Revista da Sociedade Brasileira de Medicina Tropical 45(6):707-712, Nov-Dec, 2012

Major Article

Phenotypic and molecular characterization of antimicrobial resistance and virulence factors in *Pseudomonas aeruginosa* clinical isolates from Recife, State of Pernambuco, Brazil

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ABSTRACT

Introduction: The emergence of carbapenem resistance mechanisms in *Pseudomonas aeruginosa* has been outstanding due to the wide spectrum of antimicrobial degradation of these bacteria, reducing of therapeutic options. **Method**s: Sixty-one clinical strains of *P. aeruginosa* isolated from five public hospitals in Recife, Pernambuco, Brazil, were examined between 2006 and 2010, aiming of evaluating the profiles of virulence, resistance to antimicrobials, presence of metallo-β-lactamase (MBL) genes, and clonal relationship among isolates. **Results**: A high percentage of virulence factors (34.4% mucoid colonies; 70.5% pyocyanin; 93.4% gelatinase positives; and 72.1% hemolysin positive) and a high percentage of antimicrobial resistance rates (4.9% **pan**-resistant and 54.1% multi-drug resistant isolates) were observed. Among the 29 isolates resistant to imipenem and/or ceftazidime, 44.8% (13/29) were MBL producers by phenotypic evaluation, and of these, 46.2% (6/13) were positive for the bla_{SPM-1} gene. The bla_{IMP} and bla_{VIM} genes were not detected. The molecular typing revealed 21 molecular profiles of which seven were detected in distinct hospitals and periods. Among the six positive bla_{SPM-1} isolates, three presented the same clonal profile and were from the same hospital, whereas the other three presented different clonal profiles. **Conclusions**: These results revealed that *P. aeruginosa* is able to accumulate different resistance and virulence factors, making the treatment of infections difficult. The identification of bla_{SPM-1} genes and the dissemination of clones in different hospitals, indicate the need for stricter application of infection control measures in hospitals in Recife, Brazil, aiming at reducing costs and damages caused by *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa.* Carbapenemase. Multidrug resistance. Virulence factors.

INTRODUCTION

Pseudomonas aeruginosa is a bacterium of environmental origin considered an essentially opportunistic pathogen infecting hospitalized and immune-compromised patients[1,2]. In Brazil, *P. aeruginosa* is an important cause of nosocomial infections and is considered the first cause of nosocomial pneumonia and the third cause of bloodstream primary infection in intensive care units (ICU)[3,4].

Some virulence factors favor this pathogen's infection, such as the formation of pyocyanin, hemolysin, gelatinase, and biofilm, which act increasing tissue damage and protecting *P. aeruginosa* against the recognition of the immune system and the action of antibiotics[5-7]. The pathogenesis of *P. aeruginosa* infections is multifactorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium. Pili, lipopolysaccharide (LPS), flagella, elastase, alkaline protease, siderophores, siderophore uptake systems and extracellular protein toxins (exoenzyme S and exotoxin A) are examples of other virulence factors[5].

Some regulatory mechanisms of virulence factors has excelled as the loss-of-function mutations in *mucA* that blocks the production of many invasive virulence factors (including type III secretion system [T3SS], exotoxin A, protease IV, and type IV pili [TFP]) by inhibiting cAMP-Vfr-dependent signaling (CVS) at the level of *vfr* expression involving AlgU and the response regulator AlgR[8,9]. Furthermore, together, AlgU and AlgR activate the transcription of genes encoding the biosynthetic enzymes for alginate production, resulting in the mucoid phenotype[8].

Pseudomonas aeruginosa can also harbor several mechanisms of resistance, which generates multi-drug resistant isolates (resistance to three or more classes of anti-*Pseudomonas* agents), or pan-resistant isolates (resistance to all antimicrobials clinically used)[4,10].

An alternative treatment to infections caused by multi-drug resistant organisms is the use of carbapenems, such as the imipenem, meropenem, and more recently the doripenem; however, they are not indicated as the first choice for empirical treatment of hospital or community infections. Moreover, the indiscriminate use of carbapenems in the hospital environment leads to the selection of resistant strains to this class of antimicrobials[11,12].

The production of carbapenemase enzymes became the mechanism of greater relevance towards carbapenem resistance due to the growing enzyme diversity, especially featuring the metallo-β-lactamases (MBL). These enzymes have high versatility and wide hydrolytic activity over almost all β-lactam antibiotics, with the exception of monobactams[13,14].

Nine subclasses of MBL have been described yet, IMP-1 (Imipemenase), VIM-1 (Verona imipenemase), SPM-1 (São Paulo metallo-β-lactamase), GIM-1 (German imipenemase), SIM-1 (Seoul imipenemase), AIM-1 (Australian imipenemase) KHM-1 (Kyorin Hospital metallo-β-lactamase), NDM-1 (New Delhi metallo-β lactamase), DIM-1 (Dutch imipenemase)[13], of which only the first three were identified in different Brazilian states[45].

In addition to the research of resistance genes, another indispensable tool in controlling infections is the application of

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Received in 12/03/2012
Accepted in 23/10/2012

genotyping methods for the determination of genetic relationships among the microorganisms, thus allowing mapping the dynamics of infection transmission[16,17]. The utilization of the enterobacterial repetitive intergenic consensus sequence by polymerase chain reaction (ERIC-PCR) has demonstrated its efficacy, efficiency, and usefulness for epidemiological surveillance[17-19].

This study characterized, phenotypically and genotypically, *P. aeruginosa* isolates obtained from public hospitals in Recife, Brazil, with the objective of identifying resistance and virulence markers and establishing the clonal dissemination of the isolates.

METHODS

Bacterial isolates

Were analyzed 61 *P. aeruginosa* isolates from various sites of infection (blood, urine, tracheal secretions, wound, eye discharge and ulcer) of hospitalized patients from five teaching hospitals in Recife, between 2006 and 2010. Among the microbiological characteristics were investigated colony morphology, pigment production, hemolysin production[2] and gelatinase production[20].

Pigments production and colony morphology

The strains (pure culture) were streaked on Cetrimide agar and incubated for 24h at 37°C to visual analysis of pigment production (pyocyanin, pioverdin, piorubin and piomelanin) and colony morphology (mucoid and not mucoid)[2].

Antimicrobial susceptibility

The antibiotic susceptibility testing was evaluated by agar diffusion using amikacin, gentamicin, ciprofloxacin, ticarcillin/clavulanic acid, aztreonam, cefepime, ceftazidime, imipenem, meropenem and polymyxin, as recommended by the Clinical and Laboratory Standards Institute (CLSI)[21]. Imipenem and / or ceftazidime resistant isolates were investigated for metallo-β-lactamases (MBLs) producing, using disk approximation test described by Arakawa hard et al.[22], performed in duplicate for each isolate.

Metallo-β-lactamase PCR

To search the related carbapenemase-encoding genes, the total DNA of isolates was obtained using Brazol kit (LGC Biotecnologia), according to manufacturer's instructions, and quantified by spectrophotometry (Ultraspec 3000, Pharmacia Biotech) at wavelengths 260 to 280nm. The MBL genes were surveyed using the following primers: bla_{SPM-1} F (5'-CCTACAATCTAACGGCGA CC-3'), bla_{SPM-1} R (5'-TCGCCGTGTCCAGGTATAAC-3')[9], bla_{IMP} F (5'-GGAATAGAGTG GCTTAATTCTC-3') and bla_{IMP} R (5'-CGTGTGATGCYCCAAYTTCACT-3'), bla_{VIM} F (5'-CAGATTGCCGATGGTGTTTGG-3') and bla_{VIM} R (5'-AGGTGGGCCATTCAGCCAGA-3')[23]. Amplification reactions were prepared in a total volume of 25µl per tube, comprising: 25ng of genomic DNA, 10pmol of primers, 1x buffer, 100µM of each deoxyribonucleotide triphosphate (Ludwig Biotec), 1.5mM MgCl2 and 1.0U Taq DNA polymerase (Promega). In each round of amplification positive and negative controls for genes bla_{SPM-1} (*P. aeruginosa* PSA319), bla_{IMP} (*P. aeruginosa* 48-1997A) and bla_{VIM} (*P. aeruginosa* VIM-1) were included. The cycles parameters were 95°C for 5minutes, followed by 30 cycles of denaturation at 95°C for 1minute, annealing for 1min at 50.6ºC, 55.3°C and 62ºC, to bla_{IMP}, bla_{SPM-1} and $bla_{VIM,}$ respectively; extension at 68°C for 1minute, and finally 10minutes at 68°C. PCR products were stained with blue-green (LGC Biotecnologia - São Paulo) and subjected to electrophoresis on agarose gel 1% in TBE buffer (Tris-borate 0.089 M EDTA and 0.002M) under constant voltage of 100V[11].

ERIC-PCR

To assess the genetic relationship of the isolates, ERIC-PCR was carried out. Amplification reactions were prepared in a total volume of 25µl per tube, comprising: 100ng of genomic DNA, 10pmol of primers (ERIC-1 [5'-ATGTAAGCT CCTGGGGATTCAC-3']; ERIC-2 [5'-AAGTAAGTGACTGGGGTGAGCG-3'])[12], 1x buffer, 200µM of each deoxyribonucleotide triphosphate (Promega), 1.5mM MgCl2 and 1.0U Taq DNA polymerase (Promega). The amplification parameters used were 95°C for 3minutes, followed by 30 cycles of denaturation at 92°C for 1minute, annealing at 36°C for 1minute and extension at 72°C for 8minutes, followed by a final extension 16minutes at 72°C. PCR products were stained with blue-green (LGC biotec) and subjected to electrophoresis on agarose gel to 1.5% in TBE buffer under constant voltage of 100V[24]. Data analysis and dendrograms were made using the Darwin 5.0 software.

RESULTS

The analysis of virulence factors revealed that out of the 61 *P. aeruginosa* isolates studied, 34.4% presented mucoid colonies, 70.5% were pyocyanin producers, 93.4% were hemolysin and gelatinase (72.1%) producers **(Table 1)**.

Antimicrobial susceptibility testing data are shown in **Figure 1**. The highest level of resistance was recorded against gentamicin and ciprofloxacin, 41% (25/61), whereas polymyxin B and aztreonam presented the lowest percentages of resistance, 13.1% (8/61) and 29.5% (18/61), respectively.

TABLE 1 - Frequency of the microbiological characteristics of *Pseudomonas aeruginosa* isolates from hospitals in Recife, Brazil, obtained from years 2006 to 2010.

Characteristic	Number /Sample	Percentage (%)
Smooth colonies	40/61	65.6
Mucoid colonies	21/61	34.4
Pyocyanin production	43/61	70.5
Pioverdin production	17/61	27.9
Piorubin production	0/61	0.0
Piomelanin production	1/61	1.6
Hemolysin production	57/61	93.4
Gelatinase production	44/61	72.1
Metallo-β-lactamase production	13/29	44.8
Three antimicrobial agents degraded MDR	5/33	15.2
Four antimicrobial agents degraded MDR	4/33	12.1
Five antimicrobial agents degraded MDR	3/33	9.1
Seven antimicrobial agents degraded MDR	5/33	15.2
Eight antimicrobial agents degraded MDR	4/33	12.1
Nine antimicrobial agents degraded MDR	9/33	27.3
Ten antimicrobial agents degraded MDR	3/33	9.1

Number: number of isolates presenting a particular characteristic; **Sample:** sample size used for the study of a particular characteristic; **MDR:** multidrug-resistant isolates.

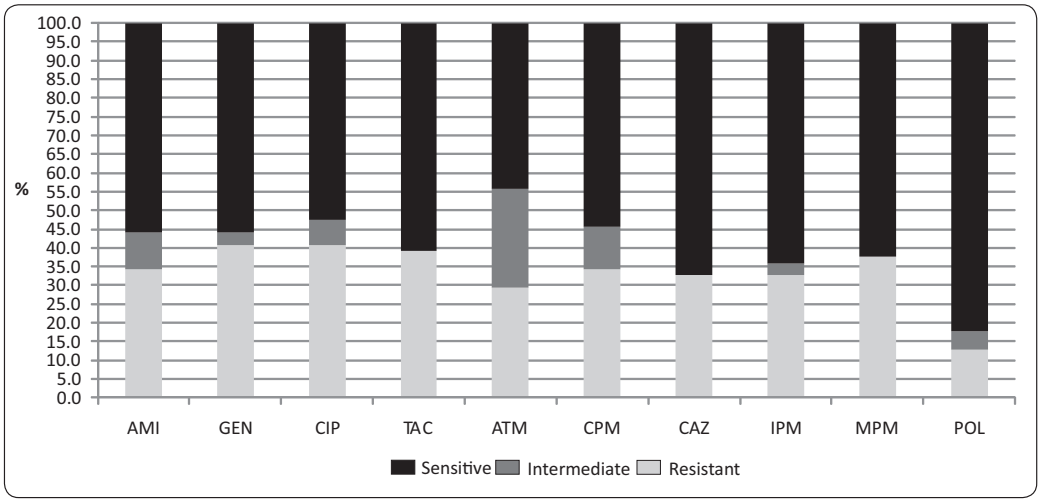

FIGURE 1 - Antimicrobial susceptibility profile of *Pseudomonas aeruginosa* isolates from five public hospitals in Recife, State of Pernambuco, Brazil, between years 2006 and 2010. **AMI**: amikacin; **GEN**: gentamicin; **CIP**: ciprofloxacin; **TAC:** ticarcillin/clavulanic acid; **ATM:** aztreonam; **CPM:** cefepime; **CAZ:** ceftazidime; **IPM:** imipenem; **MPM:** meropenem; **POL:** polymyxin.

The multidrug resistance profile was observed in 54.1% (33/61) of the isolates, of which, 27.3% (9/33) were susceptible only to polymyxin B and 9.1% (3/33) revealed the pan-resistant profile, as described in **Table 1**.

Among the 29 isolates resistant to imipenem and/or ceftazidime, 44.8% (13/29) were MBL producers, of which, 46.2% (6/13) revealed the bla_{SPM-1} gene; two of them (P7A, P15A) were isolated from the same hospital, and were collected in 2006. The other ones (P6BL, P3R, P4R, P7R) were collected in 2010 and originated from two other hospitals. The bla_{IMP} and bla_{VIM} genes were not detected.

The molecular typing of the isolates revealed 21 genetic profiles with predominance of clones A, B, and C, as shown in the dendrogram **(Figure 2)**. Among the SPM-1 positive isolates, three shared the same clonal profile (clone D), while the other three presented distinct clonal profiles (clones C, G, and P). Seven clones (A, B, C, D, E, J, and K) were detected in more than one hospital and were detected in distinct periods.

DISCUSSION

The phenotypic characterization performed in this study revealed a high percentage of virulence factors among the studied isolates, with the exception of the mucoid phenotype, associated with the formation of biofilm present in only 34.4% of the colonies. Deptuła and Gospodarek[23] observed even smaller frequencies, when comparing the frequency of virulence factors among multi-drug susceptible and multidrug-resistant (MDR) isolates with biofilm formation in 9.3% and 8%, respectively.

The biofilm is composed of alginate and confers a mucoid consistency to *P. aeruginosa* isolates, acting as a protecting niche for the bacterium against the recognition of the immune system and the action of antibiotics, thus increasing the possibility of infection chronicity[6]. The biofilm production has been well studied in patients with cystic fibrosis because this is a chronic pulmonary infection, allowing verifying the transformation from non mucoid to mucoid isolates during the process of disease evolution[6,26].

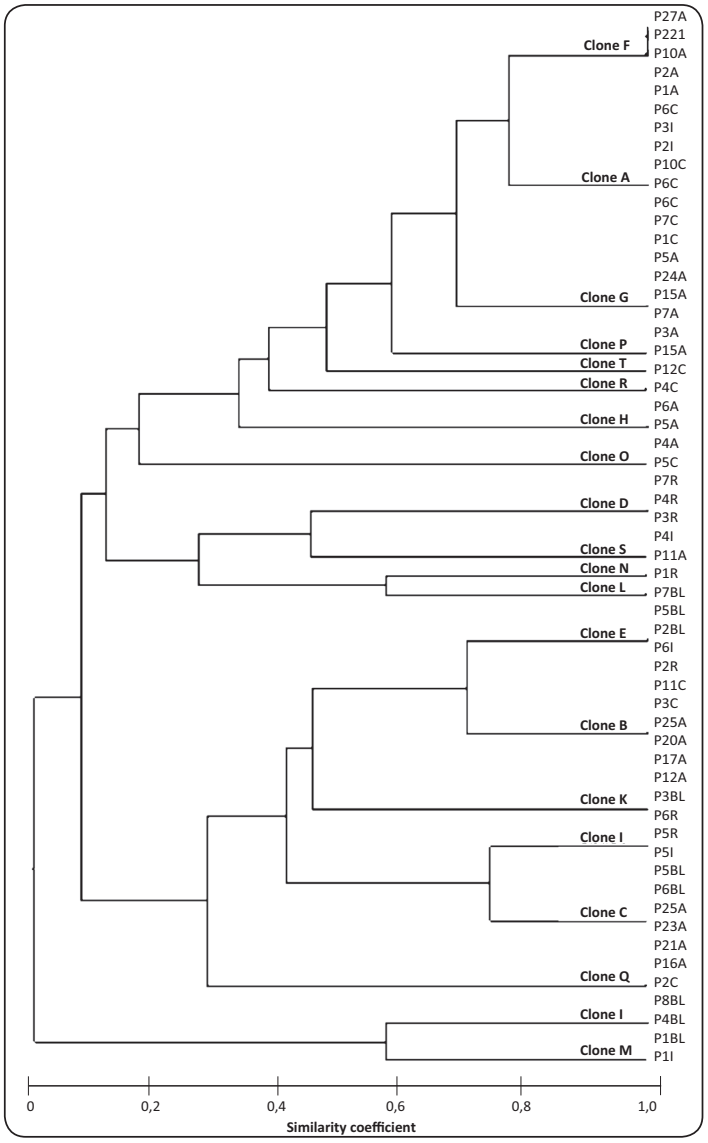

FIGURE 2 - Dendrogram estimated by the enterobacterial repetitive intergenic consensus sequence by polymerase chain reaction for 61 *Pseudomona aeruginosa* isolates from five public hospitals in Recife, State of Pernambuco, Brazil, isolated between years 2006 and 2010.

The production of pyocyanin was observed in 70.5% of the studied isolates. This virulence factor consists of a blue phenazinic pigment produced exclusively by *P. aeruginosa* and related to tissue damage through the formation of reactive hydroxyl radicals and superoxides, mainly in the respiratory epithelium[6,27]. Some studies report the production of pyocyanin in *P. aeruginosa* isolates ranging between 41.3% and 81.5%[28,29]. The association of this production with the production of biofilm[26] and the presence of multidrug resistance were also investigated in these studies[25], and no significant difference in either of the two cases was reported.

The production of hemolysin and gelatinase, which accounted for 93.4% and 72.1%, respectively, correspond to other virulence factors associated with tissue injury. Stehling et al.[31], in an analytical study to assess the production of virulence factors among non-mucoid and mucoid isolates, did not observe significant difference in the production of hemolysin (p = 0.5911) and gelatinase (p = 0.5542), reporting average frequencies of 53.6% and 37.5%, respectively.

The antimicrobial susceptibility profiles obtained in this study showed that the most active drug against *P. aeruginosa* was polymyxin B (82%), followed by ceftazidime (67.2%), imipenem (63.9%), and meropenem (62.3%). Pires et al.[32-35,38] reported, in a study conducted between January and June of 2009 in the *Hospital das Clínicas de Pernambuco*, Brazil, the observation that the most active antimicrobials against *P. aeruginosa* were amikacin (84.6%), imipenem (81.8%), meropenem (79.3%), and aztreonam (74.4%). Nevertheless, this comparative evaluation between antimicrobials was impaired because there was variation in the choice of antibiotic tested in the different studies. Figueiredo et al.[33] investigated 304 isolates of *P. aeruginosa,* from two public hospitals in Recife (*Hospital das Clínicas de Pernambuco* and *Hospital Agamenon Magalhães*), in the period between September 2004 and January 2006, and observed that aztreonam (56.5%), amikacin (55.9%), meropenem (55.7%), and imipenem (51.7%) showed increased activity. In these studies, the carbapenems were among the four most active antimicrobials, with susceptibility percentages ranging from 51.5% to 79.3%. On the other hand, both Pires et al.[32] and Figueiredo et al.[33] observed higher antimicrobial susceptibility compared to our results, where aztreonam showed the lower antimicrobial activity, with more expressive resistance rates over the past two years.

A high percentage of multi-drug resistant isolates observed in this study, is in agreement with previous findings of multidrug resistance in *P. aeruginosa* in the same municipality[33-35]. However, several studies have reported lower rates of multi-drug resistant organisms[3,10,38], which highlights the importance of the results revealed in our study.

The detection of *P. aeruginosa* isolates, producers of MBL, has been described worldwide with a predominance of the IMP and VIM types[14]. In Brazil, the most prevalent enzyme is the SPM-1[38], which description remained restricted to the Brazilian territory until 2010 when the first report of an SPM-1 isolate, identified in 2007, in Europe, from a Swiss patient who had been in a university hospital in Recife-PE, Brazil, was published[37].

The bla_{SMP-1} gene has been reported in *P. aeruginosa* isolates from the northeast, midwest, southeast and southern states in Brazil, with prevalences varying from 3.1% to 77.1% in isolates resistant to imipenem and/or ceftazidime[11,13,30,40-51]. Despite not having been identified in this study, the $bla_{IMP-type}$ genes have already been detected in others brazilian states: Rio de Janeiro, Brasília, João Pessoa, São Paulo, Porto Alegre, and Santa Catarina[40,43,45,48,51-53], whilst, the bla_{VIM-2} gene has been reported only in São Paulo [48,49].

Reconciling research including the identification of genes for the production of MBL and molecular typing for the identification of clonal relationships between isolates of *P. aeruginosa* has been quite frequent in the recent years. This approach has been considered an important methodology because it allows verifying cross transmission in cases of outbreaks[54]. Lee et al.[55], observed the production of MBL in 55% of imipenem resistant isolates from Taiwan where the isolates involved in the outbreak were not related. Mansour et al.[54], in Tunisia, detected the production of MBL in only 7% of isolates resistant to imipenem, with prevalence of a unique genetic profile, indicating that the outbreak was caused from the same source of infection. In this study, a clonal dissemination of one isolate of *P. aeruginosa* carrying the bla_{SMP-1} gene was identified involving three inpatients in the HR in the second half of 2010. These isolates showed the same virulence factors investigated as well as the same resistance profile and being susceptible to polymyxin B. On the other hand, the other bla_{SMP-1} positive isolates were not genetically related and presented distinct virulence and resistance profiles.

Out of the total sample set, 21 genetic profiles were identified, with dissemination of seven clones in more than one hospital and during different periods. Similar results are frequent, particularly in studies involving patients with serious and chronic infection, which represent favorable conditions for the occurrence of random non-lethal genetic mutations[56].

This study concluded that *P. aeruginosa* is a pathogen able to accumulate several virulence factors, which are often associated with multidrug resistance and pan-resistance, making the treatment of infections caused by this bacterium difficult. The identification of bla_{SPM-1} genes detected in more than one hospital (interhospital dissemination), suggests the implementation of the infection control measures in hospitals in Recife, Brazil, aiming at reducing costs and damages caused by this invasive microorganism especially in patients presenting serious health conditions.

ACKNOWLEDGMENTS

We gratefully acknowledge Prof. Dr. Marcia M.C. Morais from the Microbial Resistance Laboratory, Institute of Biological Sciences, University of Pernambuco for kindly providing the positive controls for detection of metallo-β-lactamases genes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work was supported by FACEPE (*Fundação de Amparo Ciência do Estado de Pernambuco*) and CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico*) granted in APQ 0579-2.12/07, and PROPESQ (*Pró-Reitoria para Assuntos de Pesquisa e Pós-Graduação*) granted in *Edital de Multiusuários*/PROPESQ-2007.

ABSTRACT IN PORTUGUESE

Caracterização fenotípica e molecular de fatores de resistência a antimicrobianos e virulência de isolados clínicos de *Pseudomonas aeruginosa* de Recife, Estado de Pernambuco, Brasil

Introducão: A emergência de mecanismos de resistência aos carbapenêmicos em *Pseudomonas aeruginosa* tem se destacado devido ao amplo espectro de degradação de antimicrobianos, reduzindo as opções terapêuticas. **Métodos**: Sessenta e um isolados de *P. aeruginosa* procedentes de cinco hospitais públicos de Recife, Pernambuco, Brasil, entre 2006 e 2010, foram analisadas, com o objetivo de avaliar o perfil de virulência, resistência aos antimicrobianos, a presença de genes metalo-β-lactamase (MBL) e a relação clonal entre os isolados. **Resultados:** Foi observada uma elevada produção de fatores de virulência na amostra (34,4% colônias mucoides; 70,5% piocianina; 93,4% gelatinase e 72,1% hemolisina), bem como um elevado percentual de resistência (4,9% isolados panresistentes e 54,1% multirresistentes). Dentre os 29 isolados resistentes ao imipenem e/ou ceftazidima, 44,8% (13/29) apresentaram MBL por meio da pesquisa fenotípica, e destes, 46,2% (6/13) foram positivos para o gene bla_{SPM-1}, não havendo detecção dos genes bla_{IMP} e bla_{VIM}. A tipagem molecular revelou 21 perfis genéticos dos quais sete foram detectados em hospitais e períodos distintos, e dos isolados bla_{SPM-1} positivos, três apresentaram o mesmo perfil clonal e foram procedentes do mesmo hospital, enquanto que os outros três isolados bla_{SPM-1} positivos apresentaram perfis clonais distintos. **Conclusões:** Estes resultados revelam que a *P. aeruginosa* é capaz de acumular diferentes fatores de virulência e resistência, dificultando o tratamento das infecções. A identificação de genes bla_{SPM-1} e disseminação de clones sugere a necessidade de aplicação mais rigorosa de medidas de controle de infecção nos hospitais de Recife, visando reduzir custos e danos provocados por este tipo de infecção.

Palavras-chaves: *Pseudomonas aeruginosa.* Carbapenemase. Multirresistência. Fatores de virulência.

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