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Virulence attributes in Brazilian clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for causing a huge variety of acute and chronic infections with significant levels of morbidity and mortality. Its success as a pathogen comes from its genetic/metabolic plasticity, intrinsic/acquired antimicrobial resistance, capacity to form biofilm and expression of numerous virulence factors. Herein, we have analyzed the genetic variability, antimicrobial susceptibility as well as the production of metallo-β-lactamases (MBLs) and virulence attributes (elastase, pyocyanin and biofilm) in 96 strains of *P. aeruginosa* isolated from different anatomical sites of patients attended at Brazilian hospitals. Our results revealed a great genetic variability, in which 86 distinct RAPD types (89.6% of polymorphisms) were detected. Regarding the susceptibility profile, 48 strains (50%) were resistant to the antimicrobials, as follows: 22.92% to the three tested antibiotics, 12.5% to both imipenem and meropenem, 11.46% to ceftazidime only, 2.08% to imipenem only and 1.04% to both ceftazidime and meropenem. Out of the 34 clinical strains of *P. aeruginosa* resistant to both imipenem and meropenem, 25 (73.53%) were MBL producers by phenotypic method while 12 (35.29%) were PCR positive for the MBL gene *SPM-1*. All *P. aeruginosa* strains produced pyocyanin, elastase and biofilm, although in different levels. Some associations were demonstrated among the susceptibility and/or production of these virulence traits with the anatomical site of strain isolation. For instance, almost all strains isolated from urine (85.71%) were resistant to the three antibiotics, while the vast majority of strains isolated from rectum (95%) and mouth (66.67%) were susceptible to all tested antibiotics. Urine isolates produced the highest pyocyanin concentration (20.15 ± 5.65 μg/ml), while strains isolated from pleural secretion and mouth produced elevated elastase activity (1441.43 ± 303.08 FAU) and biofilm formation (OD_{590} 0.676 ± 0.32), respectively. Also, MBL-positive strains produced robust biofilm compared to MBL-negative strains. Collectively, the production of site-dependent virulence factors can be highlighted as potential therapeutic targets for the treatment of infections caused by heterogeneous and resistant strains of *P. aeruginosa*.

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium able to exploit numerous environmental niches and colonize/infect distinct organisms (Kung et al., 2010; Balasubramanian et al., 2013). In this context, *P. aeruginosa* is an opportunistic human pathogen, which causes a plenty variety of acute and chronic infections, such as bloodstream infection in intensive care units, surgical site infection, hospital-acquired pneumonia and urinary tract infections, mainly in patients with severe burn wounds, AIDS, lung cancer, chronic obstructive pulmonary disease, bronchiectasis and cystic fibrosis (Williams et al., 2010; Jefferies et al., 2012; Balasubramanian et al., 2013). In these immune-debilitating conditions, *P. aeruginosa* causes a significant level of morbidity and mortality (Balasubramanian et al., 2013; Mudau et al., 2013; Muraki et al., 2013).

The emergence of multiple drug resistance has become a major threat for current medical treatment of nosocomial infections caused by *P. aeruginosa* around the world (Mudau et al., 2013; Muraki et al., 2013). The available therapeutic options to treat *P. aeruginosa* infections are limited to a handful of compounds belonging to three major antimicrobial classes: antipseudomonal β-lactams, antipseudomonal fluoroquinolones and aminoglycosides (Hong et al., 2013). According to the surveillance study conducted by the International Nosocomial Infection Control Consortium (INICC) of 36 countries in Latin America, Asia, Africa and Europe, 47.2% of *P. aeruginosa* isolates were reported to be resistant to imipenem (Rosenthal et al., 2012). In the same way, two Chinese nationwide surveillance programs, named Surveillance by Etest and Agar Dilution of Nationwide Isolate Resistance (SEANIR) and Study for Monitoring Antimicrobial Resistance Trends (SMART), reported that 32.4% of *P. aeruginosa* isolated in the period of 2008–2010 presented resistance to ceftazidime (Xiao et al., 2012). The Brazilian SCOPE (Surveillance and Control of Pathogens of Epidemiological Importance) found that 35.8%, 36.8% and 36.6% of *P. aeruginosa* isolates were resistant to meropenem, imipenem and ceftazidime, respectively (Marra et al., 2011). It is well known that antimicrobial resistance is an important determinant to maintain *P. aeruginosa* in the hospital environment, but the virulence machinery of this microorganism is very important as well (Jimenez et al., 2012).

The pathogenic potential of *P. aeruginosa* is due to its metabolic and genetic versatility, both intrinsic and acquired antibiotic resistance (e.g., production of metallo-β-lactamases (MBLs), which are characterized by their ability to inactivate most β-lactam antibiotics including carbapenems), ability to form biofilm and production of an arsenal of virulence attributes, including cell-associated determinants (e.g., lipopolysaccharide, pili and flagellum) and soluble secreted factors (e.g., extracellular polysaccharides, exotoxins, proteases and pyocyanin) (Kung et al., 2010; Balasubramanian et al., 2013; Muraki et al., 2013). *P. aeruginosa* cells are able to modulate its gene expression in response to environmental conditions, controlling the secretion of virulence factors and phenotype switching (Balasubramanian et al., 2013). For instance, changes in growth medium components, temperature and aeration can greatly affect the amount of elastase secreted by *P. aeruginosa* (Olson and Ohman, 1992). The adaptability and variability of *P. aeruginosa* are due to its pangenome, which is composed of the core genome (genes present in nearly all strains that encode a set of metabolic and pathogenic factors) and the accessory genome (genes found only in some strains, to which they confer specific phenotypes that are advantageous under certain selective conditions) (Kung et al., 2010).

Globally, few studies have reported the expression and/or production of virulence factors in *P. aeruginosa* isolates, mainly in clinical strains from Brazilian hospitals. In the present work, we have analyzed the genetic variability, the antimicrobial susceptibility to ceftazidime, meropenem and imipenem, the detection of MBLs as well as the production of three major virulence attributes (biofilm, elastase and pyocyanin) in 96 clinical strains of *P. aeruginosa*, which were isolated from different anatomical sites of patients attended at hospitals located in three States of Brazil (Espírito Santo, Minas Gerais and Rio de Janeiro).

Materials and methods

Clinical strains

A total of 96 non-duplicated strains of *P. aeruginosa* were used in all parts of the present work, which were originally isolated between 1999 and 2010 from independent studies in different Brazilian hospitals, as follows: 57 strains were recovered from Clinical Hospital of Federal University of Uberlandia – Minas Gerais State; 18 strains from Meridional Hospital – Espírito Santo State; 15 strains from Marcílio Dias Naval Hospital – Rio de Janeiro State; and the remaining 6 strains were obtained from Biology Institute of the Army, Santa Rita de Cassia Hospital and Silvestre Adventist Hospital, Rio de Janeiro (Kokis et al., 2005; Rocha et al., 2008). Each *P. aeruginosa* strain used in the present study came from only one patient; consequently, each strain was isolated from only one anatomical site regarding each individual. According to the anatomical site, the *P. aeruginosa* strains were isolated from rectum ($n = 20$), tracheal aspirate ($n = 19$), mouth ($n = 18$), blood ($n = 8$), urine ($n = 7$), venous catheter ($n = 6$), pleural secretion ($n = 5$), eschar ($n = 4$), lung of cystic fibrosis ($n = 4$), sputum ($n = 3$) and nasal secretion ($n = 2$) of patients hospitalized in intensive treatment units of the previously cited Brazilian hospitals. The reference strain of *P. aeruginosa* ATCC 27853 was used as a control in all experiments.

Random amplification of polymorphic DNA (RAPD)

In order to extract the bacterial DNA, the clinical strains were grown on trypticase soy agar (TSA; Merck, Darmstadt, Germany) for 18 h at 37 °C. Afterward, three colonies were suspended in 1 ml of 0.1 M phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2) and centrifuged at 12,000 rpm for 5 min. Supernatants were discarded and the pellets were resuspended in 100 μl of ultrapure water, boiled for 8 min and then remained on ice for 30 min. The mixtures were centrifuged at 12,000 rpm for 5 min and the pellets were discarded, while the supernatants containing the DNA were stored at −20 °C prior to polymerase chain reaction (PCR) (Pellegrino et al., 2002). For PCR amplification, the reaction mixes (25 μl) were set up as follows: 14.6 μl H_2O (DNA-free), 2.5 μl of PCR buffer 10× without Mg^{2+}, 1.5 μl $MgCl_2$ (50 mM), 2.5 μl dNTP mix (5 mM), 2.5 μl primer 272 (5′-AGCGGGCCAA-3′) (Mahenthiralingam et al., 1996) diluted 1:4 (100 pmols/μl), 0.4 μl Taq polymerase (5 U/μl) and 1 μl DNA template (100 ng). Then, the PCR mixtures were subjected to the following thermal cycling parameters in a MJ Research PTC-100 Thermal Cycler (Harlow Scientific, Arlington, USA): (i) denaturation step at 94 °C for 2 min; (ii) annealing step with 30 cycles, each one consisting of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min; (iii) extension step at 72 °C for 10 min and final temperature at 4 °C. The PCR products (10 μl) were separated by electrophoresis in a 1% agarose gel with 0.5× Tris–borate–EDTA (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) running buffer at 100 V for 1–2 h, then stained with 0.5 mg/ml of ethidium bromide and detected under UV transillumination (Bio Rad Gel Doc 2000 with UV Trans Iluminator). The amplicons were analyzed based on the fragment sizes. Dendrogram was constructed with assistance of the GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) (Pellegrino et al.,

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