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Virulence factors of *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains involved in the etiology of cardiovascular infections

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ABSTRACT

The purpose of tihis study was to investigate by *in vitro* methods the capacity of adhesion and invasion to cellular and inert substrata, as well as the invasive properties of some *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains isolated from various clinical specimens in patients with cardiovascular infections. Strains isolation and identification was performed using API galleries and the automatic Vitek II system. The selected strains have been phenotypically evaluated for the adherence to the cellular substrate represented by HeLa cells, adherence to the inert substrate quantified by slime test and a serios of soluble virulence factors (lechitinase, lipase, amylase, caseinase, gelatinase, DN-ase, haemolysins). The isolated strains exhibited an evident tendency of colonizing the cellular and inert substrate, also harboring enzymes with role in the invasion of host tissues and thus susceptible to produce systemic infections.

Keywords: cellular substrate; prosthetic devices; virulence factors; bacterial adherence; invasion;

1. Introduction

Infections associated with cardiovascular tissue and prosthetic devices are an important cause of discomfort, disability and severe evolution leading to high mortality rates [1, 2], so that prevention and treatment strategies must target the complex mechanisms of interaction among infectious agents, prosthetic devices and the host [3-6]. Our previous studies have demonstrated that the etiology of cardiovascular devices associated infections is dominated by non-fermentative, Gram-negative bacilli, especially nonfermentative species (*Pseudomonas aeruginosa* and *Acinetobacter baumannii*) followed by Gram-positive cocci (staphylococci, streptococci, enterococci) and yeasts. The purpose of this study was to study the pathogenicity and virulence factors of some selected *Ps. aeruginosa* and *Staphylococcus aureus* strains isolated from cardiovascular devices associated infections.

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2. Experimental section

Bacterial strains have been isolated from different clinical specimens from patient admitted for cardiovascular surgenry at the Institute for Heart Diseases Prof. C.C. Iliescu. Isolation was obtained on nutrient and selective culture media, and identification was done by serology, API galleries and Vitek II automatic system. For the investigation of the pathogenic potential, there were selected 16 strains of *S. aureus* isolated from bronchial secretions, wound secretions and blood and 9 strains of *Ps. aeruginosa* isolated from bronchial secretions, wound secretions and nasal swabs. Cravioto modified method and slime test were used to investigate the bacterial adherence to the cellular and inert substratum, as well as the invasion of HeLa cells. The ability of the tested strains to colonize the central venous catheter sections was visualized by CLSM. In this purpose, 1 cm catheter sections exposed for 24 h to microbial contamination, were removed from the liquid nutrient broth, washed three times with PBS, briefly fixed with cold methanol and dried before microscopic examination. Samples were visualized in reflection mode by using a Leica TCS-SP CSLM, equipped with PL FLUOTAR (40X NA0.5, electronic zoom 1) and an He-Ne laser tuned on 633 nm wavelength. A lateral resolution of about 600 nm was achieved. Leica software was used for surface topography and for statistical analysis [7]. For the investigation of soluble virulence factors, the microbial strains have been spotted on specific media containing different substrata for different enzymatic virulence factors: plate haemolysis on blood agar plates containing 5% (vol/vol) sheep blood, protease activity on 15% soluble casein and, respectively, 3% gelatine agar, DNA-se production on DNA agar medium, lecithinase production on 2.5% yolk agar, lipase production on Tween 80 agar [7, 8].

3. Results section

S. aureus and *Ps. aeruginosa* are two of the most frequently isolated bacteria in nosocomial infections, being opportunistic pathogens responsible for serious infections in immunocompromised patients. *S. aureus* is a versatile human pathogen which has the ability to cause a wide variety of human diseases, ranging from skin lesions such as abscesses and impetigo to invasive and more serious infections such as osteomyelitis, septic arthritis, pneumonia, and endocarditis. The mortality due to *S. aureus* bacteraemia still remain very high (20-40%) despite the large access to efficient antibiotics. The *S. aureus* strains isolated from ICUs as well as invasive strains isolated all over the world are becoming more and more resistant to many antibiotics: penicillin, methicillin, quinolones, vancomycin. The initial step in the pathogenesis of *S. aureus* infection is the attachment of the organism to the human cell surfaces and implanted devices. Adhesion of *S. aureus* may be mediated by specific cell-surface proteins, or be the result of interactions of cell-surface proteins with host proteins such as von Willebrand factor, fibronectin, fibrinogen and collagen [Foster & Hook, 1998]. Bacterial survival during an infection is dependent on the ability of the organism to circumvent the host's defense mechanisms. *S. aureus* produces a number of toxins and exoproteins that act against host defense mechanisms. Thus, nearly all strains secrete a group of enzymes and cytotoxins which includes four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase [9]. The pathogenesis of *Ps. aeruginosa* infections is also multifactorial, as suggested by the large number of cell-associated and extracellular virulence determinants possessed by the bacterium, some of these factors helping colonization, whereas others

facilitate bacterial invasion. The first step in *Ps. aeruginosa* infections is colonization of altered epithelium. Adherence of *Ps. aeruginosa* to epithelium is mediated by fimbriae, type 4 pili and flagella. After colonization, *Ps. aeruginosa* produces several extracellular virulence factors [proteases (alkaline protease, staphylolytic protease, elastase, protease IV), heat-labile and heat-stable haemolysins, phospholipases C and exotoxins A, S, T, U and Y], that can cause extensive tissue damage, bloodstream invasion, and dissemination as well as resistance to phagocytosis and the host immune defenses [10]. In human *Ps. aeruginosa* infections implicating the bacterial adherence and biofilm development on medical devices, as well as its resistance to biocides are all regulated by complex mechanisms dependent on cell density. Isolated production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response neutralizing these compounds. However, the coordinated expression of virulence genes by an entire bacterial population once a certain density has been reached might allow *Ps. aeruginosa* to secrete extracellular factors only when they can be produced at high enough levels to overcome host defenses. These factors could alter the precarious balance between host defenses and production of bacterial toxins, leading to invasion of blood vessels, dissemination, systemic inflammatory-response syndrome, and finally death. Even appropriate antibiotic therapies are often unable to stop this course; therefore, the process must be blocked early, before virulence gene expression can be coordinated. *S. aureus* and *Ps. aeruginosa* species are in the top of the etiology of cardiovascular devices associated infections. Many studies showed the involvement of virulence factors in different type of infections, but clear correlations between a certain virulence profile and the initial severity and outcome of clinical infections are still missing. The aim of the present work was to characterize the virulence profiles of *S. aureus* and *Ps. aeruginosa* isolated from cardiovascular devices associated infections, in order to establish the association between the presence of some virulence factors and the outcome of infections caused by these bacteria. In this purpose, we used phenotypic tools in order to detect the cell-wall associated and the extracellular virulence factors of these strains in relation with a certain substratum, either an inert or cellular one.

The majority of the tested strains exhibited a significant adherence index to the inert substrate, highlighted by their capacity to secrete an extracellular exopolycsacharide quantified by the *slime* test (Table 1) and confirmed by direct examination in CLSM (Fig. 1-2), indicating a pronounced capacity of bacterial strains to colonize the central venous catheters, with the subsequent formation of bacterial biofilms, difficult to remove by conventional therapeutic means [11-14].

Table 1. Adhesion to inert substrate of *S. aureus* and *Ps. aeruginosa* strains (slime test), quantified by a semiquantitative assay

Bacterial strain	Slime test	Bacterial strain	Slime test
S. aureus 91/2007	±	*Ps. aeruginosa* 1443	++
S. aureus 1378		*Ps. aeruginosa* 1562	±
S. aureus 10936	+	*Ps. aeruginosa* 2527	+++
S. aureus 120/2007	++	*Ps. aeruginosa* 1561	++
S. aureus 128/2007	+	*Ps. aeruginosa* 1442	++
S. aureus 11327	++	*Ps. aeruginosa* 1094	++
S. aureus 11323	++	*Ps. aeruginosa* 20	++
S. aureus 11573	-	*Ps. aeruginosa* 111	+
S. aureus 11325	++	*Ps. aeruginosa* 1558	++

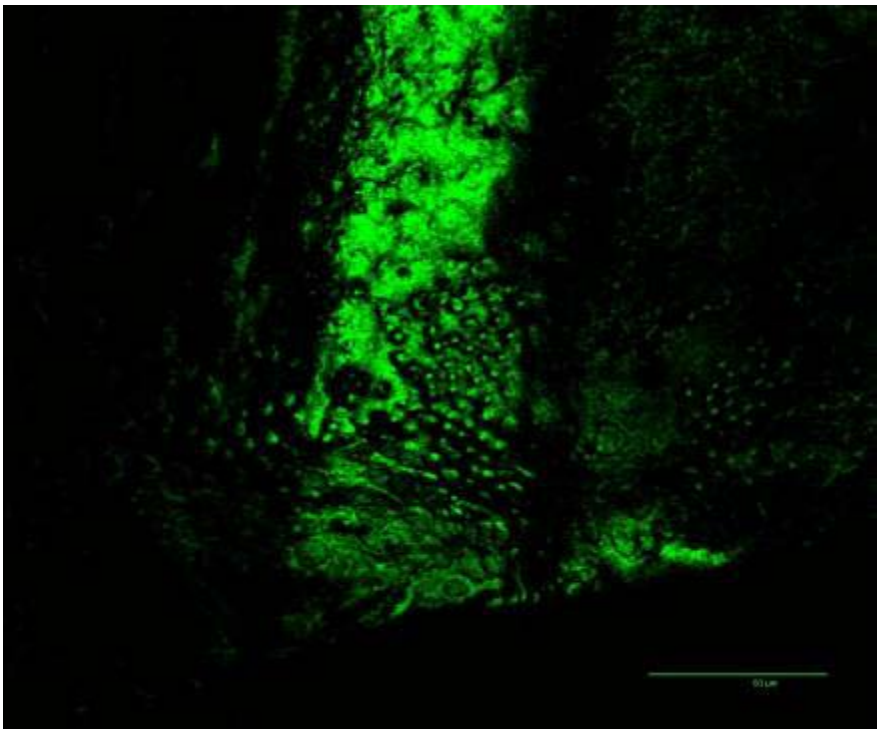

Figure 1. CLSM image of a central venous catheter (CVC) internal section colonized with *S. aureus* 11325 strain (Obj. 10x, zoom 4x)

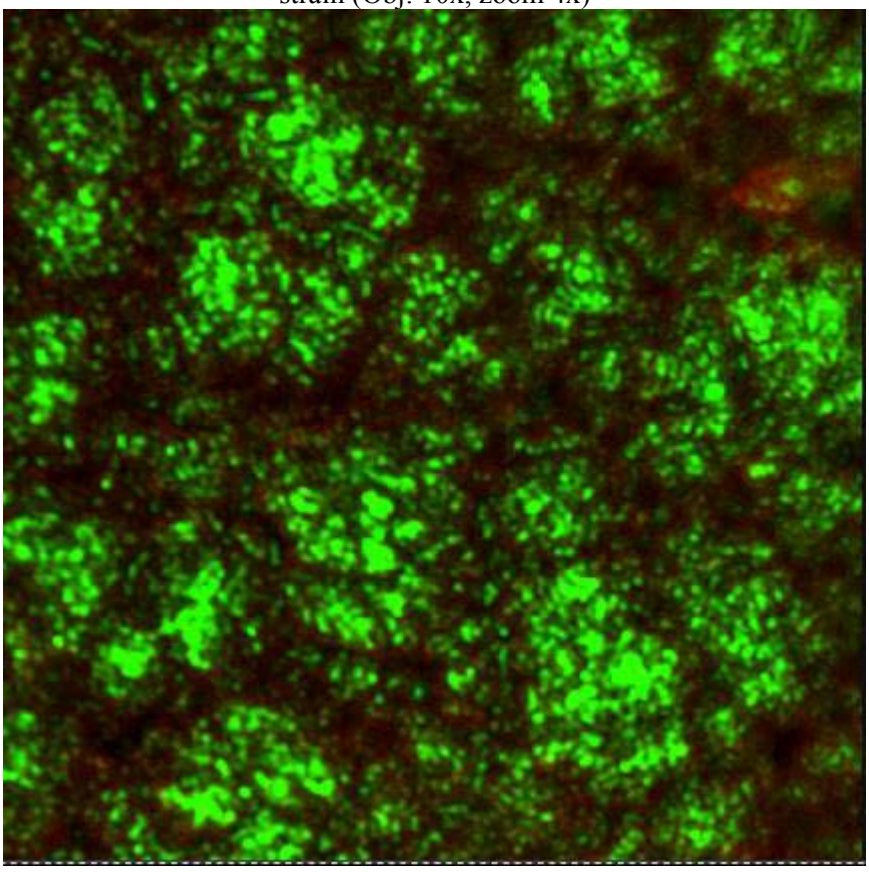

Figure 2. CLSM image of an internal surface of CVC (green) colonized with *Ps. aeruginosa* 2527 strain (red) especially at the level of surface irregularities (Obj. 10 x 0.3)

Adherence to the cellular substrate was very high in both *S. aureus* and *Ps. aeruginosa* tested strains, and surprinsingly all tested strains exhibited the ability to invade and multiply with differrent rates in HeLa cells. Our results are in concordance with other literature data, which demonstrated the intracellular survival of these opportunistic bacteria (considered by now prototypes for the extracellar infections) in mammalian cells, proving the need to create new models for the study of specific infection stages, including the intracellular one [15, 16]. It is well known that the turnover of mucosal cells is less than 48 hours, so the bacteria need to attack and multiply fast enough to not be removed. Success in achieving an optimal density in the infected host depends on bacterial adherence and tissue colonization and invasion ability, sometimes followed by intracellular multiplication and dissemination or persistence in host tissues [17]. The presence of enzymes involved in invasion could explain the ability of the analyzed strains to infect and disseminate into the host cells and tissues and to determine systemic infections.

4. Conclusions

The above mentioned results demonstrate the complexity of the interactions established between the opportunistic bacterial strains and the prosthetic devices isolated from patients with cardiovascular disease. The majority of the tested strains exhibited a strong tendency to colonize inert and cellular substrata and to produce extracellular enzymes, responsible for invasive infections.

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