Evaluation of heteroresistance to polymyxin B among carbapenem-susceptible and -resistant *Pseudomonas aeruginosa*

Djuli M. Hermes,1 Caroline Pormann Pitt,2 Larissa Lutz,3 Aline B. Teixeira,1 Vanessa B. Ribeiro,4 Bárbara Netto,1 Andreza F. Martins,5 Alexandre P. Zavascki1,6 and Afonso L. Barth1,4,7

Correspondence
Afonso L. Barth
albarth@hcpa.ufrgs.br

One hundred and twenty-four *Pseudomonas aeruginosa* isolates were selected for antimicrobial susceptibility testing with anti-pseudomonal agents, MIC determination for polymyxin B and metallo-beta-lactamase detection (genes \(\text{bla}_{\text{SPM}}\), \(\text{bla}_{\text{VIM-1}}\), \(\text{bla}_{\text{NDM-1}}\) and \(\text{bla}_{\text{IMP}}\)). According to the imipenem and/or meropenem susceptibility profile, a set of randomly selected isolates (12 isolates carbapenem-susceptible and 12 isolates carbapenem-resistant) were evaluated for heteroresistance to polymyxin B. Heteroresistance testing was performed by plating the isolates onto increasing concentrations of polymyxin B (from 0 to 8.0 mg l\(^{-1}\)). The population analysis profile (PAP) was defined as the ratio of the number of colony-forming units on the plate with the highest concentration of polymyxin B at which bacterial growth occurred against the number of colony-forming units on the plate without antibiotic. Isolates presenting subpopulations that exhibited growth at polymyxin B concentrations \(\geq 2\) mg l\(^{-1}\) were considered heteroresistant. Isolates containing subpopulations that grew at polymyxin B concentrations at least twice as high as the original MIC but \(<2\) mg l\(^{-1}\) were considered heterogeneous. Antimicrobial susceptibility testing results indicated a variable degree of susceptibility: high levels of resistance to gentamicin (30.6 %) and imipenem (29.0 %); low levels of resistance to aztreonam (1.6 %) and ciprofloxacin (4.8 %). All isolates were susceptible to polymyxin B: MIC\(_{50}\) and MIC\(_{90}\) were 1 mg l\(^{-1}\) and 2 mg l\(^{-1}\), respectively. Thirty-seven isolates (30 %) were carbapenem-resistant. Four isolates resistant to carbapenems were positive for \(\text{bla}_{\text{IMP}}\). There were no heteroresistant subpopulations in the carbapenem-susceptible group, but three isolates presented heterogeneous subpopulations. The PAP frequency ranged from 2.1\(\times 10^{-4}\) to 6.9\(\times 10^{-8}\). In the carbapenem-resistant group, one isolate was heteroresistant. Six isolates in this group presented heterogeneous subpopulations. In the resistant population, the PAP frequency ranged from 2.1\(\times 10^{-7}\) to 2.6\(\times 10^{-4}\). In this study, polymyxin B heteroresistance in *P. aeruginosa* was uncommon and occurred in only one carbapenem-resistant isolate, despite the fact that several isolates presented heterogeneous subpopulations with increased polymyxin B MICs.

**INTRODUCTION**

Therapeutic options for *Pseudomonas aeruginosa* infection are limited due to the diversity of resistance mechanisms developed by this pathogen and due to the high frequency of multidrug-resistant isolates. Carbapenems are the main class of antibiotics used in the treatment of *P. aeruginosa* infection. Despite this fact, carbapenem-resistant isolates have been frequently reported worldwide. The main...
mechanism involved in this resistance is the production of carbapenemases such as metallo-beta-lactamases (MBL), which prevent the activity of beta-lactam drugs (Poole, 2011). Thereby, polymyxin B remains an option for treatment of carbapenem-resistant P. aeruginosa infections (Zavascki et al., 2007).

The resistance generated by mechanisms such as MBL production usually occurs homogeneously among the bacterial population. However, a distinct resistance phenotype, named heteroresistance, has been reported. This phenomenon can be defined as the growth of resistant subpopulations among an antimicrobial-susceptible population (Pourmaras et al., 2005, 2008; Li et al., 2006; Hawley et al., 2008; Ikonomidis et al., 2009, Wing-Yau et al., 2009).

The clinical impact of heteroresistance has yet to be elucidated. Some authors suggest that this phenotype may account for unexplained treatment failures (Rinder, 2001; Pourmaras et al., 2007). As there is little information about heteroresistance and no investigation has addressed polymyxin B in P. aeruginosa, the aim of the present study was to investigate this phenomenon to polymyxin B in a set of carbapenem-susceptible and -resistant P. aeruginosa isolates.

METHODS

Bacterial isolates and susceptibility testing. One hundred and twenty-four P. aeruginosa isolates were randomly obtained at the Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil, throughout the year 2011. Antimicrobial susceptibility testing was performed by disc diffusion for all isolates against amikacin, ceftazidime, cefepime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin/tazobactam, ticarcillin/clavulanic acid, tobramycin and aztreonam, according to the Clinical and Laboratory Standards Institute (CLSI, 2012). The susceptibility to carbapenems (imipenem and meropenem) and to cephalosporins (cefepime and ceftazidime) was also evaluated by the determination of MIC. The susceptibility to polymyxin B was determined only by MIC for all isolates.

Phenotypic and genotypic characterization of MBL. Phenotypic detection of MBL was performed as previously described by Arakawa et al. (2000). Isolates were evaluated for the presence of blaIMP, blaKPC, blaSHV, blaTEM and blaNDM genes by qualitative PCR (Martins et al., 2007; Monteiro et al., 2012). Water free of DNases and RNases was used as negative control, and the positive controls used were: P. aeruginosa 48-1997As (SPM-1+), P. aeruginosa 395 (IMP+), P. aeruginosa 81-11963A (VIM+) and K. pneumoniae NCTC BAA2146 (NDM-1+), kindly provided by the Laboratório Especial de Microbiologia Clínica (LEMC), Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil.

Isolates with positive results for carbapenemase production by PCR were confirmed by genome sequencing. PCR products were purified using ExoStar kit (GE Healthcare) and sequenced using a BigDye Terminator version 3.1 and an ABI 3500 Genetic Analyzer (Applied Biosystems), according to the manufacturer’s instructions.

Population analysis. A total of 24/124 (12 carbapenem-susceptible and 12 carbapenem-resistant) epidemiologically unrelated isolates were randomly selected for polymyxin B population analysis. Isolates were tested in duplicate using serial dilutions using a 0.5 McFarland standard inoculum on Mueller–Hinton agar plates containing increasing concentrations of polymyxin B (0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg l⁻¹). P. aeruginosa ATCC 27853 was used for quality control. The population analysis profile (PAP) was defined as the ratio of the number of colony-forming units on the plate with the highest concentration of polymyxin B in relation to the control plate (with no antibiotic). Colonies that grew at the highest concentrations of polymyxin B were passed through 5 days in antibiotic-free Mueller–Hinton agar for further determination of polymyxin B MICs. Isolates containing subpopulations that grew at polymyxin B concentrations >2 mg l⁻¹ were considered heteroresistant. Isolates containing subpopulations that grew at polymyxin B concentrations at least twice as high as the original MIC but ≤2 mg l⁻¹ were considered heterogeneous.

RESULTS

All 124 isolates were susceptible to polymyxin B, with MIC₅₀ and MIC₉₀ of 1 mg l⁻¹ and 2 mg l⁻¹, respectively. There was variable susceptibility to other antimicrobials: high level of resistance to gentamicin (30.6 %) and imipenem (29.0 %), and low level of resistance to aztreonam (1.6 %) and ciprofloxacin (4.8 %). Thirty-seven isolates (30 %) were resistant to carbapenems. These isolates also presented high MIC₉₀ values (32 mg l⁻¹) for cefepime and ceftazidime. Overall, 16 of these 37 isolates (43 %) presented positive results in the phenotypic screening for MBL. However, in only four (10 %) isolates was an MBL gene detected by PCR. This gene was identified as blaIMP, which was confirmed by sequencing.

The two groups [12 carbapenem-susceptible isolates (group S) and 12 carbapenem-resistant isolates (group R)] presented a marked difference in the susceptibility profile: isolates in group R were mostly resistant to other antimicrobials regardless of the susceptibility method (Table 1). In group S, there was no heteroresistant subpopulation, whereas three isolates (isolates A, F and G) presented heterogeneous subpopulations (Table 2). The PAP ranged from 6.9 × 10⁻⁸ to 2.1 × 10⁻⁴. In group R, one isolate was characterized as heteroresistant (isolate M) and six other isolates (isolates N, O, P, T, V and X) presented heterogeneous subpopulations. The PAP growth frequency ranged from 2.1 × 10⁻⁷ to 2.6 × 10⁻⁴ (Table 2).

In the heterogeneous subpopulations and the single heteroresistant isolate, MICs remained stable even after 5 days of passage through an antibiotic-free medium. Population analysis of group S and group R isolates of polymyxin B presented distinct subpopulations. Only two carbapenem-susceptible isolates exhibited subpopulation growth at a polymyxin B concentration of 2 mg l⁻¹. Conversely, five of the 12 carbapenem-resistant isolates exhibited subpopulation growth at a polymyxin B concentration of 2 mg l⁻¹ (Figs 1 and 2). Of the 12 isolates selected to the population
analysis, only one (isolate U) was positive for the \textit{bla}_{IMP} \text{ gene.}

**DISCUSSION**

Heteroresistance is characterized by the development of an antimicrobial-resistant subpopulation within an otherwise susceptible bacterial population. This phenomenon is dependent on various factors such as the micro-organism, the susceptibility profile, local epidemiology, resistance phenotypes and testing methods (Tomasz et al., 1991; Yamazumi et al., 2003; Plipat et al., 2005). Among the non-fermenting Gram-negative bacteria, \textit{Acinetobacter baumannii} has been tested for heteroresistance to carbapenems (Pournaras et al., 2005; Ikonomidis et al., 2009) and colistin (Li et al., 2006; Hawley et al., 2008; Wing-Yau et al., 2009). There has been little investigation of heteroresistance in \textit{P. aeruginosa}. Some of these few studies detected distinct subpopulations exhibiting heteroresistance to carbapenems and to piperacillin/tazobactam (Pournaras et al., 2007, 2008).

The present study assessed heteroresistance and heterogeneous susceptibility to polymyxin B among carbapenem-susceptible and carbapenem-resistant \textit{P. aeruginosa} isolates. Among the 24 isolates evaluated, only one was detected as heteroresistant, suggesting that this phenomenon is less frequent to polymyxin B than to carbapenems for \textit{P. aeruginosa} isolates (Pournaras et al., 2007). On the other hand, several isolates presenting heterogeneous subpopulations were detected and this phenotype was mainly associated with the group R (50% of the isolates compared to 25% in group S). Moreover, the higher MICs of heterogeneous subpopulations were confirmed after passing through antibiotic-free medium, indicating

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{Isolate} & \textbf{Cystic fibrosis} & \textbf{Origin} & \textbf{Susceptibility (MIC, mg l$^{-1}$)*} & \textbf{Resistance profile†} \\
& & & \textbf{IPM} & \textbf{MEM} & \textbf{FEP} & \textbf{CAZ} & \\
\hline
\textbf{Group S} & & \textbf{Ascitic fluid} & S (0.5) & S ($\leq 0.5$) & S (1) & S (1) & – \\
A & No & Sputum (trap) & S (0.5) & S (0.5) & S (2) & S (1) & – \\
B & Yes & Blood & S (0.5) & S ($\leq 0.5$) & S (1) & S (1) & – \\
C & No & Sputum & S (0.5) & S ($\leq 0.5$) & S (2) & I (16) & TZP, TIM \\
D & Yes & Blood & S ($\leq 0.5$) & S (0.5) & S (1) & S (1) & – \\
E & Yes & Sputum & S (0.5) & S (0.5) & S (1) & S (1) & – \\
F & Yes & Sputum & S (0.5) & S ($\leq 0.5$) & S (1) & S (1) & – \\
G & Yes & Sputum & S (0.5) & S ($\leq 0.5$) & S (1) & S (1) & – \\
H & Yes & Sputum & S (0.5) & S (0.5) & S (1) & S (1) & – \\
I & Yes & Sputum & S (0.5) & S (0.5) & S (1) & S (1) & TZP, SAM \\
J & Yes & Sputum & S (1) & S ($\leq 0.5$) & S (4) & S (0.5) & – \\
K & Yes & Sputum & S (1) & S (0.5) & R (32) & R (32) & – \\
L & Yes & Sputum & S ($\leq 0.5$) & S (0.5) & S (1) & S (1) & AMK, GEN, SAM \\
\hline
\textbf{Group R} & & & & & & & \\
M & Yes & Sputum & R (16) & R (16) & R (32) & R (32) & AMK, CIP, GEN, TZP, TIM \\
N & Yes & Sputum & R (8) & R (16) & R (32) & R (32) & AMK, CIP, GEN, TZP, TIM, ATM \\
O & Yes & Sputum (trap) & R (32) & R (64) & R (64) & R (64) & CIP, GEN, TIM \\
Q & Yes & Sputum & R (8) & S (1) & S (1) & S (1) & GEN, TIM \\
R & Yes & Sputum & R (16) & R (16) & R (32) & R (32) & AMK, GEN, TZP, TIM, SAM \\
S & Yes & Urine & R (8) & S (1) & R (64) & R (64) & AMK, CIP, GEN, TZP, SAM \\
T & Yes & Urine & R (8) & I (4) & S (8) & S (8) & SAM \\
U & Yes & Secretion & R (16) & R (8) & S (8) & S (8) & GEN, SAM \\
V & Yes & Blood & R (8) & R (8) & S ($\leq 1$) & S ($\leq 1$) & – \\
X & Yes & Ascitic fluid & R (8) & R (8) & S (1) & S (1) & TZP, SAM \\
Z & Yes & Sputum & R (8) & R (16) & S (8) & S (8) & AMK, GEN, TZP, TIM \\
\hline
\end{tabular}
\caption{Profile of carbapenem-susceptible (group S) and carbapenem-resistant (group R) \textit{P. aeruginosa} isolates}
\end{table}

* S, Susceptible (carbapenems, MIC $\leq 2$ mg l$^{-1}$; cephalosporins, MIC $\leq 8$ mg l$^{-1}$); I, intermediate (carbapenems, MIC=4 mg l$^{-1}$; cephalosporins, MIC=16 mg l$^{-1}$); R, resistant (carbapenems, MIC $\geq 8$ mg l$^{-1}$, cephalosporins, MIC $\geq 32$ mg l$^{-1}$).
† AMK, Amikacin; ATM, aztreonam; CIP, ciprofloxacin; GEN, gentamicin; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid. Disk diffusion method in accordance with CLSI.
Table 2. Populational analysis of *P. aeruginosa* carbapenem-susceptible (group S) and carbapenem-resistant (group R) to polymyxin B

PMB, polymyxin B; PAP, population analysis profile.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Original PMB MIC (µg mL⁻¹)</th>
<th>Highest PMB concentration at which bacterial growth occurred (µg mL⁻¹)</th>
<th>Classification*</th>
<th>PAP frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.25</td>
<td>1.0</td>
<td>H</td>
<td>3.0 x 10⁻⁴</td>
</tr>
<tr>
<td>B</td>
<td>1.0</td>
<td>0.5</td>
<td>–</td>
<td>2.0 x 10⁻⁶</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>1.0</td>
<td>–</td>
<td>5.2 x 10⁻⁶</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>0.5</td>
<td>–</td>
<td>2.1 x 10⁻⁴</td>
</tr>
<tr>
<td>E</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
<td>2.2 x 10⁻⁵</td>
</tr>
<tr>
<td>F</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>4.0 x 10⁻⁷</td>
</tr>
<tr>
<td>G</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>6.7 x 10⁻⁶</td>
</tr>
<tr>
<td>H</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
<td>1.7 x 10⁻⁵</td>
</tr>
<tr>
<td>I</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
<td>7.5 x 10⁻⁶</td>
</tr>
<tr>
<td>J</td>
<td>1.0</td>
<td>0.5</td>
<td>–</td>
<td>4.1 x 10⁻⁶</td>
</tr>
<tr>
<td>K</td>
<td>1.0</td>
<td>0.5</td>
<td>–</td>
<td>1.1 x 10⁻⁵</td>
</tr>
<tr>
<td>L</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
<td>5.0 x 10⁻⁶</td>
</tr>
<tr>
<td>Group R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0.25</td>
<td>4.0</td>
<td>HR</td>
<td>2.9 x 10⁻⁵</td>
</tr>
<tr>
<td>N</td>
<td>0.25</td>
<td>1.0</td>
<td>H</td>
<td>4.0 x 10⁻⁵</td>
</tr>
<tr>
<td>O</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>2.6 x 10⁻⁴</td>
</tr>
<tr>
<td>P</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>2.2 x 10⁻⁵</td>
</tr>
<tr>
<td>Q</td>
<td>1.0</td>
<td>2.0</td>
<td>–</td>
<td>1.5 x 10⁻⁵</td>
</tr>
<tr>
<td>R</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
<td>3.0 x 10⁻⁵</td>
</tr>
<tr>
<td>S</td>
<td>0.5</td>
<td>0.5</td>
<td>–</td>
<td>4.1 x 10⁻⁵</td>
</tr>
<tr>
<td>T</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>2.0 x 10⁻⁷</td>
</tr>
<tr>
<td>U</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
<td>5.0 x 10⁻⁶</td>
</tr>
<tr>
<td>V</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>5.3 x 10⁻⁶</td>
</tr>
<tr>
<td>X</td>
<td>0.5</td>
<td>2.0</td>
<td>H</td>
<td>8.0 x 10⁻⁶</td>
</tr>
<tr>
<td>Z</td>
<td>2.0</td>
<td>2.0</td>
<td>–</td>
<td>9.0 x 10⁻⁶</td>
</tr>
</tbody>
</table>

*H, Isolates with heterogeneous subpopulations to polymyxin B; HR, isolate heteroresistant to polymyxin B; –, absence of heterogeneous or heteroresistant population.

Fig. 1. Population analysis profile for group S (carbapenem-susceptible isolates).
a stable phenotype. This stability seems to be peculiar to *Pseudomonas* spp., as similar results were already reported for isolates tested against carbapenems (Pournaras et al., 2007). In contrast, *Acinetobacter* spp. and *Klebsiella* spp. heteroresistant isolates tend to present subpopulation MICs that returned to the lower values after a period of absence of drug exposure (Pournaras et al., 2005). Considering that the use of polymyxin B may lead to the selection of resistant mutants, the presence of a heterogeneous subpopulation may represent a serious concern, due to the fact that this antimicrobial is frequently used as the drug of choice to treat carbapenem-resistant *P. aeruginosa* infection. However, as we did not evaluate the clinical outcomes of patients who presented heteroresistance or the heterogeneous *P. aeruginosa* subpopulation, the clinical impact of these phenomena remains to be investigated. Interestingly, no relationship was found between the carbapenemase production and the resistance phenotypes observed. To the best of our knowledge, this is the first evaluation of polymyxin B heteroresistance in *P. aeruginosa* isolates.

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