	VAN	LZD	C	OXA	VAN	LZD	C	OXA	VAN	LZD	C	OXA	VAN	LZD	
SH-9N/neonate/MDR	-	-	-	-	-	++	++	++	+	+	+++	+	+	+	+	+	+	*fbp*,*atl*, and *ica*A
SH-3754/adult/MDR	+	+	+	+	+	++	++	++	++	+++	+++	+++	+++	+	+	+	+	*fbp*, *atl*, and *aap*

MDR- multidrug resistance; C-control; OXA– Oxacillin; VAN – Vancomycin; LZD- Linezolid; Adherence polystyrene: weakly (+), moderately (++), strongly (+++), non-adherent (-); Adherence to glass: (-) absence of adherence, (+)weakly adherent bacteria appeared as a ring at the interface between the medium and the air, moderately (++) bacteria attached on the side of the glass tubes), strongly (+++) bacteria attached on the side of the glass tubes and on the interface between the medium and the air. BP patches: positive on biofilm formation (+).

DISCUSSION

During the last decades, the biomaterials sector has grown in development and availability. The impact on improving the quality of life is undeniable and its future contribution should be numerically higher. BPAM has been used extensively in varied surgical procedures, including cardiovascular and thoracic surgery, iatrogenic injury of the trachea, ligament and tendon augmentation, penile prosthesis implants, pelvic organ prolapse, and guided bone regeneration in implantodontics. (Gupta *et al.* 2004; Rossouw & de Villier, 2005; Shin & Sohn, 2005; Lopes *et al.* 2007; Trabuco *et al.* 2007; Barbetakis *et al.* 2008; Athar *et al.* 2014; Costa *et al*. 2016; Lauterio *et al.* 2017; Zapater *et al.* 2019; Joyce *et al.* 2019).

The low rate of definitive infection linked to BPAM was previously reported. The authors suggested that BP biomaterials may be resistant to infection, and consequently, considered it appropriate to be used in infected patients (McMillan *et al.* 2012). Later, BPAM was also described as an alternative material in the treatment of infected vessels of immunocompromised transplant patients at high risk for ongoing or recurrent infections (Aroz *et al.* 2017). However, infection is always a problem of concern when dealing with the implantation of artificial materials. Some studies did not verify significant differences between synthetic and BPP in relation to the incidence of postoperative surgical-site infections (Derksen *et al.* 2008). Human pathogens were found involved in biomaterials-associated infections, including *Staphylococcus* spp. (Carballo, Ferreirós and Criado, 1992; Garcia-Bengoechea *et al.* 1995). Observing whether the patient has an additional source of infection that may contribute to the contamination of BPP is of extreme significance. Moreover, methods available for sterilization of BPPs may be problematic since they may partially inactivate contaminating bacteria and/or affect tissue integrity (Shamis *et al.* 2009). The low number of investigations reported in available literature related to the possibility of contamination of BPP by human pathogens indicated that further studies are needed. In the present study, BPPs contamination was demonstrated by MDR*S. Haemolyticus* blood isolates from neonate and adult hospitalized patients with catheter-related infections. The ability of biofilm formation stimulated bacterial colonization and persistence on BPP by *S. haemolyticus* strains. The influence of phenotypic and genotypic virulence features in the ability of biofilm formation on BPP by*S. haemolyticus* were also analyzed.

The extracellular matrix (ECM) of mammals comprises approximately 300 proteins, including collagen, proteoglycans, and complex glycoproteins. The ECM participates in multiple processes in host cells, as well as, bacterial colonization, the key step for establishing an infection. In addition to connective tissues, collagen proteins are also found as a major component of the skin, bone, cartilage, tendon, and blood vessels. Previous investigations showed that human pathogens can establish themselves on collagenous tissues through collagen-binding proteins (CBPs). Infection linked to BPPs may contribute to the persistence and dissemination of pathogens through the bloodstream to tissues and organ systems. Commercially available BPPs are essentially composed of wavy bundles of pure collagen type 1 fiber that have sufficient spaces for allowing cell infiltration (Li *et al.* 2011; Mc Millan *et al.* 2012; Bedair *et al.* 2017; Hiromoto *et al.* 2019; Jana *et al.* 2019). CBPs were found to involve the inability of biofilm formation by the invasive *Streptococcus parasanguinis* pathogen that contributed to the ability to successfully colonize and damage heart tissues, leading to endocarditis (Singh *et al.* 2010; Chen *et al.* 2020).

Biofilm formation is of significant concern in nosocomial infections because it protects pathogens from innate host response and antimicrobial agents therapies since it is an important form of growth that contributes to bacterial colonization and persistence on abiotic and biotic surfaces (Carballo, Ferreirós and Criado, 1992; Garcia-Bengoechea *et al.* 1995; Seng *et al.* 2017; Soliman *et al.* 2018). Previous studies demonstrated the ability of biofilm formation of *S. aureus* inoculated onto the surface of different implant materials placed subcutaneously in mice, including BPAM. The authors emphasized the ability of biofilm formation on the surfaces of implanted prostheses as one of the major causes of persistent infection following surgery of patients (Lorenz *et al.* 2011). During the last decades, multiple virulence factors, including antibiotic resistance, biofilm formation, and surface proteins with adhesive properties have been involved in mechanisms of CoNS survival, dissemination, and persistence in both human hosts and nosocomial environment have been investigated, mostly for CoNS (Klingenberg *et al.* 2007; Pereira *et al.* 2014; Sued *et al.* 2017).

S. haemolyticus has been reported as the second most frequently CoNS related to nosocomial infections, especially hematogenic infections and is also an increasing problem among cases related to implanted medical devices (Jain *et al.* 2004; Kumari *et al.* 2001; Klingenberg *et al.*

2007; Pereira *et al.* 2014; Czekaj *et al.* 2015; Panda & Singh, 2018; Argemi *et al.* 2019; Pereira-Ribeiro *et al.* 2019). Genomic characterization of oxacillin-resistant *S. epidermidis* (ORSE) and *S. haemolyticus* (OSRH) isolated from Brazilian medical centers demonstrated a large genetic diversity among isolates of both species and spread of some ORSE and ORSH PFGE-types among the assessed hospitals located in Rio de Janeiro (Nunes *et al.* 2005). Multidrug-resistant ORSH strains presenting susceptibility only to linezolid and vancomycin were isolated from sphygmomanometer and thermometer which are common fomites in direct contact with clinicians, staff, and patients (adults and neonates) used in intensive care units and surgical wards of a Brazilian teaching hospital. *S. haemolyticus* strains isolated from fomites expressed the ability of biofilm formation on glass, polystyrene, and polyurethane catheter surfaces. Most of the ORSH clinical isolates expressed MDR profiles and ability of biofilm formation on both polystyrene (96.7%) and glass (87%) surfaces (Pereira *et al*. 2014; Sued *et al.* 2017). Further studies verified that different clones of nosocomial *S. haemolyticus* isolated from blood samples of neonate and adult patients with catheter-related bloodstream infection expressed the ability of biofilm formation independent of the presence *ica*A and *mec*A genes. The ability of biofilm formation on abiotic surfaces by *S. haemolyticus* strains was not inhibited in the presence of different antimicrobial agents, such as vancomycin, linezolid, oxacillin, moxifloxacin, rifampicin, teicoplanin, and tigecycline. Interestingly, vancomycin and oxacillin did not inhibit the ability of biofilm formation on abiotic surfaces expressed by vancomycin-susceptible *S. haemolyticus* strains, including on polystyrene catheter surfaces. Moreover, MDR clinical isolates from neonate and adult patients with catheter-related bloodstream infections and fomites expressed a higher ability of biofilm formation in the presence of antimicrobial agents, including vancomycin. Therefore, MDR *S. haemolyticus* is a contemporary nosocomial pathogen of concern also involved in the contamination of invasive medical procedures, including the fifth largest country in the world - Brazil (Martini *et al*. 2016; Sued *et al.* 2017; Pereira-Ribeiro *et al.* 2019).

Presently, MDR *S. haemolyticus* blood isolates were found as contaminants of BP biomaterial, but at different levels. MDR *S. haemolyticus* strains obtained from neonate and adult patients expressed differences in the ability of interaction, survival, and biofilm formation within BPP. Data suggested the involvement of multiple selective activities of virulence mechanism expressed by *S. haemolyticus* during contamination, persistence, and dissemination from BPAM.

MDR *S. haemolyticus* 9N strain from neonate patient expressed a higher ability of interaction and biofilm formation on BPAM when compared to the MDR 3754 isolated from the adult patient. Data corroborated with previously reported findings of *S. haemolyticus* surface components for recognizing human adhesive matrix molecules. Interestingly, collagen-binding properties were found significantly higher for commensal strains when compared to *S. haemolyticus* clinical isolates from blood, catheter, and wounds (Paulsson *et al.* 1990; Madani *et al.* 2017; Wolden *et al.* 2020).

Biofilm formation is generally described as a multifactorial and multistep process with or without the production of extracellular polymeric substances (EPS) (Croes *et al.* 2009; Feuillie *et al.* 2017). The presence of extracellular *slime* amorphous material was not currently evident on mature biofilm composed of microcolonies of bacterial sessile forms adherent on the surface of commercially available BPPs comprised of collagen type I fibers and absence of cells produced by both *S. haemolyticus* blood isolates. MEV assays emphasized that BPAM arrangement exerted influence on adherence and biofilm formation of human pathogens, with consequent development and increase in the risk of infection by *S. haemolyticus* strains during clinical application.

Interestingly, MDR *S. haemolyticus* 9N (neonate) strain expressing higher adhesive properties, autoaggregation microcolonies, and ability of biofilm formation on BPP biomaterial was found unable of *slime* production on CRA medium. Both *S. haemolyticus* strains isolated from catheter-related infections that demonstrated the ability of biofilm formation and survival within BPP biomaterial also presented the ability of biofilm formation on abiotic hydrophilic (glass) and hydrophobic (polystyrene) surfaces, but at different levels. A higher intensity of biofilm was observed on hydrophilic (glass) abiotic material. Consequently, *S. haemolyticus* strains were currently found to express differences in mechanisms of biofilm formation and survival within BPP biomaterial. Data indicated that MDR *S. haemolyticus* strains related to cases of invasive diseases in neonates and adult patients may express a higher virulence potential, partially due to the concomitant ability of biofilm formation on varied biotic and abiotic elements present in human body tissue and/ or generally used in medical-invasive procedures and/or environmental conditions, such as BPAM, polyurethane catheter, glass surfaces, and other plastic materials.

The ability of biofilm formation on abiotic and biotic surfaces may act as a significant virulence factor that also contributes to the response to harmful and environmental conditions, including exposure to antimicrobial agents, and/or host immune response. Several studies have been investigating surface-attached communities and mechanisms of biofilm resistance to antimicrobial compounds. Biofilm formation has been related to increased expression of antimicrobial resistance by some clinical isolates, due to the acquisition of additional resistance to environmental conditions (Mah & Toole, 2001; Martini *et al,* 2016; Pereira-Ribeiro *et al.* 2019). In many opportunities, infections can often only be treated by removal of medical implants, thus increasing the trauma to the patient and the cost of treatment (Beaudoin *et al.* 2012; Stackhouse *et al.* 2012). Presently, *S. haemolyticus* biofilm formation activity on BPAM was found independent of the presence of *mec*A gene coding for oxacillin-resistance. Both BPAM-biofilm producer *S. haemolyticus* strains also expressed the ability of biofilm production in the presence of OXA, VAN, and LZD antimicrobial agents on abiotic, as shown on glass and polystyrene surfaces. SH-9N and SH-3754 strains were the ability to interact with BPP biomaterial, biofilm formation and remained viable in the presence of antibiotics. Although both *S. haemolyticus* strains were found susceptible to linezolid, they expressed the ability of biofilm formation and survival of sessile forms in the presence of the respective antimicrobial agent.

Interestingly, enhanced ability of biofilm formation was expressed by *S. haemolyticus* SH-9N strain in the presence of oxacillin, independent of the presence of *mec*A gene. Previous studies have demonstrated the multifactorial nature of virulence mechanisms of CoNS species, including the involvement of various enzymes, cytolysins, and surface substances on virulence mechanisms of *S. haemolyticus*. Intercellular adhesion mediated by the Aap and Bap proteins has been further investigated for some *Staphylococcus* pathogenic species. Additional components such as proteins, DNA, RNA, and polysaccharides other than PIA-intercellular adhesin have been also found involved in mechanisms of biofilm formation by CoNS (Klingenberg *et al.* 2007; Feuillie *et al.* 2017; Wolden *et al*. 2020). The capacity for biofilm formation is determined by many genetic factors. The main biofilm component is the Polysaccharide Intercellular Adhesin (PIA) molecule synthesized by enzymes encoded by the *ica*A gene that forms the ica ABCD operon (Nunes *et al.* 2005). Earlier investigations showed that only 42 and 47% of the MDR *S. haemolyticus* strains isolated from neonates and adult patients, respectively, harbored *ica*A gene. MDR *S. haemolyticus* MDR 3754 isolated from the

adult patient currently expressed ability of biofilm formation on BPP. Data emphasized that *S. haemolyticus* strains express the ability of biofilm formation independent of *ica*A gene presence (Pereira-Ribeiro *et al.* 2019).

Presently, SH-9N neonate strain exhibited the increased potential of biofilm formation molecules possibly favored by the presence and expression of the *ica*A gene in addition to other adhesins: autolysin (Atl), and fibrinogen binding protein (Fbp). Staphylococcal adhesins belong collectively to a group called MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) that includes collagen-binding proteins (cna) and fibrinogen-binding proteins (Fbp), which are capable of binding with various extracellular mammalian proteins and with abiotic surfaces. In CoNS, the first stage of biofilm formation takes place via proteins expressed on the bacterial cell wall, including autolysin (Atl), and fibrinogen binding protein (Fbp). The accumulation stage is characterized by the formation of polysaccharide intercellular adhesin (PIA). However, in the formation of a PIA-independent biofilm by CoNS, a significant role is played by the accumulation-associated protein (aap) (Gajewska & Chajecka-Wierzchowska. 2020). Presently, PIA-independent biofilm formation was currently expressed by MDR *S. haemolyticus* 3754 isolated from the adult patient. The genes coding for the adhesins - autolysin (Atl), and fibrinogen binding protein (Fbp) – were detected for both MDR *S. haemolyticus* strains with adhesive properties to collagen type I fibers, autoaggregation microcolonies, and biofilm formation on BPAM.

In conclusion, our study suggests that collagen-binding properties and ability of biofilm formation were currently verified as relevant virulence mechanisms that favored colonization and persistence in bovine pericardium biomaterial by MDR *S. haemolyticus* strains isolated from patients with catheter-related infections. Data emphasize the involvement of multiple selective activities of virulence mechanism of varied nosocomial pathogens in contamination, persistence, and dissemination from biomaterials, through the blood stream and tissues during human infections, such as MDR *S. haemolyticus* strains.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: Sued-Karam, BR, Pereira-Ribeiro, PMA and Vasconcelos, RS; **Performed the experiments:** Sued-Karam, BR, Pereira-Ribeiro, PMA; Vasconcelos, RS; Cabral-Oliveira, GG; Ribeiro, FC; Olivella, JGB; Nogueira, BA; Fracalanzza, SEL; dos Santos, LS. **Data analysis and draft of the manuscript were performed by** Sued-Karam, BR; Pereira-Ribeiro, PMA; Lopes-Torres, EJ and Mattos-Guaraldi, AL. All authors approved the final version of the manuscript for submission.

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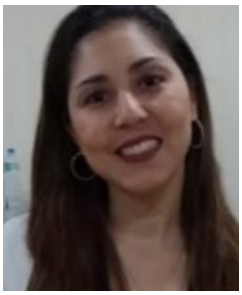
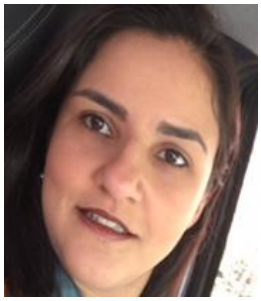
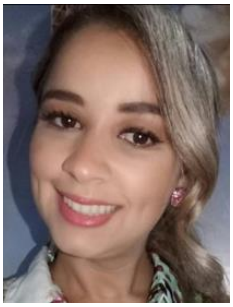
	**Bruna Ribeiro Sued Karam - *Corresponding Author*** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
	**Paula Marcele Afonso Pereira Ribeiro** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.

	**Renata da Silva Vasconcelos** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
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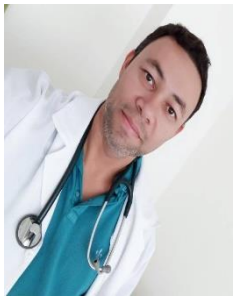	**Felipe Caldas Ribeiro** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
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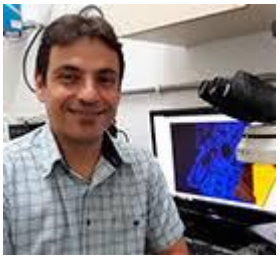	**Eduardo José Lopes-Torres** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
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