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Staphylococcus spp. present in peripheral intravenous catheters, their virulence factors and antibiotic resistance

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Nádia Isabel Almeida Osório, Professora Adjunta do Departamento de Ciências Biomédicas Laboratoriais da Escola Superior de Tecnologia da Saúde de Coimbra e coorientação da Doutora Cláudia Sofia Soares de Oliveira, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

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To my mother and my father of heart, to my three brothers, to my grandmother Margarida and to my boyfriend.

Especially the three stars that shine in the sky and that look for me - grandfather Fernando, Tiinha and Dona Irene.

o júri

presidente

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palavras-chave

Cateter Intravenoso Periférico; *Staphylococcus* spp., Fatores de Virulência; Resistência aos Antibióticos

resumo

A inserção de um cateter intravenoso periférico (CVP) é um dos procedimentos invasivos mais frequentemente realizados em ambiente hospitalar. No entanto, os CVPs falham correntemente antes da conclusão do tratamento intravenoso e aquando da sua inserção o risco de infeção aumenta exponencialmente. Existem poucos estudos que avaliam a contaminação deste dispositivo médico vascular e que caracterizam os microrganismos associados quanto à produção de fatores de virulência e resistência aos antimicrobianos.

Neste estudo fomos avaliar a contaminação microbiana de CVPs, identificando os microrganismos mais prevalentes e estudando os seus fatores de virulência e resistência a antibióticos.

Um total de 110 pontas de CVPs foram analisadas usando a metodologia de Maki *et* al. e microrganismos foram identificados. *Staphylococcus* spp. foram posteriormente estudados quanto ao perfil de susceptibilidade aos antimicrobianos pelo método de difusão em disco e com base no fenótipo de cefoxitina foram ainda classificados em estirpes resistentes à meticilina. Foi feito também um *screening* para o gene *mecA* por PCR e MIC-Vancomicina determinado por *e-test*, testou-se a atividade proteolítica e hemolítica em placa de *Skim milk* a 1% e gelose de sangue, respetivamente. A formação de biofilme foi avaliada em microplaca com leitura através de cloreto de iodonitrotetrazólio (INT).

Cerca de 30% dos CVPs estavam contaminados e o género mais prevalente foi *Staphylococcus* spp., 48.8%. Este género apresentou resistência à penicilina (91%), eritromicina (82%), ciprofloxacina (64%) e cefoxitina (59%). Detetou-se 59% de estirpes resistentes à meticilina e presença do gene *mecA* em 82% dos isolados testados. Relativamente aos fatores de virulência, 36.4% apresentaram α-hemólise e 22.7% β-hemólise, 63.6% produziam proteases e 63.6% apresentaram capacidade de formar biofilme. É de salientar que 36.4% dos isolados foram simultaneamente resistentes à meticilina e apresentaram expressão de proteases e/ou hemolisinas, formação de biofilme and MIC para vancomicina superiores a 2µg/mL.

Deste modo, o nosso estudo evidenciou contaminação de CVPs principalmente por *Staphylococcus* spp, com elevada patogenicidade demonstrada pela presença de fatores de virulência, assim como resistência a antibióticos. A produção de fatores virulência permite fortalecer a adesão e a permanência dos microrganismos no cateter. Ao associarmos ainda a resistência aos antimicrobianos, o tratamento de infeções relacionadas torna-se mais difícil e as opções de tratamento escassas. Estes dados devem ser considerados pelos profissionais de saúde que devem adotar medidas preventivas para minimização do risco de contaminação e consequente redução das infecções relacionadas ao uso de cateteres intravenosos periféricos.

keywords

Peripheral Intravenous Catheter; *Staphylococcus* spp.,Virulence Factors; Antibiotic Resistance

abstract

The insertion of a peripheral intravenous catheter (PIVC) is one of the most frequently performed invasive procedures in the hospital setting. However, PIVCs usually fail before the completion of intravenous treatment and upon insertion the risk of infection increases exponentially. There are few studies evaluating the contamination of this vascular medical device and characterizing the associated microorganisms regarding the production of virulence factors and antimicrobial resistance.

A total of 110 PIVCs ends were analyzed using the Maki *et* al. methodology and microorganisms were identified. The *Staphylococcus* spp. were subsequently studied for the antimicrobial susceptibility profile by disc diffusion method and based on the cefoxitin phenotype were further classified into strains resistant to methicillin. A screening for the *mecA* gene was also done by PCR and MIC-vancomycin as determined by E-test, proteolytic and hemolytic activity on Skim milk 1% plate and blood agar, respectively. The biofilm formation was evaluated on microplate reading through iodonitrotetrazolium chloride 95% (INT).

About 30% of PIVCs were contaminated and the most prevalent genus was *Staphylococcus* spp., 48.8%. This genus presented resistance to penicillin (91%), erythromycin (82%), ciprofloxacin (64%) and cefoxitin (59%). Thus, 59% of strains resistant to methicillin were detected. We detected the *mecA* gene in 82% of the isolates tested. Regarding the virulence factors, 36.4% presented hemolysis and 22.7% hemolysis, 63.6% presented a positive result for the production of proteases and 63.6% presented a biofilm formation capacity. About 36.4% were simultaneously resistant to methicillin and showed expression of proteases and/or hemolysins, biofilm formation and MIC for vancomycin greater than 2µg/mL.

Thus, our study evidenced contamination of PIVCs mainly by *Staphylococcus* spp., with high pathogenicity demonstrated by the presence of virulence factors, as well as resistance to antibiotics. The production of virulence factors allows to strengthen the attachment and the permanence in the catheter. When we also associate antimicrobial resistance, the treatment of the related infections becomes more difficult and the scarce treatment options. These data should be considered by health professionals who must take preventive measures to minimize the risk of contamination and consequent reduction of infections related to the use of peripheral intravenous catheters

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Abbreviations

Aap – Accumulation-Associated Protein

Agr – Accessory Gene Regulator

AIP – Autoinducing Peptide

CLSI – Clinical and Laboratory Standards Institute

CoNS – Coagulase-Negative *Staphylococci*

CRBSIs – Catheter-Related Bloodstream Infections

CVC – Central Venous Catheter

CWA – Cell Wall-Anchored

eDNA – Extracellular DNA

Embp – Extracellular Matrix Binding Protein

EPS – Exopolysaccharides

FnbpA and FnbpB – Fibrinogen Binding Proteins

HAIs – Healthcare Associated Infections

INT – Iodonitrotetrazolium Chloride 95%

LTA – Lipoteichoic Acid

MIC – Minimum Inhibitory Concentration

MRS – Methicillin-Resistant Staphylococci

MSS – Methicillin-Susceptible Staphylococci

MRSA – Methicillin-Resistant *Staphylococcus aureus*

MSCRAMMs – Microbial Surface Components Recognizing Adhesive Matrix Molecule

ORF – Open Reading Frame

PBPs – Penicillin-Binding Proteins

PBP2a – Penicillin-Binding Protein 2a

PIA – Polysaccharide Intercellular Adhesion

PICC – Peripherally Inserted Central Catheter

PNAG – Poly-N-acetylglucosamine

PSMs – Phenol-Soluble Modulins

PIVC – Peripheral Intravevous Catheter

PIVC-RBSIs – PIVC-Related Bloodstream Infections

QS – Quorum Sensing

SCC*mec* – Staphylococcal Cassette Chromosome *mec*

TA – Teichoic Acids

WTA – Wall Teichoic Acid

δ-PSM – δ-hemolysin

1. Background

The healthcare associated infections (HAIs) and the rapid adaptation of microorganisms through the expression of virulence factors and of antimicrobial resistance mechanisms are problems of growing importance worldwide (3,5). The medical devices have been identified as potential vehicles of microorganisms dissemination in an hospital environment, being often involved in the etiology of HAIs (5).

This is of concern when it comes to the need for insertion of a medical device, such as the peripheral intravevous catheter (PIVC), which gives access to the bloodstream, thus increasing the risk of infection associated with these devices (6,7).

1.1. Peripheral Intravenous Catheter (PIVC)

In healthcare, the majority of the patients admitted in a hospital requires intravenous therapy (8–11). There are several types of vascular access devices in use, such as central venous catheter (CVC), peripheral intravenous catheter (PIVC), peripherally inserted central catheter (PICC) and midline catheter. It is estimated that 2 billion of PIVCs are sold in the worldwide, being considered the most common type of vascular access device used in hospital settings (10–16).

PIVC is a small and flexible tube inserted in a peripheral vein, mainly the metacarpal, cephalic or basilica vein and secured to the skin with an adhesive dressing (11,12,17). They are typically made of polyurethane or silicone and its size can range from 26 to 14 Gauge (G) (11,12). This medical device is ideally suited for short-term use, up to 72-96 h, mainly for delivery of intravenous fluids and drugs (11,17,18).

The peripheral venous catheterization procedure is one of the most invasive in hospitalized patients, being inherent several local and systemic complications, such as thrombophlebitis, phlebitis, infiltration, catheter occlusion and catheter-associated bloodstream infection (6,10,11,13,15,16,19–23). These complications lead to the catheter failure before the end of intravenous treatment, can lead to increased mortality, morbidity and costs for healthcare system, prolonged care and hospitalization (9,11,13,19,20,23).

1.2. Catheter-Related Bloodstream Infections (CRBSIs)

The insertion of a vascular access devices is a potential pathway for the entry of microorganisms into the bloodstream, which can lead to the catheter-related bloodstream infections (CRBSIs) (6,7,17). However, some recent studies showed that the PIVC-related bloodstream infections (PIVC-RBSIs) rates (0.1%, 0.5 per 1000 catheter-days) are lower than the other intravascular devices, such as CVC (4.4%, 2.7 per 1000 catheter-days) and PICC (2.4%, 2.1 per 1000 catheter-days) (14,24,25). Despite this, the rates of PIVC-RBSIs may rise in the future due to the wide use of PIVCs (8,25,26). These are still responsible for 5% (670 per 100.000 patients) of nosocomial bacteraemia, being implicated in the aetiology of HAIs (25–27).

For the occurrence of CRBSIs, three pathways are described for the entry of microorganisms through the medical device from a non-sterile external environment into the normally sterile bloodstream (27–29). The first, is called extraluminal, where the migration of microorganisms occurs mainly from the patient's skin into the catheter tract. This process may occur during the insertion of the catheter or while the catheter is *in situ*, however it is the most common route of infection for short-term catheters. The second route is called intraluminal, involving direct contamination of catheter hubs and connectors by contact with the hands of health professionals who handle it, contaminated fluids or devices. The third contamination route is when the catheter is contaminated by microorganisms circulating in the

bloodstream, when there is already a preexisting infectious condition responsible for the contamination of the device (26,27).

These medical devices provide a surface area to which microorganisms can attach (27). The most common microorganisms involved are *Staphylococcus* species, namely coagulase-negative staphylococci (CoNS), predominantly *S. epidermidis* and *S. aureus* (25–27,30–33). Although these are commensal bacteria of the human skin and considered non-pathogenic, they have been recognized as relevant opportunistic pathogens (26,31,33). Others microorganism have been identified as the Gram-negative bacilli, *Enterococcus* spp. and fungi, such as *Candida* species (25,26,30,31).

Some of these microorganisms are associated with the hospital-acquired infections through the cross-contamination between health professionals/medical devices and the patients mainly due the incorrect disinfection technique of the catheter insertion site, poor hand hygiene practices and inefficient device maintenance (5,7,28,31).

1.3. *Staphylococcus* genus associated to PIVC-RBSIs

The *Staphylococcus* genus emerges as the main etiological agent of infections through vascular access devices. Given the increasing use of PIVCs and the global threat represented by the increase in antibiotic resistance and the virulence of these microorganisms, the treatment of these infections becomes difficult, prolonged and ineffective (3,34,35).

1.3.1. Virulence factors in *Staphylococcus* spp.

Staphylococcus species are the most common causes of indwelling device–associated infections and nosocomial and community acquired infections due to their biofilm-forming properties as well virulence factors (Table I) (3,34–36).

Table I. Virulence factors of *Staphylococcus* spp.. Adapted and modified from Diaz R. (2018) (3).

Mechanisms	Virulence Factors
Involved in evading/destroying host defenses	Microcapsule protein A coagulase Fatty acid – metabolizing enzyme Leukocidin and/or g-toxin
Involved in tissue invasion/penetration	Proteases Nucleases Lipases Hyaluronate lyase staphylokinase
Involved in toxin-mediated disease and/or sepsis	Toxic shock syndrome toxin Enterotoxins Cytolytic toxins (a, b, g, and d)
Induce specific toxinosis	Toxic shock syndrome toxin Enterotoxin Exfoliative tox
Attach to endothelial cells and basement membrane	Binding proteins for fibrinogen, fibronectin, laminin, collagen

1.3.1.1. Biofilm formation

The biofilm that anchors to abiotic or biotic surfaces is a multicellular agglomeration with a characteristic three-dimensional structure and with its own physiology, also dependent on the microorganisms present and the microenvironment (1,35,36). This matrix is constituted by polysaccharides, teichoic acids, extracellular DNA (eDNA), *staphylococcal* proteins and other components (1,35). When the microorganisms are exposed to stress conditions, the virulence genes expression, such as biofilm formation is induced as a survival strategy (34,35).

The mechanism of biofilm formation is complex and multifactorial, which has three major phases known as: the attachment, maturation and detachment (Figure 1) (1,35,36).

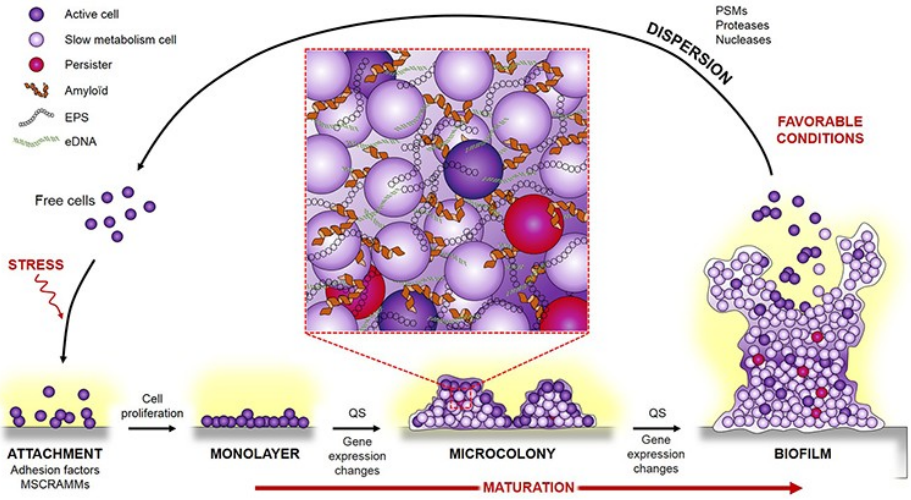

Figure 1. Biofilm formation cycle. From Reffuveille F. *et* al. (2017) (1).

1.3.1.1.1. Attachment

In any case, the adhesion of the microbial cells to a surface is essential. This phase is initially reversible and may become irreversible, and is the first step to the maturation of a future biofilm. Staphylococcal adherence depends mainly of the surface components, physico-chemical structure of the medical device (hydrophobic and electrostatic interactions, hydrodynamic forces and temperature), environmental factors and serum or tissue protein adsorption in the case of biotic surface (1,35).

The attachment of these gram-positive bacteria to the biotic surface, as human matrix proteins (fibronectin, fibrinogen, collagen and vitronectin), is due to specific interactions mediated by cell wall-anchored (CWA) proteins (1,34–36). The adhesins, better known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are one of the major groups of CWA proteins (37). MSCRAMMs have a common structure with 3 domains: binding domain, cell wall

spanning domain and domain responsible for covalent or non-covalent attachment of MSCRAMMs proteins to the bacterial surface. Covalent attachment is catalyzed by sortases which recognizes a conserved motif of the MSCRAMMs, called LPXTG motif. The variability of this type of molecule is higher in *S. aureus* compared to *S. epidermidis*. In addition to these, there are other molecules involved in this process as the several members of the serine-aspartate repeat family (Sdr proteins) and accumulation-associated protein (Aap). The non-covalent attachment of MSCRAMMs proteins to the surface of staphylococci is not well understood, but there is some evidence to suggest that autolysins are non-covalently attached to the teichoic acids. These proteins, present in great abundance in *Staphylococcus* species, have an important role in cell wall turnover and also have binding sites for human matrix proteins (1,36).

In addition to the ability to bind to human proteins, *staphylococci* can also adhere to a plastic surfaces (abiotic). The main molecules involved are teichoic acids (TA), adhesins and autolysins. TA are composed by glycerol or ribitol phosphate polymers covalently linked to the peptidoglycan through the phosphodiester bond to the C6 hydroxyl of the of N-acetyl muramic acid sugars. These may be covalently attached to the cell wall teichoic acid (WTA) or to the cell membrane through a lipid anchor known as lipoteichoic acid (LTA) (36,38). They are commonly found in Gram-positive bacteria, as is the case of *S. aureus* and *S. epidermidis* and has an important role in attachment to the plastic surfaces due to its interaction with other surface polymers. Therefore, TA have shown great importance not only in adhesion to a surfaces but also in the formation of the biofilm in species such as from *Staphylococcus* genus (1,36,38).

Other molecules are adhesins such as the cell wall-bound surface protein Bap and SasC a *S. aureus* surface protein that are involved in the adherence to a polystyrene surface. In addition to the functions of the autolysins aforementioned, they also have the ability to attach to plastics (1,35).

This ability to bind to a plastic was the basis of most *in vitro* biofilm studies performed on *staphylococci*. However, its role in the infection associated with medical devices is not clarified because soon after its insertion these devices are rapidly covered by host matrix proteins preventing the direct interaction of teichoic acids to plastic. It is believed that these interactions are overestimated *in vitro* assays (1,34,36).

1.3.1.1.2. Maturation

After the attachment to a surface, the biofilm maturation phase starts, characterized by intercellular aggregation and structuring three-dimensional and multicellularity. This phase is regulated by the increase of bacterial mediators, the slowdown of metabolism and of cell cooperation (1,35,36).

Initially, the fixed bacteria induce the biofilm process and potentiate intercellular aggregation in which adhesive proteins or usually polysaccharide-based - exopolymers intervenes. In staphylococci, polysaccharide intercellular adhesion (PIA), also known as poly-N-acetylglucosamine (PNAG), is the most described adhesive biofilm molecule and represents the major part of the extracellular matrix commonly called “slime”. This has a very important role in the organized community construction and is composed of exopolysaccharides (EPS), proteins, TA and eDNA (1,35,36,39).

The PIA is encoded by an accessory gene cluster called the intercellular adhesion (*ica*) operon, which comprises an N-acetylglucosamine transferase (icaA and icaD), a PIA deacetylase (icaB), a putative PIA exporter (icaC) and a regulatory gene (icaR) (1,34–36,39,40). Its biosynthesis is regulated by a variety of environmental factors and regulatory proteins. The PIA functions as a glue that binds bacterial cells one to each other as well as on surface by electrostatic interactions. This happens through its deacetylation, in particular beta-1-6-linked N-acetylglucosamine residues, which introduce a positive charge to the surface of the

negatively charged of the bacterial cell, releasing free amino groups making the pH neutral or acid characteristic of the natural habitat of *Staphylococcus* (1,35,36,39).

Despite being recognized as a key factor in virulence and biofilm formation of *Staphylococcus*, however it has been demonstrated that it is not expressed in all strains isolated from biofilm-associated infections suggesting that other compounds may be involved in this process (1,34–36).

In the mechanisms of biofilm formation independent of PIA, other adhesive proteins can replace the PIA. Thus, the molecules already recognized as essential are: Aap, extracellular matrix binding protein (Embp), protein A, fibrinogen binding proteins (FnbpA and FnbpB) or *S. aureus* surface protein G (SasG). Of these, the Aap, 220 kDa, appears to be the key molecule in biofilm formation independent of PIA, namely in *S. epidermidis* and in biofilm maturation by interactions with PIA, as in *S. aureus*. However, it only has the ability to induce biofilm formation when it is cleaved by proteases. SasG is responsible for intercellular aggregation to proteins present in other bacterial cells by hydrophilic interaction. Also, the SasC protein, anchored in the cell wall by having an LPXTG motif, and Bap protein both present in *S. aureus*, above referenced are also involved in intercellular aggregation and maturation of the biofilm. A homologous bap protein, named bhp was found in clinical isolates of *S. epidermidis* and it seems to contribute to a biofilm formation in this bacterial species. Particularly, in *S. aureus* the D-alanylation process of the TA is considered a very important process for the biofilm formation (1,35,36).

The three-dimensional structure typically is a “mushrooms” shape or “towers” containing fluid channels essential for the delivery of nutrients to the multiple layers of the biofilm and it is characteristic of a mature biofilm (1,35,36).

In this phase of biofilm structuring, *staphylococci* produce surfactant peptides, such as phenol-soluble modulins (PSMs) that are regulated by the global regulatory quorum sensing system through the accessory gene regulator (Agr). This was described for the first time by Seymour Klebanoff in 1999 as pro-inflammatory

“complex” agents in *S. epidermidis*. Two types of PSMs according to their length are described type α (~ 20-25 amino acids) and type β (~ 43-45 amino acids). The sequence is variable between species, but all have an amphipathic α-helix. The enhanced relative production of β-type PSMs over α-type PSMs seems to be one of the crucial factors in the biofilm maturation (volume, thickness, roughness and channel formation), as shown in *S. epidermidis*. Despite this differential production of PSMs, the mechanism by which this happens is still not understood (1,35,36,41).

Other important molecules for the biofilm structure are amyloid proteins, which confer stability to the matrix, through the binding of their fibers to the eDNA (1,27,34–36,42). As above mentioned, the Bap protein is also required at this stage for the formation of amyloid-like aggregates, taking into account environmental conditions such as calcium concentration and pH (1,35,36).

The enzymatic activity is fundamental for the occurrence of developmental and interruption events essentials during the biofilm maturation. Enzymes that degrade PIA (PIAse) and other proteases are the most important. The recognition of the importance of PIAse in this step, they have never been found in *staphylococci*. On the other hand, evidence of the role of proteases in the PIA independent pathway has already been demonstrated, namely in the development and detachment of the biofilm. These enzymes are mainly regulated by the quorum sensing (1,35,36).

1.3.1.1.3. Detachment

This last phase called detachment allows the bacterial survival and prolongation/progression of the disease. Thus, the mechanism of rupture of a mature biofilm is crucial for the systemic dissemination of bacteria, in single cells or clusters of larger cells into the bloodstream (1,35,36,43).

This process can occur in two pattways: active or passive mode. The active dispersion is based on mechanical forces such as blood flow, corosion and human intervention. On the other hand, the passive dispersion refers to the process of

detachment induced by bacteria themselves that constitute the biofilm in response to some environmental stimulus. This pathway occurs with the production of enzymes (proteases, nucleases, among others) and PSMs that will degrade structural elements of the extracellular matrix, namely proteins, eDNA and exopolysaccharides (1,35,36,43).

As we can see, molecules that act as biofilm structuring agents are also involved in their rupture when their rate of production is increased. This mechanism is controlled by the Agr system, mainly expressed in bacteria from the outer most layers of the biofilm, but is also necessary in deeper layers for the formation of the channels (1,35,36,43).

1.3.1.2. Quorum-Sensing (QS) regulating the biofilm formation

Bacteria have a regulatory system that allow the adaptation to environmental conditions such as the quorum sensing (QS) system (3,44). This is highlighted as one of the most important in the control of pathogenesis through the synchronization of genetic expression in the establishment of phenotypes such as virulence, biofilm formation and antibiotic resistance. In species of *Staphylococcus*, the main system responsible for the regulation of biofilm formation is the *Agr*, previously mentioned (1,3,35,44,45).

From the *agr* locus of 3.5 kb, two primary transcripts, RNAII and RNAIII, are generated, whose transcription is directed by the promoters P2 and P3, respectively. The P2 operon encodes four *agrB*, *agrD*, *agrC* and *agrA* genes and P3 operon encodes *hld* gene for δ-hemolysin (δ- PSM) (1,35,44).

The autoinducing peptide (AIP) is formed by 7-9 amino acids and contains a characteristic ring of thiolactone between the centrally located cysteine and the C-terminus and encoded by agrD transcript. The transmembrane endopeptidase, encoded by the *agrB* gene, is involved in the modification of propeptide through cyclic thiolactone bond between an internal cysteine and the carboxyl terminus and

also in the exportation of AIP to the extracellular medium, where it is cleaved by SspB type I signaling peptidase. The accumulation of extracellular AIP is to be detected by the histidine kinase domain of AgrC. After peptide binding to AgrC, the signal transduction cascade is activated, with histidine kinase phosphorylating which in turn regulates AgrA activity (response regulator). Upon being phosphorylated by AgrC, AgrA becomes active and is able to bind to the promoter region P2 and P3, as well as to the promoters that control the expression of the peptides PSMα and PSMβ (Figure 2) (1,2,35,44).

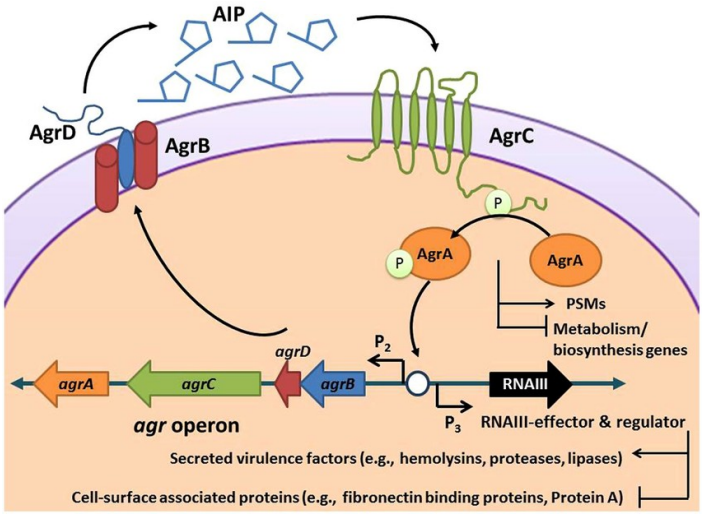

Figure 2. Representation of the *Staphylococcus* spp. accessory gene regulatory (*agr*) system. The agr locus is known to contain two divergent transcripts named RNAII and RNAIII. The RNAII transcript is an operon of four genes, agrBDCA, that encode factors required to synthesize AIP and activate the regulatory cascade. Briefly, AgrD is the precursor peptide of AIP, AgrB is a membrane protease involved in generating AIP, AgrC is a histidine kinase that is activated by binding AIP, and AgrA is a response regulator that induces transcription of RNAII and RNAIII through the P2 and P3 promoters, respectively. AgrA also directly promotes PSM production. The RNAIII transcript yields a regulatory RNA molecule that acts as the primary effector of the agr system by up-regulating extracellular virulence factors and down-regulating cell surface proteins. From Novick RP. (2003)(2).

The *agr*-QS regulator system is activated in response to cell density allowing the expression of virulence factors. During the initial phase of biofilm formation, the *agr*-quorum detection activity is weak and the surface proteins, MSCRAMMs, are highly

expressed to allow attachment. On the other hand, in the early stationary growth phase, these molecules are downregulated and Agr activity increases. There are also an overexpression of toxins and virulence factors, such as lipases, proteases and hemolysin. In the latter phase, upregulation of PSMs expression is required to promote bacterial dispersion (1–3,35,44).

The biofilm structure, regulated by QS, provides several advantages to bacteria capable of producing it by creating a selected microenvironment, protecting them from the action of antibiotics, biocides and physical challenges, and allowing the development of the stress response (33,34,45).

1.3.2. Antibiotic resistance in *Staphylococcus* spp.

The rapid acquisition of resistance by *Staphylococcus* spp. becomes a problem in the treatment of human infections caused by this microorganism. The most worrying and emerging resistances in this genus are methicillin and more recently vancomycin (3).

Methicillin was the first molecule of semi-synthetic β-lactam to be synthesized and introduced into clinical practice. Only differs from penicillin G in the substitutions at positions 2' and 6' of the benzene ring by methoxy groups, causing steric hindrance around the amide bond (46,47).

Vancomycin was the first glycopeptide antibiotic to be developed (48). It was discovered by Eli Lilly in the 1950s, when an organism, called *Amycolatopsis orientalis*, produced a brown colored substance that inhibited gram-positive organisms, becoming known as "Mississippi mud" (2,54,55). The structure is based on a central, relatively conserved heptapeptide domain in which five of the seven amino acid residues are common to all glycopeptides. They differ only in amino acids at positions 1 and 3 and in the substituents of the aromatic amino acid residues (48).

Although they are of different classes, both antibiotics have action in the synthesis of the cellular wall, namely in the synthesis of peptidoglycan (3,49).

1.3.2.1. Methicillin resistant *Staphylococcus* spp.

In 1959, the methicillin was introduced in response to increased resistance to penicillin G developed by some *Staphylococccus* strains (3,50). However, as early as the following year, the first reports of methicillin-resistant strains will be emerging. As a result of these reports, methicillin-resistant *S. aureus* (MRSA) appears as a nosocomial pathogen that is still very common worldwide until today (3,51).

1.3.2.1.1. Mechanism of resistance to methicillin

The major structural component of the bacterial cell wall is peptidoglycan. This is constituted by glycan strands made of repeating N-acetylglucosamine and N-acetylmuramic acid disaccharides linked by peptide cross-links between N-acetylmuramic acid moieties on adjacent strand. In *staphylococci*, cell wall biosynthesis begins with the β-1,4-glycosidic bond of the N-acetylglucosamine disaccharides and N-acetylmuramic acid of the reducing end of the growing peptidoglycan chain in a transglycosylation reaction. The incorporated repeating unit is cross-linked by a transpeptide reaction to a stem peptide in an adjacent peptidoglycan chain. Both the transglycosylation reaction as well as the transpeptidation reaction are performed by penicillin-binding proteins (PBPs), which are the target of β-lactams. In gram-positive bacteria, the typical composition of the stem peptide is L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala, variable between species (3,52).

Penicillin binding protein 2a (PBP2a), is encoded by the acquired *mec*A gene located in the *staphylococcal cassette chromosome mec* (SCC*mec*). These enzymes are susceptible to β-lactam antibiotic modification, leading to inhibition of bacterial cell wall biosynthesis and bacterial death. In the absence of a β-lactam antibiotic, MecI represses the transcripts of *mec*A and *mec*R1-*mec*I. If present,

MecR1 is cleaved automatically and the metalloprotease domain, located in the cytoplasmic domain of MecR1, becomes active. This metalloprotease cleaves MecI allowing transcription of *mec*A and production of PBP2a (Figure 3) (3,53).

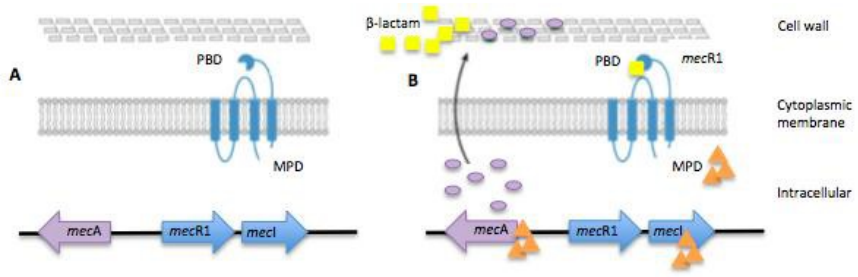

Figure 3. Model of the salient features of *mecA* regulation. (A) Absence of β-lactams: the binding of the repressor MecI to this region stops transcription from the mec operator. (B) Presence of β-Lactams: are detected by their binding to the PBD of MecR1. MecR1 is autocatalytically cleaved and the metalloprotease domain becomes active. This MPD, which is bound to the *mecA*, cleaves MecI allowing the transcription of *mecA* and the production of PBP2a. From Diaz R. (2018) (3).

Briefly, resistance to β-lactam antibiotics occurs as follows: they will function as analogs of the side chain substrates of peptidoglycan D-Ala-D-Ala, on which PBPs, such as PBP2a, act. Consequently, they form a long-acting covalent acyl-enzyme complex between β-lactam and the nucleophilic serine of the active site of PBP, inhibiting the transpeptidation step of cell wall biosynthesis. Thus, the deacylation of this complex is prevented because the active region accommodating the acceptor portion of dissociation or a potential hydrolysis water molecule is occupied by the β-lactam ring structure. PBPs are irreversibly inactivated because they lack regeneration capacity and the cross-linking phase of the cell wall is compromised, forming a defective wall that leads to cellular death (3,52).

1.3.2.1.2. SCC*mec* mobile resistance element

Pathogenic microorganisms have the ability to spread, create ecological reservoirs, colonize and cause disease. Mobile genetic elements, such as genomic

islands, bacteriophages, pathogenicity islands, chromosomal cassettes, plasmids, insertion sequences and transposons, have a key role in the dissemination of resistance and virulence (3,53).

Resistance to methicillin is conferred by a genetic element which is integrated into a resistance island known as SCC*mec*. This DNA fragment (between 15 and 60 kb) is located near the replication origin of the *Staphylococcus* chromosome and inserted at the insertion site *attB* downstream of an open reading frame (ORF), designated *orfX*. Each SCC*mec* element carries a complex of the *mec* gene and the *ccr* gene (3,53–55).

The *mec* gene complex, in addition to the *mec* genes (*mec*A, *mec*B, *mec*C), also carries the genes that control its expression: *mec*R1 (encodes a signal transducing protein MecR1) and *mec*I (encodes a MecI repressor protein). The *ccr* gene complex is composed of *ccr* genes (*ccr*A, *ccr*B and *ccr*C) and ORFs. It acts on the specific integration, orientation and excision of SCC*mec* by *ccr*AB and/or *ccr*C (8 allotypes). Furthermore, to these two essential complexes, this mobile element also has non-essential regions called J regions (Junkyard-region), namely J1, J2 and J3. Currently, eleven types of SCC*mec* are known, taking into account the variations in the different regions that constitute it (Figure 4) (3,53–56).

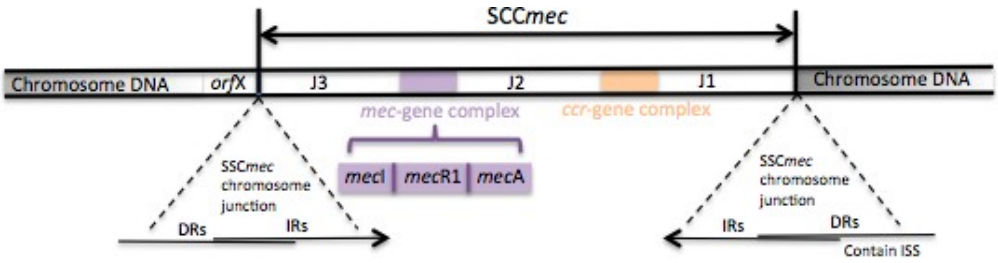

Figure 4. SCCmec is bracketed by direct repeats (DRs) that contain integration site sequence (ISS). A pair of inverted repeats (IRs) is present at the termini of SCCmec. Two critical gene complexes, ccr and mec are present, and the other regions are designated J1, J2, and J3. From Diaz R. (2018) (3).

The *mec*A gene was first identified in *S. aureus*, but since it is located in a mobile genetic element able of exchanging genetic information between strains, this gene has also been identified in several *Staphylococcus* species, such as *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. fleurettii* and among others (53,54).

1.3.2.2. Vancomycin resistant *Stpahylococcus* spp.

In 1958, vancomycin was approved for use by the Food and Drug Administration (FDA) in the USA and introduced into the clinic for the treatment of gram-positive bacteria (3,57,58). With the emergence of methicillin-resistant strains in both CoNS and *S. aureus*, vancomycin became the gold standard in the treatment of infections caused by these bacteria (3,48,57,59,60).

1.3.2.2.1. Mechanism of vancomycin action

Although vancomycin inhibits cell wall synthesis by binding to the active site of the transpeptidase of PBPs as in β-lactam antibiotics, the mechanism of action is different. In this case, vancomycin binds to the C-terminal D-Ala-D-Ala residue of the peptidoglycan precursor, forming a stable non-covalent complex which blocks the use of the precursor for the synthesis of the cellular wall. This complex is characterized by several hydrogen bonds between the peptidic component of vancomycin and the D-Ala-D-Ala residue. Vancomycin does not penetrate the cytoplasm, acting outside the cytoplasmic membrane at a late-stage of peptidoglycan biosynthesis (Figure 5) (3,4,48,57).

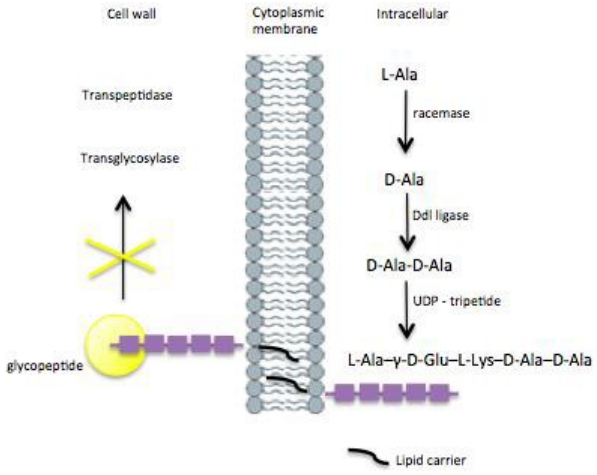

Figure 5. Peptidoglycan biosynthesis and mechanism of action of vancomycin. Binding of the antibiotic to the C-terminal d-Ala–d-Ala of late peptidoglycan precursors prevents reactions catalyzed by transglycosylases and transpeptidases. From Courvalin P. (2006) (4).

1.3.2.2.2. Mechanism of resistance to vancomycin

The activity of vancomycin is determined by the substrate specificity of the enzymes that determine the structure of peptidoglycan precursors. The presence of operons encoding enzymes leads to resistance to vancomycin. These enzymes are involved in the modification and removal of vancomycin-binding target: the C-terminal d-Ala residues are replaced by d-lactate (d-Lac) or d-serine (d-Ser), with the synthesis of precursors with low affinity and then eliminate the high affinity precursors normally produced by the host (3,4).

There are six types of vancomycin resistance: VanA, B, C, D, E and G, characterized in phenotypic and genotypic bases in enterococci. The localization of the various genes is different: the *vanA* and *vanB* operon can be found on plasmids or on the chromosome, while the *vanC*, *vanD*, *vanE* and *vanG* operons were still only found on the chromosome (3,4).

Currently, only the VanA-type has been detected in *S. aureus*. This type is characterized by high levels of resistance to vancomyne and teicoplanin. It is

mediated by transposon Tn1546 (11-kb) encoding 9 polypeptides distributed in different functional groups: transposition (ORF1 and ORF2), regulation of gene expression of resistance (VanR and VanS), d-Ala-d-Lac depsipeptide synthesis VanH and VanA) and hydrolysis of peptidoglycan precursors (VanX and VanY) (3,4).

Target modification begins with reduction of pyruvate to d-Lac by dehydrogenase (VanH) and the formation of an ester bond between d-Ala and d-Lac catalyzed by VanA-ligase. Substitution of the d-Ala-d-Ala dipeptide by the d-Ala-d-Lac depsipeptide formed in the peptidoglycan synthesis results in a considerable decrease in the affinity of the molecule for glycopeptides. VanX D,D-dipeptidase and VanY D,D-carboxypeptidase enzymes are involved in the removal of the d-Ala-terminated susceptible precursors, preventing the interaction of vancomycin with its target. VanX hydrolyzes the d-Alad-Ala dipeptide synthesized by the host d-Ala-d-Ala-ligase (Ddl) and VanY removes the C-terminal d-Ala residue of late peptidoglycan precursors when the elimination of d-Ala-d-Ala by VanX is incomplete (Figure 6) (3,4).

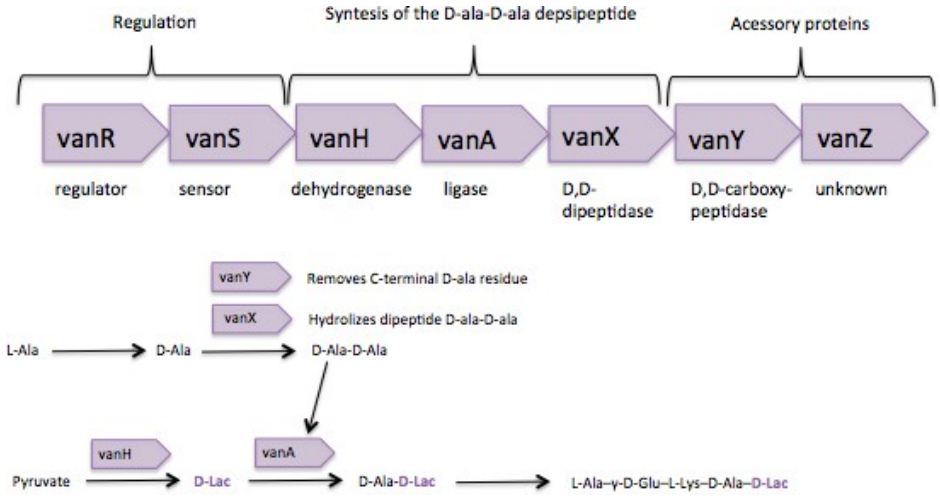

Figure 6. VanA-type glycopeptide resistance. From Diaz R. (2018) (3).

2. Aims

The insertion of a PIVC may potentiate the development of a bloodstream infection associated with this device. On the other hand, the microorganisms involved in these infections often express virulence factors and antimicrobial resistance profiles, often making it difficult to treat these infections. Despite this, it is important to point out the small number of studies that characterize these microorganisms, from their identification, production of virulence factors and antimicrobial resistance profile.

The aim of this study was to evaluate the microbial contamination of PIVCs used in the peripheral catheterization procedure, identifying the most prevalent microorganisms and evaluating risk associated with these contaminations as seen by evaluating their virulence factors and antibiotic resistance.

In addition, the specific objectives:

- To isolate the most abundant species and their identification;
- To evaluate the extracellular enzymes production: hemolysins and proteases;
- To evaluate the ability to the biofilm formation;
- To evaluate the antibiotic susceptibility profiles;
- To detect the methicillin resistance and screnning of the *mecA* gene;
- To determine the vancomycin-MIC.

3. Material and Methods

3.1. Sample characterization

The present study was performed in a service of a large tertiary hospital in the central region of Portugal. It had the approval of the Ethics Hospital Committee (reference number 0226/CES) and the authorization number 14037/2017 from the Portuguese Data Protection Authority. The patients' written informed consent was obtained, respecting the Helsinki Declaration.

A total of 110 PIVCs ends (2 cm) were collected and were storaged in sterile flasks at 4ºC, until the microbiological analysis at Coimbra Health School, Polytechnic Institute of Coimbra.

3.2. Microbiological analysis

3.2.1. Isolation and identification

The PIVCs ends samples were inoculated on Columbia agar base (supplemented with 5% of sheep blood), using the technique of Maki *et* al. (61). The cultures were incubated at 37ºC in the normal atmosphere during 18h-24h. After enumeration and macroscopic evaluation, we performed the isolations of the macroscopically different microorganisms in Tryptic Soy Agar.

Pure colonies obtained were characterized by the Gram staining and biochemical tests as catalase and/or oxidase to a primary identification. Subsequently, we used biochemical identification galleries as API Staph (REF 20500) bioMérieux®, API 20 Strepto (REF 20600) bioMérieux®, API 20 NE (REF 20050) bioMérieux®, API 20 E (REF 20100) bioMérieux®, following the manufacturer's instructions. The identification to the species was obtained using the Apiweb software as well as the score of identification.

3.2.2. Detection of extracellular enzymes

3.2.2.1. Proteases

The extracellular proteases production was determined by the plate assay in Luria Bertani (LB) (Merck, Darmstad, Germany) agar medium supplemented of 1% of skim milk (w/v). A bacterial suspension of 0.5 McFarland was prepared and subsequently inoculated (5µL), then all plates were incubated at 37 ˚C during 24h at normal atmosphere. The presence of extracellular proteases was revealed by the formation of clear halos around the colonies which were measured. The halos were classified as negative (-) in the absence of halo, as weak positive (+/-) in the presence of halo less than 11 mm, as positive (+) in the presence of halo less than 13mm and as stong positive (++) in the presence of halo less than 15mm (62).

3.2.2.2. Hemolysins

The hemolytic activity was determined by the plate assay using a Columbia Agar with 5% sheep blood (Merck, Darmstad, Germany). A bacterial suspension of 0.5 McFarland was prepared and subsequently 5µL was inoculated and plates incubated at 37˚C for 24h at normal atmosphere. The production of hemolysins was identified by the presence of clear (β-hemolysis) or diffuse (α-hemolysis) halos around the colonies (63).

3.2.3. Biofilm formation assay

From a 0.5 McFarland bacterial suspension, 10µL was inoculated into a multiwell (of 96 wells) plate with 100µL of Luria Bertani Broth (5 replicates for each strain were done) and the incubation was performed at 37 °C *overnight* with normal atmosphere. The planktonic cells were transferred and 25µL of 0.2 $g.L^{-1}$ iodonitrotetrazolium chloride 95% (INT) solution was added. Cells from biofilms were washed from multiwells with 200µL of phosphate buffered saline (PBS 1x) to remove all non-adherent cells and this process was repeated more 2 times. A 100µL of fresh media was added to the correspondent wells after rinsing followed by the

addition of 25µL of 0.2 $g.L^{-1}$ INT solution. Both plates were immediately covered with aluminum foil and incubated in the dark at 37ºC. The reading was performed at λ: 492nm after 30 min of incubation using the microplate reader (Thermo Fisher Scientific, USA). The biofilm formation results were normalized using the ratio of adherent cells at OD492nm / planktonic cells at OD492nm. The isolates which have values below 0.75 were classified moderate biofilm-formers, values between 0.75 and 1.0 were classified high biofilm-formers, values above 1.0 were classified very high biofilm-formers (64).

3.2.4. Antimicrobial susceptibility test: disk diffusion method

The evaluation of the antimicrobial susceptibility profile of the isolates obtained was performed by disk diffusion method (modified Kirby-Bauer's test). From the fresh and pure culture, a suspension of 0.5 McFarland in sterile NaCl 0.9% was obtained and then inoculated in Muller Hinton agar. The selection of antimicrobial disks for *Staphylococcus* spp. took into account its clinical application: cefoxitin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 units), tetracycline (30 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg) (Table II).

Table II. Classes of antibiotics and antibiotics tested for *Staphylococcus* isolates.

Class	Antibiotic
Penicillinase-stable penicillins	Cefoxitin
Fluoroquinolones	Ciprofloxacin
Phenicols	Chloramphenicol
Macrolides	Erythromycin
Aminoglycosides	Gentamicin
Penicillinase-labile penicillins	Penicillin
Tetracyclines	Tetracycline
Folate pathway antagonists	Trimethoprim-sulfamethoxazole

The antibiograms were incubated in normal atmosphere at 37ºC for 18-24h. The reading was based in the inhibition halos (mm) measurement, and phenotypically the strain was classified as sensitive, intermediate and resistant. The interpretation of the results was performed taking into account the Clinical and Laboratory Standards Institute (CLSI, 2018) (65).

The detection of methicillin resistance was also performed, based on the reading of the inhibition halo to cefoxitin, taking into account CLSI standards (65).

3.2.5. Determination of susceptibility to vancomycin by E-test method

The susceptibility to vancomycin was performed by the E-test method with the determination of the minimum inhibitory concentration (MIC). From the fresh and pure culture, a suspension of 0.5 McFarland in sterile NaCl 0.9% was obtained and then inoculated in Muller Hinton agar. The antibiograms were incubated in normal atmosphere at 37ºC for 18-24h. The interpretation of the results was performed according to CLSI, 2018 (65).

3.3. DNA extraction

A bacterial cell suspension, with one pure colony, in LB medium, was incubated overnight at 37°C. After, the inoculum was centrifuged for 10 min at 13000g, then the supernatant was discarded and the pellet resuspended in 200µL of Tris-EDTA (TE). The DNA extraction was made using the GeneJET Genomic DNA Purification Kit #K0721 (Thermo Fisher Scientific, USA) following the manufacturer instructions. The DNA solution obtained was stored at -20ºC.

3.4. Detection of *mec*A gene using PCR technique

The *mec*A gene amplification was performed by PCR using specific primers: *mecA*-R (5'-CAATTCCACATTGTTTCGGTC-3') and *mecA*-F (5'-

GAAATGACTGAACGTCCGATA-3') (Metabion International AG, Germany). The primers used were designing using the nucleotide sequence databases from NCBI. Amplification was done using 12.5µL DreamTaq PCR Master Mix (2X) (Thermo Scientific, EUA), 10pmol of the forward and the reverse primers, 9.5µL of the nuclease-free water and 1µL of the bacterial DNA. The PCR reactions were performed using a MyCycler Thermal Cycler (Bio-Rad, USA). The amplification conditions consisted of an initial denaturation step (95 °C for 5 min), followed by 30 amplification cycles consisting of denaturation (94 °C for 45 seg), annealing (53ºC for 45 seg) and extension (72°C for 1 min) and a final extension step (72°C for 10 min). The DNA of a positive and negative strains to *mecA* gene were used as a positive and negative controls previously characterized and provided by a hospital. The reaction products were separated by electrophoresis on a 1.5% (w/v) agarose gel. All gels were run in 1×TAE buffer at 80V for 80min, stained in 0.5µgmL^{-1} of ethidium bromide solution and the images were acquired with the Gel Doc XR+ System (Bio Rad, California).

4. Results

4.1. Prevalence of the PIVCs microbiological contamination and identification of the isolates

The study population had, on average, 79 years with a standard deviation of ± 11 years. At 110 PIVCs ends, 30% were contaminated from which 45 macroscopically different isolates were obtained. The most prevalent genus was *Staphylococcus* spp. in 48.8%. Belonging to this genus, isolated bacterial species were *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lentus*, *S. warneri* and *S. xylosus*. Other clinically relevant microorganisms were found as *Enterococcus* spp., *Escherichia coli*, *Klebsiella pneumonia* and *Stenotrophomonas maltophilia*. The remaining 40% corresponded to other microorganisms whose identification was not possible (Table III).

In 8.2% of the infected PIVCs, more than one isolate was observed.

Table III. Prevalence of isolated microorganisms in PIVCs.

Isolated Microorganisms		Isolates	Prevalence (%)
***Staphylococcus* spp.**	*S. aureus*	12,17	4.4
	S. epidermidis	4-7,11,13-15,18,20-22	26.7
	S. haemolyticus	2,8-10,19	11.1
	S. lentus	3	2.2
	S. warneri	16	2.2
	S. xylosus	1	2.2
***Enterococcus* spp.**			4.4
Escherichia coli			2.2
Klebsiella pneumonia			2.2
Stenotrophomonas maltophilia			2.2
Others (molds, yeasts and not other bacteria)			40

4.2. Virulence factors in *Staphylococcus* isolates: proteolytic and hemolytic activity and biofilm formation

Concerning proteases production, the majority of the isolates displayed a positive phenotype. However, 40.9% of the isolates presented higher levels of proteolytic activity (Table IV).

Table IV. Proteolytic activity in *Staphylococcus* isolates.

Protease Production	Isolates	Prevalence Rates (%)
-	1-3,8,11,12,17,22	36.4
+/-	6,9,13-15	22.7
+	4,5,7,10,18,19,21	31.8
++	16,20	9.1

In the haemolytic activity different phenotypes were observed, some strains were negative (40.9%), 36.4% presented α-hemolysis and 22.7% presented β-hemolysis (Table V).

Table V. Hemolytic activity in *Staphylococcus* isolates.

Hemolytic Activity	Isolates	Prevalence Rates (%)
-	1,3,7,13-16,20,21	40.9
α-hemolysis	4-6,18,22	36.4
β-hemolysis	2,8-12,17,19	22.7

All strains demonstrated the ability to produce biofilm, with ratios ranging from capacity 0.45 to 1.7. However, it was found that the majority (63.8%) had high to very high capacity (Table VI, Figure 7). It was also observed that of this majority, 50% of the isolates correspond to *S. epidermidis*.

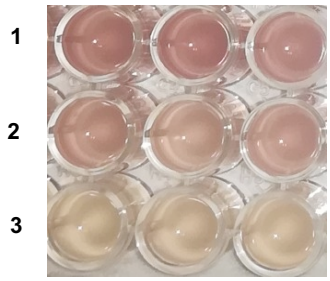

Figure 7. Bacterial cell culture in microplate showing the biofilm cells after INT incubation; 1 – Higher ability to form a biofilm; 2 – Moderate ability to form a biofilm; 3 – Lower ability to form a biofilm.

Table VI. Biofilm formation in *Staphylococcus* isolates.

Ratio of adherent/planktonic cells		Isolates	Prevalence Rates (%)
< 0.75	+	3,8-10,12,15,17,19	36.4
≥ 0.75 – < 1	++	2,7,13,16,20-22	31.8
≥ 1	+++	1,4-6,11,14,18	31.8

4.3. Antibiotic resistance in *Staphylococcus* isolates: susceptibility profile and presence of *mecA* gene

The main resistances found among *Staphylococcus* isolates were penicillin (91%), erythromycin (82%), ciprofloxacin (64%) and cefoxitin (59%). The antimicrobial agents for which they presented greater sensitivity (showing a largest halos) were ciprofloxacin (95%), tetracycline (86%), gentamicin (73%) and trimethoprim-sulfamethoxazole (59 %) (Figure 8).

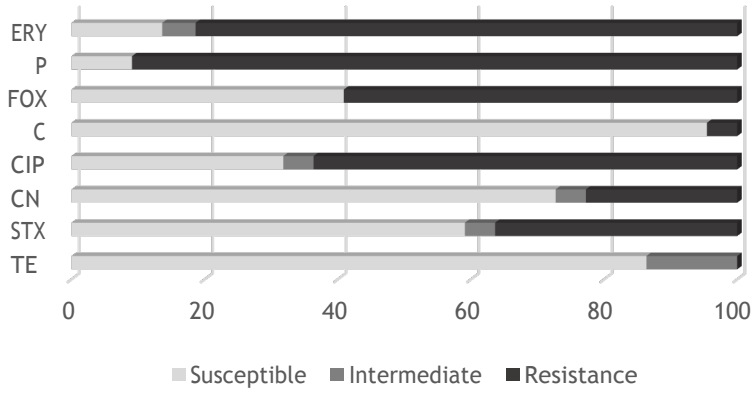

Figure 8. Antibiotic susceptibility profile of *Staphylococcus* isolates; TE – Tetracycline; STX – Trimethoprim-Sulfamethoxazole; CN – Gentamicin; CIP – Ciprofloxacin; C – Chloramphenicol; FOX – Cefoxitin; P – Penicillin; ERY – Erythromycin.

According with CLSI (2018) we found 59% of methicillin resistance in *Staphylococcus* isolates (65). However, when we evaluated the presence of the genetic determinant *mecA* among methicillin resistant isolates, putatively enconding PBP2a, we found 82% of positive strains (Figure 9).

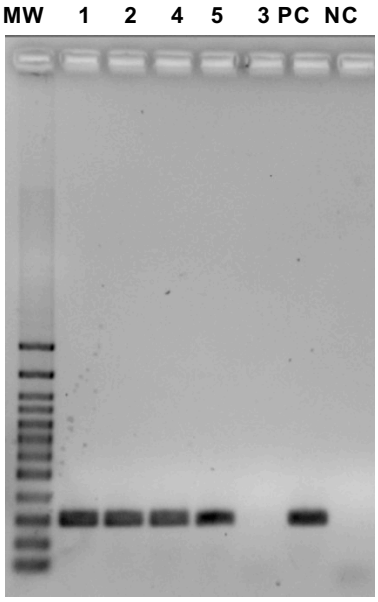

Figure 9. Genotypic profiles in *Staphylococcus* isolates; MW – Molecular Weight; 1,2,4 and 5 – *Staphylococcus* isolates positive to the presence of the *mecA* gene; 3 - *Staphylococcus*

isolates negative to the presence of the *mecA* gene; PC – Positive Control and NC – Negative Control.

Relatively to the vancomycin susceptibility profile, we found sensitivity in all isolates, according the CLSI classification and had been into account the identifition as *S. aureus* or CoNS (65). However, when we observed the MIC levels of sensitivity, the majority of the isolates presented values higher than to 2µg/mL (Table VII, Figure 10).

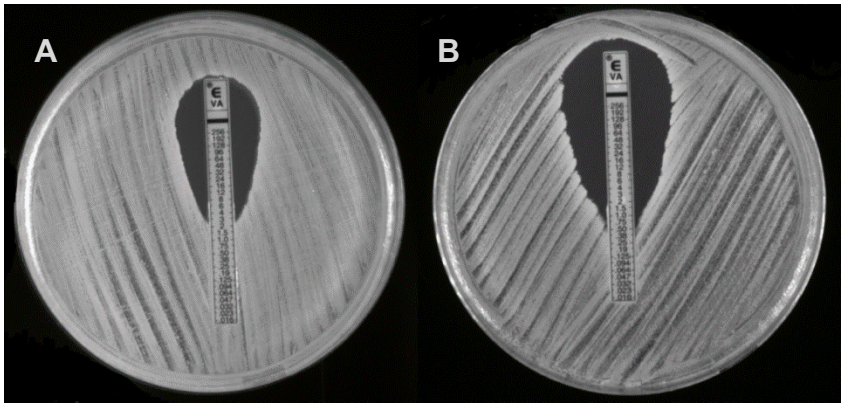

Figure 10. Example of vancomycin-sensitive isolates with differents MIC, where (A) has MIC ≥ 2 µg/mL and (B) MIC < 2 µg/mL.

Table VII. Antibibiotic susceptibility profile: MIC-Vancomycin.

MIC-Vancomycin (µg/mL)	Isolates	Prevalence Rates (%)
< 2	2,8,11,12,17,19,20,21	36.4
≥ 2	1,3-7,9,10,13-16,18,22	63.6

In addition, it is noted that about 45.5% of methicillin-resistant staphylococci (MRS) and 9.1% methicillin susceptible staphylococci (MSS) has MIC for vancomycin greater than 2µg/mL.

5. Discussion

The present investigation was carried out in order to act at prevent level PIVCs related infections, with evaluation of the contamination of the devices, identification of the microorganisms, as well as to study some of its virulence factors and antimicrobial resistance.

In this study it was verified that 30% of the analyzed PIVCs were contaminated. We consider this rate very relevant, given the high use of this medical device in a hospital context. Some of the variables to be considered with a significant impact on the contamination of these devices are the degree of compliance with aseptic care during insertion and maintenance, the number of manipulations, the type of dressing applied in their fixation and their length of stay (66). However, there are no pre-existing studies in this field, there are only studies that report the rate of infection of the bloodstream associated with PIVCs. In these studies rates of infection were varied, ranging from 35 to 70% (30,67). In this present study the preexisting infectious condition, responsible for the contamination of the device, can not be discarded, since this variable was not controlled.

Regarding the identified microorganisms, it was verified that the most prevalent genus was *Staphylococcus* in 48.8%, predominantly CoNS (44.4%), of which *S. epidermidis* was the most common (27.7%). Also in these studies the genus *Staphylococcus* is responsible for 58% of the infections caused by PIVCs, of which about 25% are coagulase negative (25,30). Other identified microorganisms belonging to this genus were also found in the present investigation: *S. aureus*, *S. haemolyticus*, *S. lentus*, *S. warneri* and *S. xylosus*.

The presence, even punctual, of *Enterococcus* spp., *E. coli*, *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia* was also observed. The low incidence can be explained by the fact that they are not part of the normal microbiota of the skin, however the presence of these microorganisms in a hospital environment can allow their presence in a transient microbiota (26). However, we

highlight the relevance of these microorganisms found in the context of HAIs as opportunistic pathogens (3). There are reports of studies on the presence of these species associated with PIVCs infections also with low prevalence (25,26,68).

The presence of more than one isolate occurred in 8.2% of contaminated PIVCs, this result being similar to that found by Guembe *et* al. which found only 2.9% of PIVC-RBSI polymicrobials episodes (68).

The fact of the higher prevalence of *S. epidermidis* in this medical devices can be due to it´s commensal feature of the human skin that can be associated with procedures during the insertion and manipulation of PIVC that can lead to an infection. In addition, these microorganisms produce virulence factors involved in processes such as attachment to the catheter with increased expression of TA, adhesins and autolysins and to the human matrix with increased expression of MSCRAMMs and tissue invasion with the production of extracellular enzymes such as proteases and hemolysins (1–3,35,44). This fact justifies the results obtained in this study for the proteolytic and hemolytic activity of the isolates, in which protease production was observed in 40.9%, α-hemolysis production in 36.4%.

After adaptation to the catheter, the production of biofilm by these microorganisms is quite common, as verified in 63.8% of the studied isolates that revealed high capacity of biofilm production. In addition, one of the species that demonstrated to have this capacity was *S. epidermidis* in 50% of the isolates. This microorganism is described as the major nosocomial pathogen associated with implanted medical device infections due to its ability to form the extracellular polysaccharide membrane which allows its own protection and strengthens attachement to the catheter (33,34). The investigation by Hashem *et* al. shows that 55% of *S. epidermidis* associated with CRBSI are biofilm producers, being in agreement with the one observed in our study (69).

Due to the protective nature of the biofilm, associated bacteria are intrinsically resistant to many antibiotics, which can increase up to 1000 times. The main reasons for this may be the difficulty of biofilm penetration by antibiotics, low growth

rate of bacteria and the presence of antibiotic degradation mechanisms (70,71). In addition, biofilm promotes horizontal gene transfer between bacteria, causing the spread of drug resistance determinants and other virulence factors (70,72). This association has already been demonstrated by some studies, such as Belbase *et* al. and Ghasemian *et* al., who found higher rates of resistance to multiple drugs and resistance to methicillin in biofilm producing strains compared to non-biofilm producing strains (70,73). The present study corroborated this information, with 59% of isolates being methicillin resistant *Staphylococcus* and 82% of strains positive for the presence of the *mecA* gene. The presence of this genetic determinant in the majority of the isolates suggests the propensity for the dissimination of resistance genes between bacteria living in biofilm, as evidenced by other studies (33,70,73). Kitao *et* al. also indicates that an increase of *S. epidermidis* resistant to methicillin with the presence of the *mecA* gene and capable of biofilm formation has occurred in many cases of CRBSIs (33).

In addition, isolates of *Staphylococcus* spp. studied have an antimicrobial multiresistance profile: penicillin (91%), erythromycin (82%), ciprofloxacin (64%) and cefoxitin (59%). This type of profile leads to difficulties in treatment, resulting in prolonged treatment, extended duration of hospital admission, development and persistence of chronic infectious diseases in local and/or distant organs, or even mortality (33).

When methicillin resistance was detected in *Staphylococcus* spp., the glycopeptide antibiotics, such as vancomycin, were selected as gold standard for the treatment of serious infections caused by these microorganisms. However, it was observed a slow but steady increase in vancomycin MIC in recent years (3). Although in this study all the isolates showed a sensitivity profile to vancomycin. Yet, it is noted that 63.6% presented values higher than 2µg/mL, which are close to an intermediate phenotype. Thus, this result is in agreement with the study conducted by Cherifi *et* al. who also did not observe any resistance to this antibiotic glycopeptide (74). In clinical context, vancomycin is used as the treatment of choice and the last resort for infections caused by *Staphylococcus* spp.. However, its

excessive intravenous use has allowed the adaptation of these microorganisms, causing strains with greater sensitivity to vancomycin (3).

It is noted that 36.4% of the strains showed methicillin resistance, MIC-vancomycin greater than 2µg/mL, the ability to produce at least one extracellular enzyme and biofilm production. This is rather worrying when we think that these highly adapted and virulent strains are in a catheter that has access to the bloodstream in an already immunocompromised patient.

In this context, in order to minimize the contamination of PIVCs and consequent CRBSIs, health professionals should have an active role, through proper hand hygiene before, during and after any procedure associated with peripheral venous catheterization, to take preventive measures during to the insertion and maintenance of these medical devices. Other authors have already mentioned that this control is very relevant with regard to the minimization of infections related to vascular medical devices, as is the case of PIVCs (7,25,26,75).

6. Conclusions

This study is innovative at a preventive level, in the sense that the risk associated with the use of PIVCs was evaluated, one of the most frequently used devices in healthcare, and may play an important role in HAIs.

This investigation allowed to identify the microorganisms relevant to colonizing PIVCs, to know their profile of antimicrobial susceptibility and their virulence factors.

It was verified that the main microorganisms found belong to the genus *Staphylococcu*s. CoNS were the most prevalent, with the presence of *S. epidermidis* in 27.7%. In fact, these microorganisms are able to remain in the catheter, often due to failures of hand hygiene of health professionals, usually nurses, and failures in insertion and maintenance of the device.

In addition, they produce virulence factors such as proteases, hemolysins and biofilm that facilitate their adhesion and permanence in the catheter, often leading to CRBSIs. Associated with these virulence factors, they develop resistance to antimicrobials, making it difficult to choose the trait and reducing the available therapeutic options.

Since it is widely recognized that health professionals play an important role in the context of hospital infections and considering the high frequency of intravascular device insertion and the associated risks, it is urged to raise awareness of this problem so that new strategies can be tested preventive measures.

For the future prevention of HAIs associated with PIVCs, we sensitize the scientific community for more extensive investigations, with more samples and with continuous characterization of the microorganisms.

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