Molecular epidemiological survey of the quinolone- and carbapenem-resistant genotype and its association with the type III secretion system in Pseudomonas aeruginosa

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This study evaluated the predictors of mortality and the impact of inappropriate therapy on the outcomes of patients with bacteremia and ventilator-associated pneumonia (VAP). Additionally, we evaluated the correlation of the type III secretion system (TTSS) effector genotype with resistance to carbapenems and fluoroquinolones, mutations in the quinolone resistance-determining regions (QRDRs), metallo-β-lactamase and virulence factors. A retrospective cohort was conducted at a tertiary hospital in patients with multidrug-resistant (MDR) P. aeruginosa bacteremia (157 patients) and VAP (60 patients). The genes for blaiMP, blaiIM, blaiSM, blaiGM and blaiSPM and virulence genes (exoT, exoS, exoY, exoU, lasB, algD and toxA) were detected; sequencing was conducted for QRDR genes on fluoroquinolone-resistant strains. The multivariate analyses showed that the predictors independently associated with death in patients with bacteremia were cancer and inappropriate therapy. Carbapenem resistance was more frequent among strains causing VAP (53.3%), and in blood we observed the blaiSPM genotype (66.6%) and blaiIM genotype (33.3%). The exoS gene was found in all isolates, whilst the frequency was low for exoU (9.4%). Substitution of threonine to isoleucine at position 83 in gyrA was the most frequent mutation among fluoroquinolone-resistant strains. Our study showed a mutation at position 91 in the parC gene (Glu91Lys) associated with a mutation in gyrA (Thr83Ile) in a strain of extensively drug-resistant P. aeruginosa, with the exoT+ exoS+ exoU+ genotype, that has not yet been described in Brazil to the best of our knowledge. This comprehensive analysis of resistance mechanisms to carbapenem and fluoroquinolones and their association with TTSS virulence genes, covering MDR P. aeruginosa in Brazil, is the largest reported to date.

INTRODUCTION

Severe infections such as bloodstream infection and ventilator-associated pneumonia (VAP) due to multidrug-resistant (MDR) Pseudomonas aeruginosa result in greater morbidity and mortality, longer hospitalization and higher cost than infections caused by susceptible bacteria (Morales et al., 2012; Suárez et al., 2010; Voor In’t Holt et al., 2014), and aspects related to epidemiology and the outcome of patients with these infections may change, resulting in high rates of resistance and as consequence difficulties in treatment (Kang et al., 2005; Lodise et al., 2007).

Today, the emergence of MDR P. aeruginosa is a global problem (Sader et al., 2001; Van der Bij et al., 2011, 2012), resulting in the ability of this pathogen to develop resistance to almost all available antibiotics, either by selection of mutations in chromosomal genes or from horizontal gene transfer (Breidenstein et al., 2011). In Brazil this problem is highly significant and some studies show a very high density of antibiotic use, especially of carbapenems and fluoroquinolones (Moreira et al., 2013; Porto et al., 2013). The resistance of P. aeruginosa to carbapenems is higher than 60% in some Brazilian hospitals (Baumgart et al., 2010; Sader et al., 2005) and the production of metallo-β-lactamase (MBL) encoded by several
genes, including blsSPM, blsVIM and blsAMP, is considered the main mechanism of resistance to these antibiotics (Sader et al., 2005).

Resistance to fluoroquinolone has become an increasing problem and now has the highest resistance rates in Latin American countries (Andrade et al., 2006; Sader et al., 2001), and is associated with mutations in the quinolone resistance-determining regions (QRDRs), especially the genes encoding DNA gyrase (gyrA) and topoisomerase IV (parC) (Higgins et al., 2003; Lee et al., 2005; Mouneimné et al., 1999).

Recent studies have shown that a large number of genes expressed by P. aeruginosa clinical strains are implicated directly in the pathogenesis of severe infections caused by this micro-organism (Aldred et al., 2014; Bleves et al., 2010; Golovkine et al., 2014). Among these is the type III secretion system (TTSS), which delivers effector toxins (ExoS, ExoT, ExoY and ExoU) directly into host cells (Galán & Collmer, 1999; Hauser, 2009). Depending on the anatomical site of the disease or the genetic background, the exoS genotype is found at a higher frequency than genes that encode the ExoU cytotoxin, which are present in variable traits among clinical strains (Feltman et al., 2001; Roy-Burman et al., 2001).

According to the literature, factors related to adherence to the host cell, such as alginate (encoded by the algD gene) and those that facilitate the disruption of epithelial integrity, such as LasB elastase and exotoxin A (encoded by the lasB and toxA genes, respectively) are also important (Lanotte et al., 2004).

This study was performed to identify the predictors of mortality in patients with bacteraemia and VAP caused by MDR P. aeruginosa. In addition, we also aimed to determine whether there was a correlation between TTSS effector genotype (ExoU, ExoS, ExoT and ExoY) and: (i) the prevalence and degree of carbapenem and fluoroquinolone resistance; (ii) mutations in target genes conferring fluoroquinolone resistance; (iii) the presence of genes associated to MBL production; and (iv) the virulence genes algD, toxA and lasB.

METHODS

Patients and setting. The database at our clinical microbiology laboratory was reviewed to identify patients with P. aeruginosa bacteraemia from May 2009 to December 2012 and patients with VAP from March 2011 to November 2012 at Uberlandia University Hospital (Brazil), a 530-bed tertiary-care university hospital. Only the first episode was analysed for those patients with more than one episode of bacteraemia or VAP.

Study design and data collection. A retrospective observational cohort study was employed to identify the predictors of mortality and the impact of inappropriate therapy on the outcomes of patients with P. aeruginosa bacteraemia (throughout the hospital) and VAP (only in the adult intensive care unit [ICU]). The main outcome was in-hospital mortality, and the measure used was a 30-day mortality rate.

We also assessed secondary outcomes, including the duration of hospital stays, admission to the ICU and the use of invasive procedures. For each patient studied, the following characteristics were recovered from their clinical records: age; gender; length of total hospital stay; admission to the ICU (except for patients with VAP); surgery; invasive procedures such as mechanical ventilation (MV), central venous line, urinary catheter, tracheostomy, haemodialysis, catheter enteral or gastric nutrition, and surgical drain during the current hospitalization; underlying conditions such as diabetes mellitus, chronic renal failure, heart failure and cancer; sources of bacteraemia; antibiotic use during the current hospitalization; and cases of inappropriate antimicrobial therapy.

Definitions. VAP was defined as pneumonia developing at least 48 h after ICU admission and initiation of MV along with at least one of the following criteria: purulent endotracheal secretion, temperature >38 °C or <35 °C, leukocytosis or leukopenia, new and/or progressive radiographic infiltrate, and positive quantitative culture of the endotracheal aspirate count ≥10⁶ c.f.u. ml⁻¹ (Edwards et al., 2007; Peleg & Hooper, 2010). VAP occurring 4 days after initiation of MV was classified as late-onset VAP (Rello et al., 2006).

The clinical pulmonary infection score (CPIS) was calculated for patients clinically suspected of VAP on the same date as endotracheal secretion collection, and all those with a CPIS of ≥6 were included (Zilberberg & Shorr, 2010). The Charlson co-morbidity index was also calculated for these patients, as defined previously (Needham et al., 2005).

According to the Centers for Disease Control and Prevention (CDC), bacteraemia can be defined as the presence of viable bacteria in the blood, documented by a positive blood culture result. Bacteraemia was considered to be nosocomial if the infection occurred >48 h after admission and no clinical evidence of infection on admission existed (Garner et al., 1996). Bacteraemia was classified as primary when it was unrelated to another focus of infection or when it was related to an intravenous catheter, and secondary when it was clinically related to infection in another anatomical site (Baumgart et al., 2010; CDC, 2002).

The average severity of illness score (ASIS) was also recorded for each patient with bacteraemia using the CDC National Nosocomial Infections Surveillance (NNIS) System criteria (Rosenthal et al., 2006).

MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories; extensively drug-resistant (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remained susceptible to only one or two categories) (Magiorakos et al., 2012).

The antimicrobial therapy was considered to be ‘appropriate’ if the initial antibiotics, which were administered within 24 h of acquisition of a blood culture sample, included at least one antibiotic that was active in vitro (Gilbert et al., 2007).

The 30-day mortality was considered as the number of deaths of patients with bacteraemia or VAP during hospitalization that occurred within 30 days of the diagnosis of infection (Lodise et al., 2007).

Bacterial isolates and clinical microbiological testing. Cultures were processed using a BACT/Aler (bioMérieux). Microbial identification and antimicrobial susceptibility testing were performed on a VITEK II automated system (bioMérieux) for the following antimicrobials: aminoglycosides (gentamicin, amikacin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillins plus β-lactamase inhibitors (piperacillin-tazobactam), monobactams (aztreonam) and polymyxins (colistin, polymyxin B). P. aeruginosa strains that showed intermediate susceptibility were considered to be resistant.
Quality-control protocols were used according to the standards of the Clinical and Laboratory Standards Institute (CLSI, 2014).

**Molecular testing.** All PCR primers and reaction conditions used in this study are described in Table S1 (available in the online Supplementary Material). To assess the presence of the MBL genes (bladmp, bladTMD, bladTIM and bladSIM) in *P. aeruginosa* strains, a multiplex PCR was performed, as described previously (Woodford, 2010). The presence of the TTSS virulence genes (*exoS*, *exoY*, *exoT* and *exoU*) was determined by simplex PCR, as described previously (Jabalameli et al., 2012); and to assess the presence of the virulence factors (*algD*, *toxA* and *lasB*) a simplex PCR was performed, as described by Lanotte et al. (2004). All PCRs were carried out in a Mastercycler Personal (Eppendorf).

**Detection of mutations in QRDRs (gyrA and parC).** The target regions were amplified by PCR using the primers and conditions described in Table S1 (Lee et al., 2005). Purified PCR-amplified DNA was sequenced directly using an automatic sequencer ABI-PRISM 3100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems). The nucleotide sequence information to determine the fragments that included the possible mutations in QRDRs was obtained from the *Pseudomonas* genome project (http://www.pseudomonas.com) and from GenBank [accession numbers L29417 (gyrA) and AB003428 (parC)].

**Statistical analysis.** Student’s *t*-test was used to compare continuous variables, and *χ*² or Fisher’s exact tests were used to compare categorical variables. To determine independent risk factors for 30-day mortality, a multiple logistic regression model was used to control for the effects of confounding variables. Variables with *P*<0.05 in the univariate analysis were candidates for multivariate analysis. All *P* values were two tailed, and *P* values of <0.05 were considered statistically significant.

**Ethical approval.** The research Ethics Committee of the Federal University Uberlandia evaluated and approved our study design.

## RESULTS

### Study population and mortality predictors

From 1 May 2009 to 31 December 2012 and from 1 March 1 2011 to 31 November 2012, a total of 157 and 60 non-repetitive patients with *P. aeruginosa* bacteraemia and VAP, respectively, at the University Hospital were identified and included in the study.

The detailed information on factors associated with death and the relevant demography and clinical characteristics of the study population are summarized in Tables 1 and 2. Compared with the cohort of patients with bacteraemia, the mean age of patients was 51.3 ± 25.1 years (range 0–89) and the majority of the patients were from surgical and medical wards (32.5 and 28.6 %, respectively). The sources of infection included bacteraemia of unknown origin in 62.4 % of cases and bacteraemia associated with the lungs in 17.2 %. The results of multivariate analyses for the association between risk factors and 30-day mortality showed that predictors independently associated with death were patients with severe underlying disease, such as cancer, as well as patients who received inappropriate therapy. The mean length of a hospital stay after admission was 76.9 days for survivors and 30.9 days for non-survivors. The Kaplan–Meier cumulative survival estimates (Fig. 1) for patients with inappropriate versus appropriate therapy showed that the first group had a lower probability of survival (*P*=0.0007). The 30-day mortality rate of the first group was 47.1 %, whereas that of the second group was 33.3 %. The severity of the patients was accessed using ASIS scores (Table 1), and no significant difference was found between survivors and non-survivors.

Regarding the cohort of patients with VAP, no risk factor was associated with mortality; however, the value of the odds ratio was higher among patients who had had surgery, use of MV for more than 40 days and those who had chronic renal failure or chronic obstructive pulmonary disease as the underlying disease (Table 2).

### Distribution of genes encoding metallo-ß-lactamase (MBL) and TTSS, and degree of antimicrobial resistance

Table 3 summarizes the characterization of *P. aeruginosa* strains in terms of the production of MBL, resistance profile, TTSS genotype and virulence factors of carbapenem- and fluoroquinolone-resistant clinical isolates included in the study.

According to PCR, the TTSS genotype for the genes encoding the cytotoxins *ExoU*, *ExoS*, *ExoY* and *ExoT* revealed that all 32 clinical isolates carried *exoS*, *exoT* and *exoY* genes (78.1 %), and *exoU* in 87.5 % of strains. The genotype *exoS*<sup>+</sup>*exoT*<sup>+</sup>*exoY*<sup>+</sup> was observed in 68.7 % of strains. Three isolates were positive for the two effector genes *exoU* and *exoS* (9.4 %), the *exoU* gene was observed only in 9.4 % of strains and a low frequency was observed for isolates encoding the four TTSS genes.

All isolates of *P. aeruginosa* recovered from blood were more susceptible to carbapenems than those recovered from lung, unlike fluoroquinolone resistance. To summarize, there was a relationship between high levels of resistance to carbapenems and fluoroquinolones and lower rates of resistance to amikacin. The strains recovered from blood were more resistant to cefepime, amikacin and piperacillin/tazobactam than those recovered from the lung.

The resistance rates were similar among isolates with *exoT*, *exoS*, *exoU* and *exoY*, with high frequencies to carbapenems (100 %), ciprofloxacin (100 %) and gentamicin (93.7 %). In general, the strains showed a MDR profile (100 %).

MBL multiplex PCR was conducted on 56 carbapenem-resistant *P. aeruginosa* isolates from blood and 29 isolates from lung, which showed that 16.1 % (9/56) and 3.4 % (1/29) of isolates, respectively, presented an amplicon consistent with MBL genes identified as *bla*<sub>SPM-1</sub>-type in 66.6 % (6/9), *bla*<sub>IMP</sub>-type in 33.3 % (3/9) and *bla*<sub>NDM</sub>-type in 3.4 % (1/29), the latter detected in the lung.

Comparing the detection of the irulence genes analysed (*lasB*, *toxA* and *algD*) between the two groups (blood and lung) of *P. aeruginosa* isolates, we observed the
### Table 1. Risk factors associated with 30-day mortality in patients with bacteraemia caused by *P. aeruginosa*

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Total [n=157 (%)]</th>
<th>Non-survival [n=68 (%)]</th>
<th>Survival [n=89 (%)]</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tbody>
<tr>
<td></td>
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<td>Odds ratio (CI 95 %)</td>
<td>P value</td>
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<td>–</td>
<td>0.065</td>
</tr>
<tr>
<td>Age (years, mean)</td>
<td>51.36 ± 25.10</td>
<td>56.10 ± 22.74</td>
<td>47.73 ± 26.31</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Male</td>
<td>105 (66.8)</td>
<td>44 (64.7)</td>
<td>61 (68.5)</td>
<td>0.84 (0.43–1.64)</td>
<td>0.737</td>
</tr>
<tr>
<td>Female</td>
<td>52 (33.1)</td>
<td>24 (35.3)</td>
<td>28 (31.5)</td>
<td>1.18 (0.60–2.31)</td>
<td>0.737</td>
</tr>
<tr>
<td>Length of hospital stay (mean days)</td>
<td>57.01 ± 56.93</td>
<td>30.90 ± 26.34</td>
<td>76.97 ± 65.48</td>
<td>–</td>
<td>0.0001*</td>
</tr>
<tr>
<td>ICU</td>
<td>74 (47.1)</td>
<td>32 (47.1)</td>
<td>42 (47.2)</td>
<td>0.99 (0.52–1.87)</td>
<td>0.884</td>
</tr>
<tr>
<td>Surgery</td>
<td>69 (43.9)</td>
<td>30 (44.1)</td>
<td>39 (43.8)</td>
<td>1.01 (0.53–1.91)</td>
<td>0.9</td>
</tr>
<tr>
<td>Invasive procedures</td>
<td>139 (88.5)</td>
<td>61 (89.7)</td>
<td>78 (87.6)</td>
<td>1.22 (0.44–3.35)</td>
<td>0.88</td>
</tr>
<tr>
<td>MV</td>
<td>89 (56.7)</td>
<td>40 (58.8)</td>
<td>49 (55.0)</td>
<td>1.16 (0.61–2.21)</td>
<td>0.756</td>
</tr>
<tr>
<td>Tracheostomy</td>
<td>70 (44.6)</td>
<td>32 (47.1)</td>
<td>38 (42.6)</td>
<td>1.19 (0.63–2.25)</td>
<td>0.701</td>
</tr>
<tr>
<td>Urinary catheter</td>
<td>101 (64.3)</td>
<td>48 (30.6)</td>
<td>53 (59.5)</td>
<td>1.63 (0.83–3.19)</td>
<td>0.206</td>
</tr>
<tr>
<td>Central venous catheter</td>
<td>125 (79.6)</td>
<td>57 (83.8)</td>
<td>68 (76.4)</td>
<td>1.60 (0.71–3.59)</td>
<td>0.345</td>
</tr>
<tr>
<td>Surgical drain</td>
<td>24 (15.3)</td>
<td>12 (17.6)</td>
<td>12 (13.5)</td>
<td>1.37 (0.57–3.28)</td>
<td>0.62</td>
</tr>
<tr>
<td>Enteral probes/gastric nutrition</td>
<td>109 (69.4)</td>
<td>53 (33.7)</td>
<td>56 (62.9)</td>
<td>2.08 (1.01–4.26)</td>
<td>0.064</td>
</tr>
<tr>
<td>Haemodialysis</td>
<td>39 (24.8)</td>
<td>21 (13.4)</td>
<td>18 (20.2)</td>
<td>1.76 (0.84–3.65)</td>
<td>0.178</td>
</tr>
<tr>
<td>Co-morbidity conditions</td>
<td>123 (78.3)</td>
<td>61 (89.7)</td>
<td>62 (69.6)</td>
<td>3.79 (1.53–9.36)</td>
<td>0.004*</td>
</tr>
<tr>
<td>Heart failure</td>
<td>35 (22.3)</td>
<td>18 (26.5)</td>
<td>17 (19.1)</td>
<td>1.52 (0.71–3.24)</td>
<td>0.365</td>
</tr>
<tr>
<td>Cancer</td>
<td>32 (20.4)</td>
<td>21 (13.4)</td>
<td>11 (12.3)</td>
<td>3.16 (1.40–7.15)</td>
<td>0.007*</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>21 (13.4)</td>
<td>11 (16.2)</td>
<td>10 (11.2)</td>
<td>1.52 (0.60–3.83)</td>
<td>0.506</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>40 (25.5)</td>
<td>22 (32.3)</td>
<td>18 (20.2)</td>
<td>1.88 (0.91–3.89)</td>
<td>0.122</td>
</tr>
<tr>
<td>ASIS score ≥ 4</td>
<td>81 (51.6)</td>
<td>39 (57.3)</td>
<td>42 (57.2)</td>
<td>1.50 (0.79–2.84)</td>
<td>0.270</td>
</tr>
<tr>
<td>Primary bacteraemia</td>
<td>119 (75.8)</td>
<td>46 (67.6)</td>
<td>73 (82.0)</td>
<td>0.45 (0.21–0.96)</td>
<td>0.057*</td>
</tr>
<tr>
<td>Central line catheter related</td>
<td>21 (13.8)</td>
<td>6 (8.8)</td>
<td>15 (16.8)</td>
<td>0.47 (0.17–1.30)</td>
<td>0.219</td>
</tr>
<tr>
<td>Unknown</td>
<td>98 (62.4)</td>
<td>40 (58.8)</td>
<td>58 (65.3)</td>
<td>0.76 (0.39–1.46)</td>
<td>0.517</td>
</tr>
<tr>
<td>Secondary bacteraemia</td>
<td>38 (24.2)</td>
<td>22 (32.3)</td>
<td>16 (17.9)</td>
<td>2.18 (1.03–4.58)</td>
<td>0.06</td>
</tr>
<tr>
<td>Respiratory tract</td>
<td>27 (17.2)</td>
<td>17 (25.0)</td>
<td>10 (11.2)</td>
<td>2.63 (1.11–6.20)</td>
<td>0.040*</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>6 (3.8)</td>
<td>2 (2.9)</td>
<td>4 (4.4)</td>
<td>0.64 (0.11–3.62)</td>
<td>0.698</td>
</tr>
<tr>
<td>Others†</td>
<td>5 (3.2)</td>
<td>3 (4.4)</td>
<td>2 (2.2)</td>
<td>2.00 (0.32–2.36)</td>
<td>0.652</td>
</tr>
<tr>
<td>Inappropriate therapy</td>
<td>49 (31.2)</td>
<td>32 (47.1)</td>
<td>17 (19.1)</td>
<td>3.76 (1.84–7.66)</td>
<td>0.0003*</td>
</tr>
</tbody>
</table>

CI, Confidence interval.  
*Statistically significant (P≤0.05).  
†Ascitic fluid, cavity abscess, wound secretion, ocular secretion or liquor.
presence of virulence genes at a high percentage in all isolates, independent of their resistance to carbapenems and fluoroquinolones.

Table 4 summarizes the comparison of target site mutations in the \( \text{gyrA} \) and \( \text{parC} \) genes by TTSS effector genotype. Substitution of threonine for isoleucine at position 83 in \( \text{gyrA} \) was the most commonly noted mutation among the clinical isolates of \( P. \text{aeruginosa} \), and was found in similar proportions among \( \text{exoS} (92\%) \), \( \text{exoY} (76\%) \) and \( \text{exoT} (76\%) \) isolates. Mutations in \( \text{exoU} \) strains included an amino acid change in \( \text{gyrA} \) at position 83 and a \( \text{parC} \) substitution at position 91 (Glu91Lys). Nineteen \( P. \text{aeruginosa} \) isolates showed alterations in QRDRs of the \( \text{parC} \) gene, all with a Ser87Leu substitution, and no strain showed a mutation only in the \( \text{parC} \) gene.

**DISCUSSION**

Healthcare-associated infections are recognized as a severe global problem with serious implications for hospitalized patients.
### Table 3. Characterization of carbapenem- and fluorquinolone-resistant *P. aeruginosa* with regard to the production of MBL, TTSS genotype, resistance profile and virulence factors

<table>
<thead>
<tr>
<th>Site</th>
<th><em>P. aeruginosa</em> carbapenem resistant: no. total FQ resistant/total (%)</th>
<th>No. analysed/no. MBL positive (%)</th>
<th>PCR product size (bp)/gene (no. isolates)</th>
<th>Analysed TTSS genotype (n/total)</th>
<th>TTSS profile (no. isolates)</th>
<th>Resistance profile (%)</th>
<th>Virulence profile (<em>lasB, toxA, algD</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>71/162 (43.8)</td>
<td>56/9 (16.1)</td>
<td>271/bla&lt;sub&gt;SPM-1&lt;/sub&gt; (6)</td>
<td>19/162</td>
<td>exoU (2)</td>
<td>FEP (50.0) GEN&lt;sup&gt;a&lt;/sup&gt; (100) AMK (0) TZP&lt;sup&gt;b&lt;/sup&gt; (50.0)</td>
<td>lasB&lt;sup&gt;+&lt;/sup&gt;/toxA&lt;sup&gt;+&lt;/sup&gt;/algD&lt;sup&gt;+&lt;/sup&gt; 19/19 (100)</td>
</tr>
<tr>
<td></td>
<td>65/162 (40.1)</td>
<td></td>
<td>390/bla&lt;sub&gt;VDM-type&lt;/sub&gt; (3)</td>
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<tr>
<td>Lung</td>
<td>32/60 (53.3)</td>
<td>29/1 (3.4)</td>
<td>188/bla&lt;sub&gt;IMP-type&lt;/sub&gt; (1)</td>
<td>13/60</td>
<td>exoU (1)</td>
<td>FEP (100) GEN (100) AMK (100) TZP (0)</td>
<td>lasB&lt;sup&gt;+&lt;/sup&gt;/toxA&lt;sup&gt;+&lt;/sup&gt;/algD&lt;sup&gt;+&lt;/sup&gt; 12/13 (92.3)</td>
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<td></td>
<td>13/60 (21.6)</td>
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</table>

FQ, Fluoroquinolone; FEP, cefepime; GEN, gentamicin; AMK, amikacin; TZP, piperacillin-tazobactam.

<sup>a</sup> FEP (94.7) GEN (94.7) AMK (52.6) TZP (84.2)
<sup>b</sup> FEP (93.7) GEN (93.7) AMK (56.2) TZP (81.2)
<sup>c</sup> FEP (93.3) GEN (93.3) AMK (40.0) TZP (80.0)
<sup>d</sup> FEP (83.3) GEN (75.0) AMK (41.6) TZP (66.6)
<sup>e</sup> FEP (50.0) GEN (100) AMK (0) TZP (50.0)
<sup>f</sup> FEP (76.9) GEN (92.3) AMK (15.4) TZP (23.1)
<sup>g</sup> FEP (75.0) GEN (91.6) AMK (16.6) TZP (25.0)
<sup>h</sup> FEP (72.7) GEN (100) AMK (9.1) TZP (18.2)
<sup>i</sup> FEP (70.0) GEN (100) AMK (10.0) TZP (20.0)
<sup>j</sup> lasB<sup>+</sup>/toxA<sup>+</sup>/algD<sup>+</sup> 1/13 (7.7)
patients, especially in developing countries, and yet they have not received attention commensurate with the impact on public health (Gupta et al., 2011; Roberts et al., 2010; Rosenthal et al., 2006). Allied to this is the rapid emergence and dissemination of antimicrobial-resistant microorganisms, especially non-fermentative, Gram-negative bacilli, such as P. aeruginosa (Breidenstein et al., 2011; Sader et al., 2001; Voor In’t Holt et al., 2014).

To the best of our knowledge, this work represents the first comprehensive study in Brazil of bacteraemia and VAP caused by carbapenem- and fluoroquinolone-resistant P. aeruginosa. MBL production, QRDR mutations, TTSS virulence and other virulence factors. In addition, we also evaluated the impact of inappropriate therapy and death in a tertiary-care population.

Bacteraemia caused by MDR P. aeruginosa is a serious infection and is usually hospital acquired, occurring in patients with severe underlying diseases and raising the mortality rate (Morales et al., 2012; Suárez et al., 2010; Voor In’t Holt et al., 2014) as a result of inappropriate antimicrobial therapy (Kang et al., 2005; Lodise et al., 2007). VAP is the most important infection in the ICU associated with significant increases in mortality, cost and length of hospital stay (Bassi et al., 2010; Davis, 2006; Park, 2005) and is responsible for about 6–52 % of ICU infections (Davis, 2006). In this study, we investigated a cohort of 157 patients with bacteraemia and VAP in the first group, 42 % had a MDR profile and 24.8 % had an XDR profile. Among studies that have evaluated the mortality of patients with P. aeruginosa bacteraemia, the results demonstrate the importance of antimicrobial resistance, inappropriate therapy and the severity of the underlying disease associated with mortality rate (Kang et al., 2005; Lodise et al., 2007; Morales et al., 2012; Suárez et al., 2010; Voor In’t Holt et al., 2014). Our data are similar to those, and showed that patients with cancer and those who received inappropriate antimicrobial therapy were independent factors associated with mortality.

Among patients admitted to the ICU with VAP, even after adjusting for the severity of illness and the underlying condition, we did not find any risk factors significantly associated with mortality; this can be explained by the lower number of patients with VAP. However, we observed high values of the odds ratio in surgical patients, in patients with use of MV for more than 40 days and in those with underlying diseases such as chronic renal failure and chronic obstructive pulmonary disease.

P. aeruginosa has a wide spectrum of intrinsic and acquired antimicrobial resistance mechanisms (Breidenstein et al., 2011; Morales et al., 2012). Carbapenem resistance frequently results from the production of β-lactamases, particularly β-lactamases that hydrolyse carbapenems, such as MBL (Breidenstein et al., 2011; Sader et al., 2005; Van der Bij et al., 2011, 2012). Our data showed that carbapenem-resistant P. aeruginosa was prevalent throughout the hospital. However, when comparing bloodstream infection and VAP, carbapenem resistance was more frequent among VAP strains (53.3 %), but without detection of the MBL gene, except for an isolate characterized as being blaIMP genotype, in contrast to what was observed in blood, where the frequency of positive strains for MBL genes was higher (16.1 %), with 66.6 % being blaSPM genotype and 33.3 % being blaVIM genotype. This study suggests that other resistance mechanisms co-exist in these strains such as loss of the OprD porin and overexpression of efflux systems, which were not evaluated in this study. The MBL-producing isolates showed the XDR phenotype (77.8 %). The emergence of P. aeruginosa MBL-producing strains in Brazil is largely caused by spread of the dominant clone (ST-277) (Galés et al., 2003). Our data suggested that, in our hospital, as well as the SPM clone, there is spread of strains producing MBL of the VIM type. Sporadic reports from Brazil have suggested a similar increase in VIM-positive P. aeruginosa isolates (Franco et al., 2010; Sader et al., 2005), but longitudinal studies that support these data have not been done.

Previous studies have shown that prior treatment with fluoroquinolone is a risk factor for colonization with MDR P. aeruginosa, and this resistance is associated with a poor clinical outcome for infected patients (Gasink et al., 2006; Hsu et al., 2005). Consistent with the findings from other investigations, our study identified by univariate analysis that mortality associated with fluoroquinolone resistance is significant (P=0.023) (data not demonstrated).

Indirect evidence from epidemiological studies suggests that fluoroquinolone resistance and expression of TTSS

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**Table 4. Comparison of carbapenem- and fluoroquinolone-resistant P. aeruginosa with regard to target site mutations in gyrA and parC according to TTSS effector genotype**

<table>
<thead>
<tr>
<th>QRDR mutations (n=25)</th>
<th>ExoU [n (%)]</th>
<th>ExoS [n (%)]</th>
<th>ExoY [n (%)]</th>
<th>ExoT [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA position</td>
<td>Thre83Ile (n=23)</td>
<td>1 (4)</td>
<td>23 (92)</td>
<td>19 (76)</td>
</tr>
<tr>
<td>parC position</td>
<td>Ser87Leu (n=19)</td>
<td>0</td>
<td>19 (76)</td>
<td>16 (64)</td>
</tr>
<tr>
<td>Glu91Lys (n=1)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>0</td>
<td>1 (4)</td>
</tr>
<tr>
<td>gyrA + parC position</td>
<td>Thre83Ile + Ser87Leu (n=19)</td>
<td>0</td>
<td>19 (76)</td>
<td>16 (64)</td>
</tr>
<tr>
<td>Thre83Ile + Glu91Lys (n=1)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>0 (4)</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>
virulence are independently associated with poor outcomes resulting from MDR \textit{P. aeruginosa} infections (Bleves et al., 2010; El-Solh et al., 2012; Roy-Burman et al., 2001; Wong-Beringer et al., 2008). Although this relationship was not assessed in our study, the \textit{exoS} gene was found in all clinical isolates whilst the presence of the \textit{exoT} and \textit{exoY} genes varied, and unlike other studies (El-Solh et al., 2012; Wareham & Curtis, 2007), the presence of the \textit{exoU} gene occurred at low frequency in our study (9.4 %).

Some published studies have reported that \textit{exoU}-positive \textit{P. aeruginosa} strains are most often multi-resistant compared with \textit{exoS} strains (Garey et al., 2008; Wong-Beringer et al., 2008). In our study, this relationship was not observed as the strains with the TTSS genotype were also multi-resistant, suggesting again that these bacteria could exhibit other additional resistance mechanisms that make these strains present multiple antimicrobial co-resistance. However, we also noted a high frequency of gentamicin resistance among isolates harbouring the \textit{exoS}, \textit{exoT}, \textit{exoY} and \textit{exoU} genes. This resistance appears to be drug specific rather than class specific, as our strains presented a lower frequency of resistance to amikacin.

With respect to target site mutations in fluoroquinolone-resistant \textit{P. aeruginosa}, we observed mutations consistent with those previously published in all isolates (Higgins et al., 2003; Lee et al., 2005; Mounenheim et al., 1999). Substitution of threonine by isoleucine at position 83 in \textit{gyrA} was the most frequent mutation in our fluoroquinolone-resistant isolates. A double \textit{gyrA/parC} mutation was also common (80.0 %), so our results provide further support for accumulation of a mutation mechanism. In addition, our study showed a mutation at position 91 in the \textit{parC} gene (Glu91Lys) associated with a mutation in \textit{gyrA} (Thr83Ile) in a strain of XDR \textit{P. aeruginosa} (\textit{exoT}+\textit{exoS}+\textit{exoU}+) genotype that, to the best of our knowledge, has yet to be described in Brazil.

Other investigators have demonstrated that it is mainly inappropriate antimicrobial therapy that is associated with a poor prognosis in \textit{P. aeruginosa} infection (Kang et al., 2005; Lodise et al., 2007), but our results suggest that MDR and TTSS virulence associated with other virulence factors as \textit{lasB}, \textit{toxA} and \textit{algD} should be added to the growing list of factors responsible for the increased mortality rate due to severe \textit{P. aeruginosa} infections.

In conclusion, our results confirm previous findings regarding MBL production in Brazil with the spread of clones of types SPM and VIM, and contribute additional evidence suggesting that inappropriate therapy is a crucial factor in the worst prognosis among patients with bloodstream infection caused by MDR \textit{P. aeruginosa}, independent of the genotype associated with TTSS virulence. Additionally, this study presents some novel findings: (i) isolation of a strain that showed a mutation in topoisomerase IV (Glu91Lys) associated with a \textit{gyrA} mutation (Thr83Ile) not described in Brazil to date; and (ii) the finding that the \textit{exoU} genotype was not common among isolates, even when associated with serious infections such as bacteraemia and VAP.

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