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Biofilm formation in catheter-related infections by Panton-Valentine leukocidin-producing *Staphylococcus aureus*

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Summary. The use of invasive techniques, such as intravascular catheter insertion, and the formation of biofilms in several devices by methicillin-resistant *Staphylococcus aureus* (MRSA) have contributed to the increased number of septic patients, morbidity and mortality. This study aimed to evaluate the virulence of strains through catheter colonization and identification of microbial biofilm, as well as pathological changes on the colonized skin. An experimental biofilm formation model utilized catheter fragments implanted subcutaneously in 25 Swiss mice. The technique consisted of inoculating a catheter fragment on the back of each animal, followed by intradermal inoculation of 50 µl of bacterial suspension at 1.0×10^7 colony forming units/ml. After 96 h, catheters were removed for macroscopic analysis and evaluated through culture. Local skin fragments were also extracted for histopathology analysis. *Staphylococcus aureus* can adhere to catheters, colonize and form biofilms. The high amount of viable bacterial cells colonizing catheters and virulence factors can lead to severe infections of skin and adjacent tissues. [**Int Microbiol** 19(4): 199-207 (2016)]

Keywords: *Staphylococcus aureus* · biofilms · infections · MRSA · Panton-Valentine leukocidin

Introduction

Staphylococcus aureus is commonly observed colonizing several parts of the body in healthy individuals, such as skin, nasal cavity, throat and intestine [5,9,10]. Depending on the carrier conditions, it can cause severe infections such as meningitis, endocarditis and sepsis. From primary colonization sites, it can reach other areas where natural defense barriers (skin and mucosa) are compromised by trauma or surgical procedures, thus causing infection [36,48].

Considering that these microorganisms belong to normal skin microbiota, they can cause a primary infection in the region where an intravascular catheter is inserted and then gain access to the bloodstream, consequently causing bacteremia. The infection may aggravate if the *S. aureus* strain is methicillin-resistant (MRSA) [20].

Different types of toxins produced by *S. aureus*, such as

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Panton-Valentine leukocidin (PVL), are responsible for specific inflammatory responses to infectious processes in different degrees of severity and systemic symptoms. PVL is widely associated with severe skin infections and necrotizing pneumonia [22]. This protein is encoded by the LukPV operon, which contains *lukF*-PV and *lukS*-PV genes inserted into temperate bacteriophages such as PhiSLT [7,15,19,27,44]. These phages carrying the genes for the production of PVL are more associated with strains containing the chromosomal cassette SCC*mec* type IV and represent a major virulence factor [50]. Increasingly, PVL-producing strains have been reported in hospital infections associated with intravascular and urinary catheters, thus colonizing and forming biofilms on these devices [12].

Another major factor in hospital infections is biofilm formation on surgical materials. The pathogenicity of *S. aureus* is defined as an association of microbial cells attached to biotic or abiotic surfaces involved in a complex extracellular polymeric matrix [1,43]. When a medical device is implanted, it is immediately covered with tissue matrix proteins, laminin, fibronectin, fibrinogen and collagen. The presence of *S. aureus* on medical devices prior to implementation may promote interactions with the host tissue, causing local and systemic infections through bacteremia. This is caused by the adhesion proteins covalently attached to the peptidoglycan cell wall, as well as FnBPA and FnBPB capable of binding to both fibronectin and fibrinogen, thus providing an interaction with the host tissue and causing local and systemic infection through bacteremia. These binding proteins are named as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [2,14,18,23,34,40].

The expression of *ica*ADBC gene in *S. aureus* promotes the synthesis of polysaccharide intercellular adhesin (PIA) responsible for the association and adhesion of microbial cells and formation of biofilms [33,40]. PIA structure is also responsible for the formation of a capsule around the bacterial colonies, preventing their recognition by the immune system [4,31,47]. The union of several species of bacteria in a biofilm provides a great advantage over the effectiveness of antibiotics, innate immune defense as antimicrobial peptides (AMPs) and phagocytosis by leukocytes [23,35,40].

When a biofilm reaches a boundary density the displacement of bacterial cells or small cell aggregates occurs [17] mediated by the *agr* gene (accessory gene regulator), which activates an intercellular communication system called quorum-sensing [6,40,41,47]. The *agr* gene expresses the production of peptides to break the cell junctions, allowing bacterial cells from the biofilm to remain suspended in the medium [8,24,42], and thus causing local infections [17], bacteremia, colonization of other tissues and organs, and consequently the production of more biofilms [8,24,42]. For these reasons, infections caused by bacteria forming biofilms are extremely difficult to eliminate and a great challenge for treatment [33].

The goal of this study was to evaluate the colonization and formation of biofilms in clinically isolated MRSA and PVL-producing MRSA clones through in vitro and in vivo studies. In addition, it aimed to quantify viable bacterial cells adhered to the catheter and perform anatomicopathological and histopathological analysis of the colonized skin.

Material and methods

Animals used in the study. This study was approved by the Ethics Committee on Animal Research from Federal Fluminense University under the registration number 439/2013. A total of 25 Swiss inbred mice, males and six-week-old were used in this study. They weighed approximately 34 g

Table 1. Distribution and source of bacterial samples in different experimental groups

Groups	Inoculum 1.0×10^7 CFU/ml	Genes		N
		mecA	*lukF-PV; lukS-PV*	
pvl (–) MSSA	Sample isolated from nasal colonization	(−)	(−)	5
pvl (+) MSSA	Sample isolated from nasal colonization	(−)	(+)	5
pvl (+) MRSA	Sample isolated from venous blood of patient with severe pulmonary infection	(+)	(+)	5
pvl (+) MRSA USA300 WT	Sample isolated from venous blood of septic patient	(+)	(+)	5
Control	Physiological saline (0.9% NaCl)	Absence of microorganisms		5

Note: *pvl* (+) MRSA USA300 WT samples were donate by Prof. BinhAn Diep, University of California, San Francisco, CA, USA.

each, were specific-pathogens-free (SPF) and divided in five animals per group (Table 1). The animals were kept in individual cages and received standard chow diet and filtered water *ad libitum*, maintained in light-dark cycles at 21°C (± 2).

All procedures that could result in anxiety and/or pain were conducted under anesthesia by isoflurane FORANE (2-chlorine-2-[difluorometoxy]-1.1.1-trifluor-ethane) [24,26].

Identification of *Staphylococcus aureus* and the genes of virulence and resistance. Bacterial samples belonged to the Laboratory of Molecular Epidemiology and Biotechnology, Rodolpho Albino University Laboratory from the Federal Fluminense University. Samples were conserved in brain heart infusion (BHI) plus 10% glycerol and frozen at −80°C.

Staphylococcus aureus was identified by standard microbiological procedures: Gram staining, colonial morphology, fermentation of mannitol-salt [16], catalase production [30] and coagulase production [29]. Separately, the species was confirmed by polymerase chain reaction (PCR) for 442-bp chromosomal DNA fragment, according to Martineau et al. [28].

Methicillin resistance was identified using PCR for *mecA* gene according to the protocol of Oliveira and Lencastre [32], and the virulence genes *lukF-PV* and *lukS-PV*, responsible for the production of PVL, were identified according to the protocol established by Lina et al. [27].

Bacterial samples selected for this study had the following characteristics: methicillin-susceptible and non-PVL-producing strains isolated from nasal colonization, *pvl* (−) MSSA; methicillin-susceptible and PVL-producing strains isolated from nasal colonization, *pvl* (+) MSSA; methicillin-resistant and PVL-producing strains isolated from peripheral blood of a patient with severe pulmonary infection, *pvl* (+) MRSA; methicillin-resistant and PVL-producing strains isolated from peripheral blood of patient with bacteremia, *pvl* (+) MRSA USA300 WT.

Biofilm formation and in vitro cell viability assay. Bacterial suspensions of each sample were prepared at 0.5 McFarland turbidity scale 10^8 colony forming units/ml (CFU/ml) in tryptic soy broth (TSB) with 1% glucose using mild stirring (1800 rpm) at 37 °C for 24 h. Subsequently, 200 µl of each inoculum was deposited in a 96-well polystyrene plate with flat bottom and incubated at 37°C for 24 h along with the negative control, sterile TSB. The resulted biofilm was stained with 3% crystal violet for 15 minutes. The optical density of biofilm (DOB) was performed using Optima fluorimeter Elisa Fluostart BMG Labtech at 590 nm and Optima start software, as described by Hassan et al. [21].

The reduction of tetrazolium salt XTT (2.3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5 carboxyanilide) was performed in order to determine the metabolic activity of cells composing biofilm. The analysis, performed in triplicate, was conducted at 492 nm, as described by Chaieb et al. [11].

Preparation of the bacterial inoculum. Bacterial samples were obtained from infected tissues asymptomatic or nasal colonization, preserved in brain heart infusion (BHI) containing 10% glycerol, frozen at −80°C and thawed 2 h prior to inoculum preparation.

Twenty-four hours before the study, bacterial samples were cultivated in tryptic soy agar (TSA). Colonies were suspended in sterile test tube containing 1000 µl of sterile saline (0.9% NaCl) and then serial dilutions were made up to the density of 1.0×10^7 CFU/ml.

Catheter insertion procedure. Animals were anesthetized and had the dorsolateral region of their neck shaved and decontaminated with 70% ethanol. A subcutaneous air pouch measuring about 1.5 cm was made through an incision. Thereafter, a peripheral intravenous catheter (Becton Dickinson, Argentina S.R.L) measuring 5.0 mm and 2.5 mm of diameter was introduced into the pouch under aseptic conditions [3,4,26]. The incision was closed with synthetic surgical glue Glubran2 (GEM S.r.I, Italy).

After 24 h of observation and confirmation that catheters were neither infected nor rejected, the animals were manually restrained and intradermally inoculated with bacterial suspension. The procedure kept a limit distance of 1 cm from the insertion of the catheter fragment and used disposable insulin syringe BD Ultra-FineTM (0.3 ml/30UI, needle 8 mm (5/16”) × 0.3 mm (30 G). Each animal was inoculated with 50 µl of bacterial suspension with a density of 1.0×10^7 CFU/ml in sterile saline [26], except the control group, which received only sterile saline.

Macroscopic examination of the backs of mice. After 96 h, the estimated time for consolidation of the infection and colonization of the catheter, animals were euthanized with Isoflurane FORANE through inhalation in closed campanula. Death was confirmed by cardiac and respiratory arrest, absence of corneal reflex and fall of body temperature < 25°C [26]. The backs were comparatively analyzed using the control group as standard, seeking for any morphological alteration, as well as the presence of infection and edema.

Macroscopic examination of catheter. Catheter fragments were removed from the backs of mice through incision and the adhered material was preserved. It was observed if the material adhered to the inner or outer surface of the catheter and its aspect, if viscous or liquid, with vitreous luster or opaque and the color.

Colonization and biofilm formation on catheter. The explanted catheters were separately placed in test tubes containing 1 ml of sterile saline solution (0.9% NaCl) for quantitative culture and subsequently vortexed during one minute (1800 rpm), an aliquot of 100 µl was cultivated in blood agar 5% Merckoplate (pH 6.5–7.5) using aerobic conditions at 37°C and daily examined up to 48 h. The calculation of the number of CFU was correlated with the initial dilution. The quantitative culture was reported as CFU/ml and growth $\geq 10^3$ CFU/ml (≥ 1000 colonies) confirmed the catheter colonization [4,38]. Five bacterial colonies obtained from blood agar culture were isolated to confirm the presence of *S. aureus* using the methods: Gram staining, fermentation of mannitol-salt agar, catalase production and coagulase production, as reported previously.

Histological analysis of dorsal tissue. One dorsal skin fragment measuring about 1 cm wide and 1 cm long was extracted for the preparation of histological slides. Tissue samples were stored in 10% formaldehyde with pH between 0.6 and 0.7 during 48 h and then submitted to dehydration, diafanization and inclusion in paraffin. Fragments were 3 µm thick and stained with hematoxylin and eosin (H&E). The slides were observed in optical microscope (LX 500 model) and photographed using IVM 5000 camera and ProgRes Capture Pro 2.7 software for the description of the histopathology inflammatory processes.

Statistical analysis. Statistical analysis evaluated the quantification of solutions obtained from the colonization of catheters 96 h after explanation. Multiple comparison test used graphic column. The SPSS software version 10.0 was utilized with statistical significance level $\alpha \leq 0.05$.

Results

Biofilm formation and in vitro cell viability assay. All the bacterial samples had high potential to adhere to the surface of the plates, as confirmed by biofilm formation through staining with violet crystal and Elisa Fluostar Optima-BMG Labtech fluorimeter. A large amount of metabolically active bacterial cells in biofilms were also observed by the XTT reduction in all groups in comparison with the control group (Fig. 1).

Macroscopic examination of the backs of mice. None of the animals died or presented signs of anorexia, diarrhea and behavioral changes 96 h prior to the study.

In the control groups, *pvl* (−) MSSA and *pvl* (+) MRSA USA300 WT, no evidence of infection were observed and the skin remained with normal appearance (Fig. 2A, B and E). Swelling and redness were observed at the site of catheter insertion in the group *pvl* (+) MSSA (Fig. 2C), as well as erythematous lesions where the bacterial suspension was injected. The *pvl* (+) MRSA group presented severe edema causing suture detachment (Fig. 2D).

Macroscopic observations of catheters. After 96 hour, a yellowish film was observed adhered to both the inner and outer surfaces of catheters in the groups *pvl* (+) MSSA and *pvl* (+) MRSA, confirming biofilm formation (Fig. 2H and 2K). Adhered materials were not observed in the control groups and *pvl* (+) MRSA USA300 WT (Fig. 2F and 2J), but

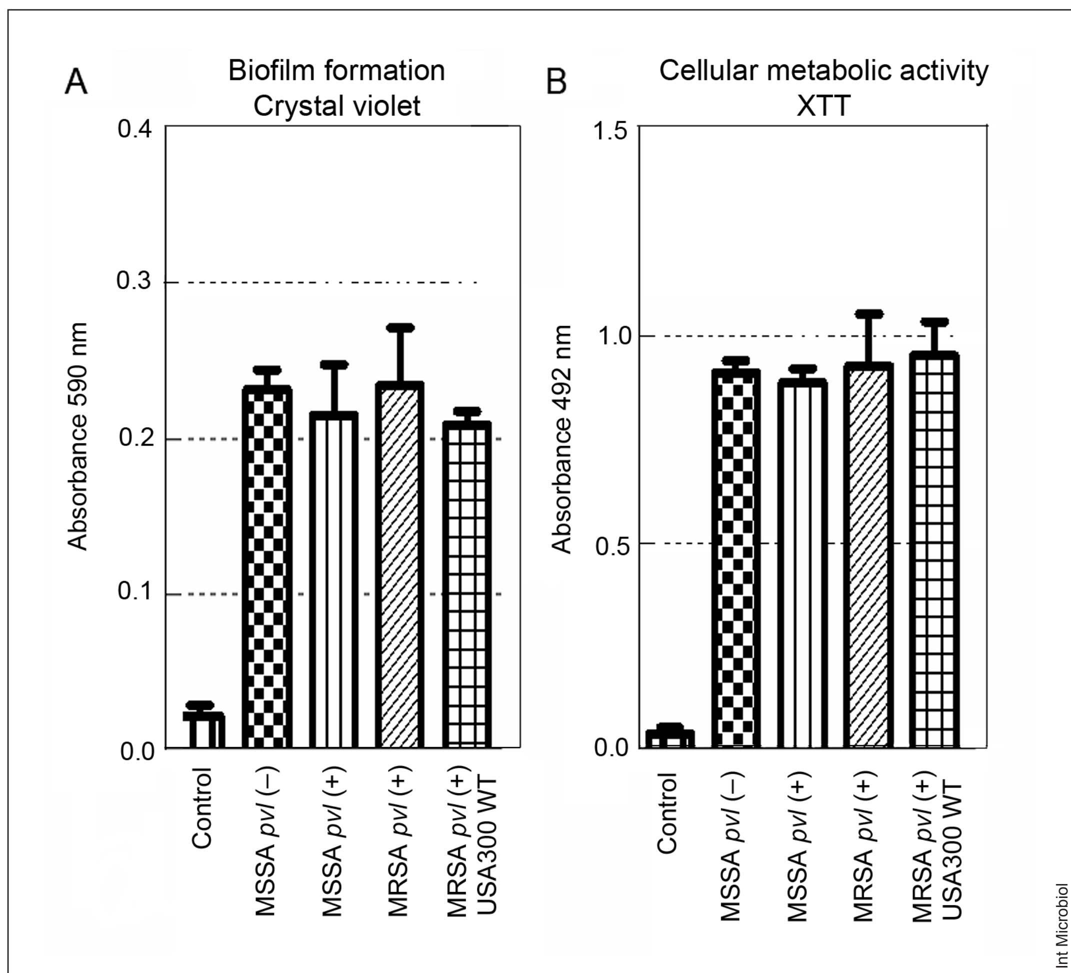


Fig. 1. **(A)** All bacterial samples were able to colonize and form biofilms through crystal violet staining method, *pvl* (−) MRSA with similar concentration values of *pvl* (+) MRSA, and *pvl* (+) MRSA with similar values of *pvl* (+) MRSA USA300 WT. **(B)** All bacterial samples presented similar amount of metabolically active cells in the biofilm: values were expressed by XTT reduction method.

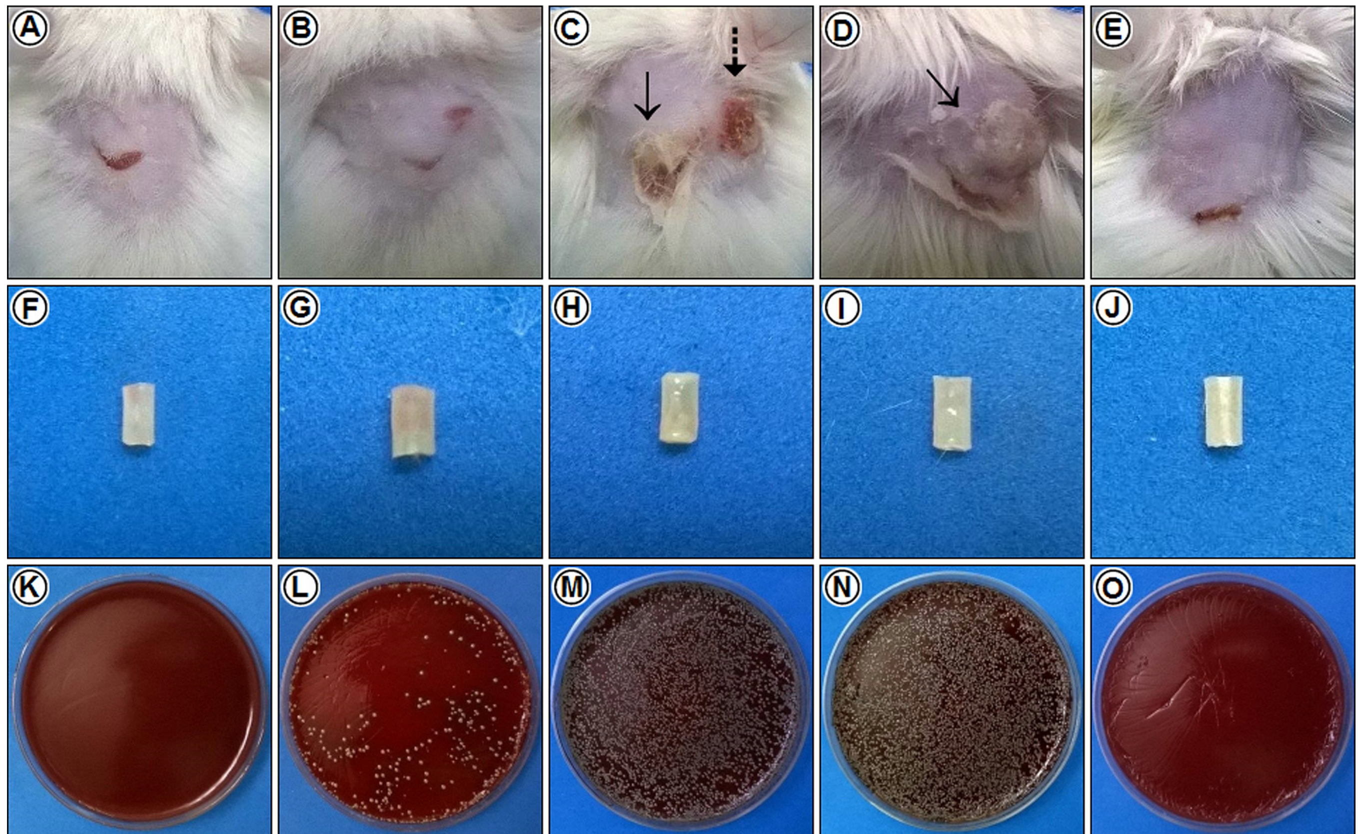


Fig. 2. The mice dorsal region, the site of insertion of the catheter. (A) control group; **(B)** *pvl* (−) MSSA and **(E)** *pvl* (+) MRSA USA300 WT: absence of local infection and morphological changes in skin; **(C)** *pvl* (+) MSSA: infection with edema and hyperemia in the skin (arrow), erythematosus lesion at inoculation site (dotted arrow); **(D)** *pvl* (+) MRSA: intense edema and hyperemia (arrow). **Catheter fragments extracted after 96 h: (H)** *pvl* (+) MSSA and **(I)** *pvl* (+) MRSA: yellowish material adhered to inner and outer surface of the catheter; **(G)** *pvl* (−) MSSA: reddish material adhered to inner surface of the catheter; **(F)** control group and **(J)** *pvl* (+) MRSA USA300 WT: absence of material adhered to catheter surfaces. **Bacterial culture obtained from material adhered to catheter: (M)** *pvl* (+) MSSA and **(N)** *pvl* (+) MRSA: cell cultures showing bacterial colonization higher than 1000 CFU/cm^2; **(L)** *pvl* (–) MSSA: 523 CFU/cm^2 colonizing the catheter; **(K)** control group and **(O)** *pvl* (+) MRSA USA300 WT: absence of bacterial colonies.

the group *pvl* (−) MSSA had a reddish material adhered to the inner surface (Fig. 2E).

Colonization and biofilm formation on catheters. Quantitative culture revealed the absence of bacterial colonies in control and *pvl* (+) MRSA USA300 WT groups (Fig. 2K, O and Fig. 3). The *pvl* (−) MSSA group showed only one catheter with 523 CFU/cm^2 (Fig. 2L and Fig. 3). Colonies counting were higher than 1000 CFU/cm^2 in all catheters from the groups *pvl* (+) MSSA and *pvl* (+) MRSA (Fig. 2M, N and Fig. 3). All colonies isolated from blood agar culture were *S. aureus*.

Histopathological analysis of dorsal tissue. Histopathological analysis of catheter fragments in the control group revealed an intact epidermis with corneal layer. Dermis had normal cellularity and conjunctive tissues with its attachments (Fig. 4A). The *pvl* (–) MSSA group preserved the epidermis and dermis. However, adipocytes in hypodermis showed increased cellularity in the inflammatory infiltrate composed of polynuclear/mononuclear leukocytes (Fig. 4B and C). The *pvl* (+) MSSA group showed intact dermis and epidermis. The hypodermis presented reduced adipocytes and intense inflammatory infiltrate composed of polynuclear/mononuclear leukocytes, fibrin and red blood cells (Fig. 4D and E). The *pvl* (+) MRSA group had normal dermis and epidermis, but the hypodermis presented edema and capillary congestion amongst adipocytes. A necrotic area was observed below the hypodermis with mixed inflammatory cell infiltrate containing polynuclear/mononuclear leukocytes and fibrin

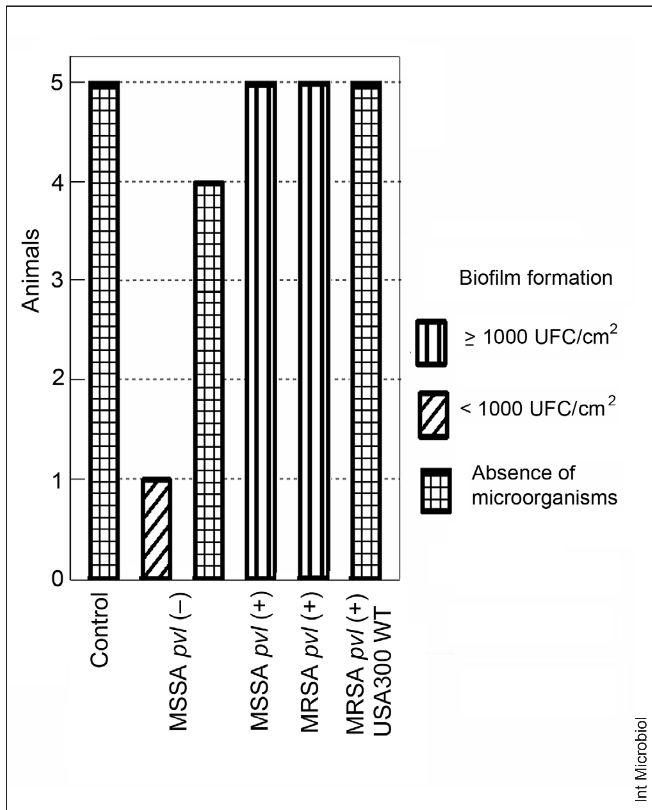


Fig. 3. The comparative colonization of catheter fragments. None of the catheters in the control group and *pvl* (+) MRSA USA300 WT showed bacterial colonies. The *pvl* (−) MSSA group showed only 523 CFU/cm^2 while the *pvl* (+) MSSA and *pvl* (+) MRSA groups presented more than 1000 CFU/cm^2 in all catheters.

(Fig. 4F and G). Finally, the histopathological examination of skins from the *pvl* (+) MRSA USA300 WT group showed preserved epidermis and dermis. A lower number of adipocytes in hypodermis was also observed, as well as mixed inflammatory cell infiltrate evolving to the dermis and capillary congestion (Fig. 4H and I).

Discussion

Approximately 45% of hospital infections are associated with contaminated materials or implanted medical devices. MRSA-related infections in catheters have been a severe complication in vascular surgery, increasing morbidity and mortality in hospitalized patients [46]. Intravascular grafts are usually susceptible to colonization by microorganisms, causing infection through direct contamination during implantation or bacteremia after surgical procedures. The diagnosis of catheter-related infections is difficult because there is no relationship between clinical and microbiological laboratory findings. Furthermore, positive cultures may be related to both catheter colonization and contamination. Prevention of this type of infection is essential because it may result in graft excision, morbidity and mortality [26,42].

The contamination of a catheter is confirmed by removing it from the site of insertion in the patient and cultivation of its distal tips. The isolation of a same microorganism from both an intravascular catheter tip and patient's blood with systemic infection suggests that the colonizing microorganism could be the cause of the disease [42]. Several methods are used for catheter tip culture, the gold standard being quantitative or semi-quantitative analysis [42] with 80% of sensitivity [39]. In the present study, the quantitative method was chosen based on sonication of catheter fragments in order to obtain the adhered microorganisms.

The absence of behavioral and physiological changes and mortality in our study indicates that the inoculation method did not cause systemic infection. The insertion of subcutaneous catheter induces local skin infection; however, in a hospital environment, microorganisms from an intravenous catheter can reach the bloodstream, causing bacteremia and systemic infection.

In groups inoculated with *pvl* (+) MSSA and *pvl* (+) MRSA strains, the infection presented severe localized edema in early inflammatory processes. Different aspects reported by Santana et al, such as the change of red skin color to cyanotic and epidermal skin detachment, suggested necrosis. These evidences suggest that the production of PVL could be associated with increased infections of skin and soft tissues [37]. Despite the fact that the USA300 strain is commonly associated with epidemic infections of skin in USA communities [25,45], no macroscopic lesions were observed in the groups *pvl* (−) MSSA and *pvl* (+) MRSA USA300 WT.

A yellowish film adhered to internal and external surfaces of explanted catheter fragments in the groups *pvl* (+) MSSA and *pvl* (+) MRSA suggested biofilm formation. Similar results were obtained by Santana et al. [37] in *S. aureus* strains susceptible and resistant to methicillin. The cultivation of the material adhered to the catheter fragment in *pvl* (−) MSSA group presented only 523 CFU/cm^2 and did not confirm the formation of biofilm, according to the criteria established by Atahan et al. [4] and Schaechter and Marangoni [38]. Nevertheless, it is still a potential site for infection, and biofilm might not have been formed in this group because it does not

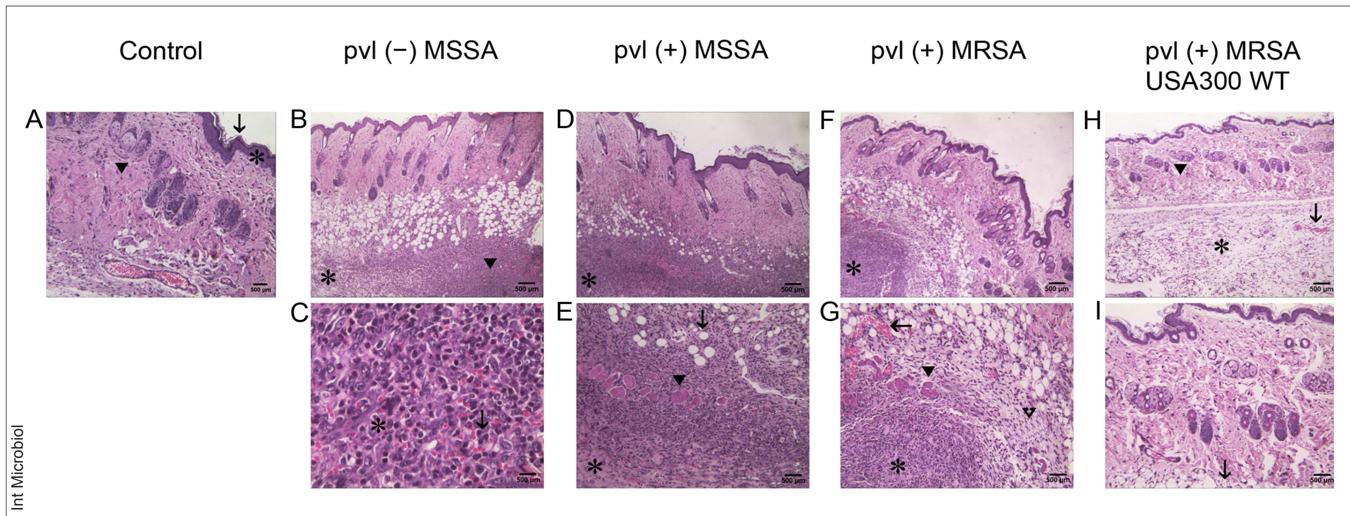


Fig. 4. Microscopy of back skin. Control group (stained with H&E): **(A)** intact epidermis (asterisk) and corneal layer (arrow), dermis with attachments (tip arrow). The *pvl* (−) MSSA group: (**B**) increased cellularity in the subcutaneous adipocytes with inflammatory infiltrate (asterisk), necrotic area (tip arrow); **(C)** large number of red blood cells (arrow), mixed inflammatory infiltrate composed of mononuclear/polynuclear cells (asterisk). The *pvl* (+) MSSA group: **(D)** inflammatory afflux (asterisk); **(E)** reduced number of adipocytes in hypodermis (arrow), mixed inflammatory influx (asterisk) and fibrin (tip arrow). The *pvl* (+) MRSA group: **(F)** subcutaneous necrotic area (asterisk); **(G)** capillary congestion (arrow), edema (tip of hollow arrow), fibrin (tip arrow), necrotic area with inflammatory infiltrate (asterisk). The *pvl* (+) MRSA USA300 WT group: **(H)** low number of adipocytes in hypodermis (tip arrow), capillary congestion (arrow), edema with inflammatory influx (asterisk); **(I)** low number of adipocytes with inflammatory influx and perfusion in the dermis (arrow).

express specific genes responsible for the production of surface proteins that recognize adhesins. *pvl* (+) MSSA and *pvl* (+) MRSA groups had a yellowish film. The culture of 100 µl solution confirmed catheter colonization in a concentration exceeding 1000 CFU/cm^2 and the formation of biofilms. The colonies isolated from bacterial cultures were confirmed as *S. aureus*, which may migrate to other sites, adhere to medical devices, and thus form biofilm and cause infections. Similar results were observed by Atahan et al. in groups without antimicrobial prophylaxis. Another study by Santana et al. using a method of scrolling also found that a film around a catheter in MSSA and MRSA groups was constituted by microorganisms at a density higher than 1000 CFU/cm^2 [37].

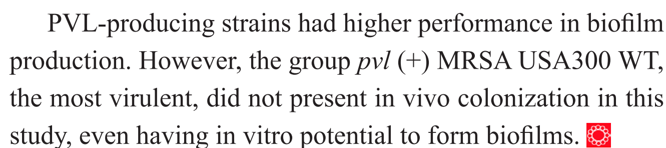
PVL-producing strains have caused severe skin infections associated with colonization and biofilm production, thus suggesting the expression of *icaC* gene. The association of biofilm production in catheter fragments and production of PVL through in vivo studies of *S. aureus* has not been described in the literature. There was no association of resistance to β-lactams and higher or lower production of biofilm in our study. The examination of the dorsal skin of animals in the control group revealed intact and preserved structures, thus we considered them as standard for comparison with other groups. The origin of inflammatory processes in the infected groups was below the hypodermis, where the catheter fragment was introduced. Although non-PVL-producing strains did not form biofilm with the same intensity as PVL-producing strains, the inflammatory processes presented similar intensities. These results corroborate that *pvl* (+) *S. aureus* tend to be more virulent than *pvl* (−) *S. aureus* and therefore associated with infections of skin and soft tissues [13]. Wardenburg et al. have utilized subcutaneous injections in the right flank of mice using bacterial suspensions of *S. aureus* at a density of 1.0×10^7 CFU/ml. The LAC and LAC*Δpvl* strains demonstrated skin abscess with dermonecrotics after 96 h of infection [49]. Similar aspects were also observed in all groups of our study.

Storti et al. [42] analyzed 118 tips of central venous catheters in adult patients by quantitative culture and correlated colony counting with initial dilution. They observed growth ≥ 10^3 CFU/ml and confirmed that 50% of catheter-related infections were caused by *S. aureus*, including four cases of bacteremia, and that the most frequently isolated microorganisms were MRSA [42]. *Staphylococcus* spp. proved to be most frequently isolated microorganism in catheter tips. The source of infection may be the patient's skin because through material handling by medical staff during surgical procedures. Therefore, data demonstrate the high level of virulence of these mi-

croorganisms and the importance of prevention and treatment [42].

Staphylococcus aureus can adhere to catheters, colonize and form biofilms. The amount of bacterial cells (colony forming units) is deeply related to higher or lower degrees of infection, including adjacent tissues. Intravascular catheter colonization by *S. aureus* can gain access to the bloodstream and cause bacteremia.

PVL-producing strains had higher performance in biofilm production. However, the group *pvl* (+) MRSA USA300 WT, the most virulent, did not present in vivo colonization in this study, even having in vitro potential to form biofilms.



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Competing interests. None declared.

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