Original article

Spread of multidrug-resistant Acinetobacter baumannii and Pseudomonas aeruginosa clones in patients with ventilator-associated pneumonia in an adult intensive care unit at a university hospital

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A B S T R A C T

Background: In Brazil, ventilator-associated pneumonia (VAP) caused by carbapenem resistant Acinetobacter baumannii and Pseudomonas aeruginosa isolates are associated with significant mortality, morbidity and costs. Studies on the clonal relatedness of these isolates could lay the foundation for effective infection prevention and control programs.

Objectives: We sought to study the epidemiological and molecular characteristics of A. baumannii vs. P. aeruginosa VAP in an adult intensive care unit (ICU).

Methods: It was conducted a cohort study of patients with VAP caused by carbapenem resistant A. baumannii and P. aeruginosa during 14 months in an adult ICU. Genomic studies were used to investigate the clonal relatedness of carbapenem resistant OXA-23-producing A. baumannii and P. aeruginosa clinical isolates. The risk factors for acquisition of VAP were also evaluated. Clinical isolates were collected for analysis as were samples from the environment and were typed using pulsed field gel electrophoresis.

Results: Multivariate logistic regression analysis identified trauma diagnosed at admission and inappropriate antimicrobial therapy as independent variables associated with the development of A. baumannii VAP and hemodialysis as independent variable associated with P. aeruginosa VAP. All carbapenem resistant clinical and environmental isolates of A. baumannii were OXA-23 producers. No MBL-producer P. aeruginosa was detected. Molecular typing
Introduction

Ventilator-associated pneumonia (VAP) is one of the most frequent intensive-care-unit (ICU)-acquired infection, with an incidence ranging from 6 to 52%\(^1\), and continues to be a major cause of morbidity, mortality, and increased financial burden in ICUs.\(^2\) The overall rate of VAP is higher in developing countries ICUs than that reported from the US ICUs, with rate of 13.6% vs. 3.3 per 1000 ventilator-days, respectively.\(^3\)

Non-fermentative Gram-negative bacilli (NF-GNB) mainly A. baumannii and P. aeruginosa, have emerged as major agents of VAP\(^4\) and the resistance of these organisms to antibiotics, particularly to carbapenems, has posed important therapeutic challenges. Currently, carbapenems are considered the antimicrobials of choice for serious infections caused by A. baumannii and P. aeruginosa\(^5\) but their efficacy is increasingly compromised by resistance as reported worldwide.\(^6\) This resistance has been attributed to the production of carbapenem-hydrolysing-\(\beta\)-lactamase enzymes of Ambler molecular class D (oxacillinases) and B (metallo-\(\beta\)-lactamases).\(^7\)

Acinetobacter species, considered organisms of low virulence, have become one of the most difficult nosocomial pathogens to control and treat with an associated mortality of approximately 30%.\(^6\) P. aeruginosa is a highly virulent microorganism and mortality rates range from 40% to more than 60%.\(^8\) It has been reported that multidrug-resistant (MDR) strains of this organism are associated with a higher rate of mortality and that mortality attributed to infection by P. aeruginosa is considerable.\(^9\)

The objectives of the present study were to investigate the risk factors of A. baumannii vs. P. aeruginosa VAP in an adult ICU. Furthermore, data on spread of MDR A. baumannii and P. aeruginosa isolates and their phenotypic and genotypic characteristics are presented in this study.

Material and methods

Setting

The Uberlandia Federal University-Hospital Clinic (UFU-HC) is a 530-bed teaching hospital and a 30-bed clinical-surgical ICU for adults.

Study design

This prospective cohort study was conducted from April 2011 to June 2012, in a 30-bed clinical-surgical ICU for adults. All the medical records of the patients with VAP by A. baumannii and P. aeruginosa admitted to ICU at the time of the study were analyzed. Adult patients (≥18 years old) with a first episode of VAP by A. baumannii or P. aeruginosa were included in the study. Clinical isolates for A. baumannii and P. aeruginosa were obtained from cultures of endotracheal aspirate (EA) of patients admitted to the ICU. Patients identified as having VAP by A. baumannii were selected for environmental sampling, and samples were collected on surfaces near patients with VAP, before and after cleaning.

Definitions

All patients included in this study were monitored for the development of VAP using clinical and microbiological criteria, until discharge or death. VAP was defined as a pneumonia occurring 48 h or more after endotracheal intubation, with new and/or progressive radiological infiltrate, and at least two of the following features: purulent sputum, temperature higher than 38.5°C or lower than 35°C, leukocyte count higher than 1000/μL or lower than 3000/μL; and positive quantitative culture of the EA (count ≥10⁶ CFU/mL).\(^10\) The Clinical Pulmonary Infection Score (CPIS),\(^11\) based on six clinical assessments, was calculated for patients clinically suspected of VAP on the same day of endotracheal secretions collection, and all those with CPIS ≥ 6 were included. Patients developing VAP within the first four days of mechanical ventilation (MV) were classified as having early-onset VAP, while those developing VAP five or more days after the initiation of MV were classified as having late-onset VAP.\(^12\) Previous antimicrobial therapy was defined as receiving a systemic antimicrobial agent for at least 48 h within two weeks preceding collection sputum for culture.\(^13\) The antimicrobial therapy was considered appropriate if all pathogens isolated from culture were sensitive to it and inappropriate if any pathogen was resistant to it.\(^13\) Multidrug-resistant (MDR) pathogens were defined as resistant to three or more classes of antibiotics.\(^14\)

Data collection, clinical and environmental samples

The demographic characteristics including age, gender, underlying disease or condition, admission diagnosis, surgery, Average Severity of Illness Score (ASIS),\(^15\) length of hospital stay, previous use of antibiotics, and discharge status were obtained from medical charts. The collection of clinical specimens obtained from EA was performed by the nursing staff and physiotherapists, early in the morning, by probe number 12, and transported in a sterile tube to the Microbiology Laboratory of UFU for processing. Environmental isolates of A. baumannii revealed a polyclonal pattern; however, clone A (clinical) and H (surface) were the most frequent among isolates of A. baumannii tested, with a greater pattern of resistance than other isolates. In P. aeruginosa the most frequent clone I was multi-sensitive.

Conclusion: These findings suggest the requirement of constant monitoring of these microorganisms in order to control the spread of these clones in the hospital environment.

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***baumannii*** were obtained while the patient occupied the room. Three surfaces in each room were sampled, before and after cleaning: bedside table, bed rail, and door knob. At each site, an area was sampled using a pre-moistened sterile wipe cloth (7 × 7 cm).

**Species identification and antimicrobial susceptibilities**

Isolates were identified by conventional techniques (environment/surfaces) and automated systems (clinical specimen), including the Vitek2® system (bioMérieux Vitek Systems Inc., Hazelwood, MO). Susceptibility testing for clinical specimens was performed using the Vitek2® system, and the disk diffusion method was employed to evaluate susceptibility of isolates from surfaces. The following antimicrobial agents were used to evaluate susceptibility of *A. baumannii* isolates: amikacin, ampicillin/sulbactam, cefepime, ciprofloxacin, polymyxin B or colistin, gentamicin, imipenem, and tigecycline. Imipenem, meropenem, ceftazidime, ciprofloxacin, gentamicin, cefepime, amikacin, polymyxin B, and piperacillin/tazobactam were used to evaluate susceptibility of *P. aeruginosa*. All the tests were done in accordance with the Clinical and Laboratory Standards Institute16 recommended practices. Since there are no breakpoints available for tigecycline for *Acinetobacter* spp., US Food and Drug Administration (FDA) tigecycline breakpoints listed for Enterobacteriaceae (≥2, 4 and ≥8 μg/mL for susceptible, intermediate and resistant strains, respectively) were applied to *Acinetobacter* spp. in this study. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as quality control strains. All isolates of *A. baumannii* were screened for carbapenemase producing phenotype by the Modified Hodge Test (MHT)16 and all isolates of *P. aeruginosa* were screened for metallo-β-lactamases producing phenotype by the double-disk synergy test (DDST).17

**PCR amplification**

To assess the presence of *blaOXA* genes in *A. baumannii* strains, the multiplex PCR was performed as previously described by Higgins et al.18 and Woodford et al.19 and for amplification of the genes encoding Metallo-Beta-lactamases (MBLs) in *P. aeruginosa* strains, the multiplex PCR was performed according to Woodford.20 Amplification of the genes was carried out with PCR using primers described in Table 1.

**Molecular typing**

Isolates were typed by pulsed-field gel electrophoresis21 (PFGE), following digestion of intact genomic DNA with *Apal* (Invitrogen) for *A. baumannii* and *SpeI* (Fermentas) for *P. aeruginosa*. DNA fragments were separated on 1% (w/v) agarose gels in 0.5% TBE [Tris–borate–ethylene diamine tetra-acetic acid (EDTA)] buffer using a CHEF DRIII apparatus (Bio-Rad, Hercules, CA) with 6 V/cm, pulsed from 5 s to 15 s, for 18 h at 14 °C for *A. baumannii* and with 6 V/cm, pulsed from 5 s to 35 s, for 17 h at 14 °C, for *P. aeruginosa*. Gels were stained with ethidium bromide and photographed under ultraviolet light. Computer-assisted analysis was performed using BioNumerics v.4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

**Comparison of the banding patterns was accomplished by** the unweighted pair-group method with arithmetic averages (UPGMA) using the Dice similarity coefficient.

**Statistical analysis**

The Chi-square tests or Fisher’s exact test were used to compare discrete variables. Fisher’s exact test was used instead of the Chi-square test when one or more expected values in the 2 × 2 contingency table were equal or less than 5. The comparison of two quantitative variables was made using the Mann–Whitney test for nonparametric variables and the Student *t* test for parametric variables. Two-sided tests were used for all analyses. Multivariate analysis was performed using multiple logistic regression and the values were included when significance was <0.05 in univariate analysis. All *p*-value <0.05 was considered statistically significant. The epidemiological data were analyzed through the programs GraphPad Prism® 5.0 (La Jolla, CA, USA) and BioStat 5.0 (Têfê, AM, Brazil).

**Ethical considerations**

The Ethics Committee for Human Research of UFU approved the project under the protocol number 228/11.

**Table 1 – Specific primers used in this study.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>blaOXA-51</em></td>
<td>F 5′-TAATGGTCTTTGATGGCGCTTG-3′</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>R 5′-TGGATCGAATCTAGCTGTTG-3′</td>
<td></td>
</tr>
<tr>
<td><em>blaOXA-23</em></td>
<td>F 5′-GATTCACTGGGAGAAACGCA-3′</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>R 5′-ATTCTGACGCCATTTCCAT-3′</td>
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<tr>
<td><em>blaOXA-24</em></td>
<td>F 5′-GCTTGAATGGGGCTCTTCAA-3′</td>
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<td>R 5′-AGTTGACGGAAAGGGGATT-3′</td>
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<tr>
<td><em>blaOXA-58</em></td>
<td>F 5′-AAGTATGCGCGCTTGTCGT-3′</td>
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</tr>
<tr>
<td></td>
<td>R 5′-CCCCCCCTGCNGCTCACATAC-3′</td>
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<tr>
<td><em>blaOXA-143</em></td>
<td>F 5′-TGGCATCTTTACAGTTCCT-3′</td>
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<td>R 5′-TAATCTGGAGGGGGCCCAAC-3′</td>
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<tr>
<td><em>blaIMP</em></td>
<td>F 5′-GAATAGRTTCTTTAATTCTC-3′</td>
<td>20</td>
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<tr>
<td></td>
<td>R 5′-CCAACAYCTATSGTATAC-3′</td>
<td></td>
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<tr>
<td><em>blaVIM</em></td>
<td>F 5′-TTTGGTGGCATATCGCAAC-3′</td>
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<tr>
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<td>R 5′-AATTGCGGACACGAGGATAG-3′</td>
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<tr>
<td><em>blaVPM</em></td>
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<td>R 5′-GCTTTTCGGCGAGCCCTTGATC-3′</td>
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<td><em>blaGIM</em></td>
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<td><em>blaSIM</em></td>
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<tr>
<td></td>
<td>R 5′-TGCCCTTGTTCCATGTAG-3′</td>
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</tbody>
</table>

**Results**

During the study period, we enrolled 30 subjects with VAP caused by *A. baumannii* and a similar number of subjects with VAP caused by *P. aeruginosa*. Three patients were excluded from the epidemiological study because the VAP were due *A. baumannii* associated to other microorganisms. The demographic and clinical characteristics, including risk factors and outcome of patients infected by these bacteria, are shown in Table 2.

The risk factors for acquiring *A. baumannii* VAP by univariate analysis (*p* ≤ 0.05) were: trauma diagnosis at admission,
score ASIS >4, MDR, and inappropriate antimicrobial therapy. However, after multiple logistic regression analysis only trauma diagnosis at admission (OR 7.21, 95% CI: 1.62–32.10, \( p = 0.001 \)) and inappropriate antimicrobial therapy (OR 17.29, 95% CI: 2.61–114.50, \( p = 0.003 \)) remained as an independent variables associated with the development of A. baumannii VAP (Table 3). For acquiring P. aeruginosa VAP, only hemodialysis (OR 0.14, 95% CI: 0.03–0.70, \( p = 0.017 \)) remained as an independent variable associated with the development of VAP (Table 4). Overall, out of 29 clinical isolates of VAP due to A. baumannii, 17 (58.62%) were imipenem-resistant and most (12, 70.59%) were positive for class D carbapenemase production (MHT). Screening for resistance genes was performed by multiplex PCR and all the 12 isolates were OXA-23 producers. No MBL-producer P. aeruginosa was detected (Table 5).

Thirty one (29.5%) of the 126 samples obtained from 21 rooms occupied by an A. baumannii VAP patient were positive for A. baumannii and the isolates were recovered mainly
from the bed rail (29.0%) after cleaning. The prevalence of MDR organisms was 74.2% (23/31) with eight (25.8%) imipenem resistant and six (75.0%) positive by the MHT. By PCR, all six isolates recovered from surface were positive for the presence of blaOXA-51 and blaOXA-23.

For operational reasons, only 23 A. baumannii and 19 P. aeruginosa strains were typed. This is the first study that evaluated the clonal relationship among samples of A. baumannii from patients and hospital environment at the ICU studied.

Twenty-three isolates of A. baumannii were analyzed by PFGE. Most of them were imipenem-resistant (20/23, 86.9%) and recovered from VAP episodes (17/23, 73.9%). Eight genotypes with 80% similarity were observed and genotype A (n = 9, 52.9% of clinical isolates) and H (n = 4, 66.6% of surface isolates) were the most frequent in the unit. In general, we observed different genotypes among environmental (H) and clinical (A) imipenem-resistant A. baumannii isolates and the three imipenem-susceptible isolates belonged to two other distinct genotypes (1, F and 2, G) (Table 5).

Based on the antibiotic susceptibility testing, eight antibiotic patterns (R1–R8) were identified among A. baumannii isolates that were genotypically positive for oxacillinases genes. Five isolates were assigned as antibiotic R1 and were resistant to all antibiotics tested, with the exception of colistin. The other antibiotics (R2–R5) fit in the MDR pattern. These antibiotics presented major pulsotypes characterized as type A in EA isolates and type H in environmental isolates (Table 5).

A total of 10 PFGE patterns of P. aeruginosa were observed among the 19 isolates analyzed, also with a polyclonal pattern, but with three major clones: I (n = 4, 21.1%), A (n = 3, 15.8%), H (n = 3, 15.8%). Based on the antibiotic susceptibility testing, nine antibiotic patterns (R1–R9) were identified among P. aeruginosa isolates that were genotypically negative for MBLs genes and the main pulsotype I presented itself as multi-sensitive or only resistant to carbapenems (Table 5).

### Discussion

Ventilator-associated pneumonia caused by A. baumannii and P. aeruginosa, particularly those resistant to the carbapenems, are associated with significant mortality and morbidity, adding considerable costs to hospital care.

Risk factors for VAP due to A. baumannii are mainly head trauma, prolonged hospital stay and prior antibiotic use, whereas VAP due to P. aeruginosa is diagnosed in immunocompromised patients, especially those with neutropenia.

In our study, the univariate analysis identified some variables as significant risk factors for acquiring A. baumannii VAP, but only trauma diagnosis at admission and inappropriate antimicrobial therapy were independent risks factors for the development of VAP by A. baumannii. Likewise, for acquiring P. aeruginosa VAP, only hemodialysis was an independent factor.

Therapeutic options are limited for A. baumannii and P. aeruginosa VAP and the antimicrobial resistance in these organisms is associated with several mechanisms, but the most important is the production of β-lactamases and has been attributed to the production of carbapenem-hydrolysing-β-lactamase enzymes of Ambler molecular class D (oxacillinases) for A. baumannii, and class B (MBLs) associated with loss of porins and efflux system overexpression for P. aeruginosa.

Currently, carbapenems are the antimicrobials of choice for treating serious infections caused by A. baumannii. In Brazil and elsewhere, carbapenem resistant isolates are mostly related to β-lactamase OXA-23, as detected in all A. baumannii imipenem resistant isolates recovered in our study.

Although the most important source of A. baumannii is the infected or colonized patient, a widespread environmental contamination is often demonstrated as seen in our study, with a more expressive contamination of the bed rail even after cleaning (29.0%), questioning the routine of this service.
<table>
<thead>
<tr>
<th>Ab&lt;sup&gt;1&lt;/sup&gt; carbapenem-resistant/susceptible N (%)</th>
<th>MHT&lt;sup&gt;2&lt;/sup&gt; isolates analyzed/positive N (%)</th>
<th>PCR&lt;sup&gt;3&lt;/sup&gt; analyzed N (%)</th>
<th>PCR product size bp/gene (isolates)</th>
<th>Antibiotyp&lt;sup&gt;3&lt;/sup&gt; (isolates)</th>
<th>Resistance profile (isolates)</th>
<th>Major Ab&lt;sup&gt;1&lt;/sup&gt; pulsortype/N (%)</th>
<th>Non-major Ab&lt;sup&gt;1&lt;/sup&gt; pulsortype/N (%)</th>
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<tr>
<td><strong>Endotracheal aspirate</strong></td>
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<td>17/29 (58.6)/12/29 (41.4)</td>
<td>17 (100.0)/12 (70.6)</td>
<td>&lt;sup&gt;a&lt;/sup&gt;12/17 (70.6)</td>
<td>353pb/bla&lt;sub&gt;OXA-51&lt;/sub&gt; (12)</td>
<td>R1 (5) AN; SAM; GEN; IPM; TGC</td>
<td>A/4 (80.0)</td>
<td>G/Y (20.0)</td>
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<td></td>
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<td>&lt;sup&gt;b&lt;/sup&gt;02/17 (11.8)</td>
<td>501pb/bla&lt;sub&gt;OXA-23&lt;/sub&gt; (12)</td>
<td>R2 (3) SAM; GEN; IPM; TGC</td>
<td>A/2 (66.7)</td>
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<td>D/Y (33.3)</td>
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<td>&lt;sup&gt;c&lt;/sup&gt;03/17 (17.6)</td>
<td>353pb/bla&lt;sub&gt;OXA-51&lt;/sub&gt; (02)</td>
<td>R3 (1) AN; SAM; GEN; IPM</td>
<td>A/Y (100.0)</td>
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<td>501pb/bla&lt;sub&gt;OXA-23&lt;/sub&gt; (02)</td>
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<td>R5 (2) SAM; GEN; IPM</td>
<td>A/2 (100.0)</td>
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<td>R6 (2) SAM; IPM</td>
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<td>R7 (3) SAM</td>
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<td>R8 (2) Multi-sensitive</td>
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<tr>
<td><strong>Environment</strong></td>
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<tr>
<td>8/31 (25.8)/23/31 (74.2)</td>
<td>8 (100.0)/6 (75.0)</td>
<td>6 (75.0)</td>
<td>353pb/bla&lt;sub&gt;OXA-51&lt;/sub&gt; (6)</td>
<td>R2 (5) SAM; GEN; IPM; TGC</td>
<td>H/Y (100.0)</td>
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<td>501pb/bla&lt;sub&gt;OXA-23&lt;/sub&gt; (6)</td>
<td>R5 (4) SAM; GEN; IPM</td>
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<td>G/Y (25.0)</td>
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<td>R6 (1) SAM; IPM</td>
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<td>E/Y (100.0)</td>
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<tr>
<td>**Pa&lt;sup&gt;4&lt;/sup&gt; carbapenem-resistant/susceptible N (%)</td>
<td>DDST&lt;sup&gt;5&lt;/sup&gt; isolates analyzed/positive N (%)</td>
<td>PCR&lt;sup&gt;3&lt;/sup&gt; analyzed N (%)</td>
<td>PCR product size bp/gene (isolates)</td>
<td>Antibiotyp&lt;sup&gt;3&lt;/sup&gt; (isolates)</td>
<td>Resistance profile (isolates)</td>
<td>Major Pa&lt;sup&gt;4&lt;/sup&gt; pulsortype/N (%)</td>
<td>Non-major Pa&lt;sup&gt;4&lt;/sup&gt; pulsortype/N (%)</td>
</tr>
<tr>
<td>15/30 (50.0)/15/30 (50.0)</td>
<td>14 (93.3)/11 (78.6)</td>
<td>14 (100.0)</td>
<td>353pb/bla&lt;sub&gt;OXA-51&lt;/sub&gt; (6)</td>
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<td>H/Y (100.0)</td>
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<td>R7 (2) IPM</td>
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<td>R9 (4) Multi-sensitive</td>
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</table>

<sup>1</sup>Acinetobacter baumannii; <sup>2</sup>Modified Hodge Test; <sup>3</sup>Polymerase chain reaction; <sup>4</sup>Pseudomonas aeruginosa; <sup>5</sup>Double Disk Synergy Test; <sup>a</sup>12 isolates positive for carbapenemase production (MHT); <sup>b</sup>2 isolates negative for carbapenemase production (MHT); <sup>c</sup>3 isolates carbapenem-susceptible; AN, amikacin; SAM, ampicillin/sulbactam; CIP, ciprofloxacin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline; TZP, piperacillin/tazobactam.
in the ICU. As the microorganism can survive in dry conditions and persist for prolonged periods of time, surfaces may be a reservoir of epidemic strains in outbreaks in ICUs that ceased only after closing for terminal cleaning and disinfection.25

The diversity of MDR A. baumannii and P. aeruginosa isolates in our ICU could provide useful information for hospital infection control. One or more A. baumannii clones often coexist in ICUs, as seen in our investigation, but different from other authors26,27 we have demonstrated the coexistence of two major PFGE-type clones (A – clinical and H – environmental) of A. baumannii and three of P. aeruginosa (I, A, H–clinical), reinforcing the clonal diversity of A. baumannii and P. aeruginosa. Furthermore, carbapenem-resistance could be transmitted horizontally highlighting the infection control measures, such as hand hygiene, that should be strengthened to reduce the further spread of both microorganisms.9,27 There is not a single mechanism of polyclonal dissemination and although this work has not analyzed colonized patients, this is a puzzle that could indicate endogenous acquisition.28

Person-to-person transmission of carbapenem resistant A. baumannii carrying blaoxa-23 was indeed identified in isolates belonging to the same pulsotype (genotype A) recovered from different patients. These data suggest that rapid identification of blaoxa-23 by molecular methods is necessary to implement rigorous infection control programs, justifying more effective contact precautions for patients with carbapenem-resistant strains.6,27

In summary, this study demonstrates high VAP rates caused by A. baumannii and P. aeruginosa in an adult ICU of our hospital mainly associated with the severity of the underlying disease in patients with P. aeruginosa and inappropriate therapy for patients with A. baumannii. Moreover, PFGE revealed the polyclonal spread of OXA-23-producing A. baumannii, isolated also in the environment, and P. aeruginosa resistant to carbapenems. Taken together, these results suggest the requirement of an epidemiological surveillance to control the transmission of these resistance mechanisms in the hospital environment.

Conflicts of interest
The authors declare no conflicts of interest.

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