

Antimicrobial resistance profiles and oxacillinase genes in carbapenem-resistant *Acinetobacter baumannii* isolated from hospitalized patients in Santa Catarina, Brazil

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ABSTRACT

Introduction: Carbapenems are the therapy of choice for treating severe infections caused by the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. We aimed to assess the prevalence and antimicrobial susceptibility profiles of producers of distinct oxacillinases among nosocomial isolates of the *A. calcoaceticus-A. baumannii* complex in a 249-bed general hospital located in Joinville, Southern Brazil. **Methods:** Of the 139 *A. baumannii* clinical isolates with reduced susceptibility to carbapenems between 2010 and 2013, 118 isolates from varying anatomical sites and hospital sectors were selected for genotypic analysis. Five families of genes encoding oxacillinases, namely *bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-51-like}, *bla*_{OXA-58-like}, and *bla*_{OXA-143-like}, were investigated by multiplex polymerase chain reaction (PCR). **Results:** Most (87.3%) isolates simultaneously carried the *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes, whereas three (2.5%) isolates harbored only *bla*_{OXA-51-like} ones. The circulation of carbapenem-resistant isolates increased during the study period: from none in 2010, to 22 in 2011, 64 in 2012, and 53 in 2013. **Conclusions:** Isolates carrying the *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes were widely distributed in the hospital investigated. Because of the worsening scenario, the implementation of preventive measures and effective barriers is needed.

Keywords: Carbapenem resistance. *Acinetobacter*. Nosocomial infection. Oxacillinases.

INTRODUCTION

In the last decade, the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex emerged as a major nosocomial pathogen complex worldwide because of its remarkable ability to develop resistance to broad-spectrum antimicrobial agents⁽¹⁾⁽²⁾. These bacterial strains can persist for long periods on both wet and dry surfaces, as they have minimal nutritional requirements, are tolerant to various physical conditions such as humidity and temperature, and can cause frequent hospital-acquired infection outbreaks⁽³⁾. In addition, this complex is highly predominant in intensive care units (ICUs) and has been mainly involved in respiratory tract infections that are associated with mechanical ventilation, postoperative bacteremia, secondary meningitis, and urinary tract infections⁽¹⁾⁽²⁾⁽⁴⁾.

Acinetobacter baumannii has innate resistance to most antimicrobials commonly used in medical practice, including carbapenems, a class of β -lactam antibiotics⁽⁵⁾. Resistance to carbapenems involves combined mechanisms including enzymatic hydrolysis by β -lactamases, altered permeability of the outer cell membrane, antibiotic affinity to penicillin-binding proteins, and increased activity of efflux pumps⁽³⁾. The oxacillinase OXA-type carbapenemases (oxacillinases) belong to the Ambler class D β -lactamases and constitute the main resistance mechanism to carbapenems worldwide. In the genus *Acinetobacter*, oxacillinases are genetically divided into six commonly identified subgroups: *bla*_{OXA-23-like} (OXA-23, OXA-27, and OXA-49), *bla*_{OXA-24-like} (OXA-24, OXA-25, OXA-26, OXA-40, and OXA-72), *bla*_{OXA-58-like}, *bla*_{OXA-143-like} (OXA-143, OXA-231, and OXA-253), *bla*_{OXA-235}, and *bla*_{OXA-51-like}, the latter being intrinsic to *A. baumannii*⁽⁶⁾⁽⁷⁾⁽⁸⁾.

Tracking of nosocomial infections, particularly when combined with assessment of phenotypic and genotypic profiles of the most important multidrug-resistant pathogens, enables the implementation of containment measures during infection outbreaks and the development of adequate infection control practices⁽⁴⁾. Therefore, the present study aimed to assess the antimicrobial susceptibility profile and the prevalence of producers of distinct oxacillinases among nosocomial isolates in the *A. calcoaceticus-A. baumannii* complex.

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METHODS

Clinical isolates

The study was performed in a general public hospital located in Joinville, Southern Brazil, with 249 beds, 20 of which were located in the ICU. Data on the antimicrobial susceptibility profiles of the *A. calcoaceticus*-*A. baumannii* complex and *Pseudomonas* spp. were obtained from the Hospital Infection Control Service between 2010 and 2013 (n=139). A set of 118 clinical isolates of carbapenem-resistant *Acinetobacter baumannii* (CRAb) from patients hospitalized for at least 72h⁽⁹⁾ between June 2011 and August 2013 were used for phenotypic and genotypic assessment. The isolates were obtained from samples of bronchoalveolar lavage fluid, blood, urine, pericardial and abdominal fluids, and catheter tips. Microbial identification was performed exclusively on isolated colonies using the semi-automated system AutoScan[®]-4 (Siemens, Munich, Germany), and was complemented by Gram staining. The study was reviewed and approved by the Research Ethics Committee of University of Region of Joinville (UNIVILLE) (protocol 73464).

Antimicrobial susceptibility analysis

Antimicrobial susceptibility was assessed via disk diffusion according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)⁽¹⁰⁾ The antimicrobial agents tested were imipenem (10µg), meropenem (10µg), polymyxin B (300U), tigecycline (15µg), ampicillin/sulbactam (10µg/10µg), amikacin (30µg), gentamicin (10µg), ciprofloxacin (5µg), ceftriaxone (30µg), cefepime (30µg), piperacillin/tazobactam (100µg/10µg), and sulfamethoxazole/trimethoprim (1.25µg/23.75µg), all of which were purchased from Oxoid (Cambridge, England). The minimum inhibitory concentrations (MICs) for imipenem, meropenem, polymyxin B, and tigecycline were determined using Etest[®] strips according to the manufacturer's instructions (bioMérieux, Marcy-l'Etoile, France).

The sizes of the inhibition zones formed in the presence of the antimicrobials were interpreted as recommended by the CLSI⁽¹⁰⁾, except for tigecycline, for which there is no standard until date⁽¹¹⁾. In the latter case, the interpretation criteria used were those adopted by the United States Food and Drug Administration for enterobacteria when using the disk diffusion method, namely susceptible: ≥19mm, intermediate: 15-18mm, and resistant: ≤14mm (9).

Phenotypic assessment of carbapenemase-producing isolates

Isolates with inhibition zones of ≤13mm in the disk diffusion test and a MIC ≥16µg/mL in the Etest[®] were considered possible producers of carbapenemases and potentially resistant to both imipenem and meropenem. The modified Hodge test was not used in this study because the CLSI no longer recommends it for use in routine laboratory screening for carbapenemase-producing strains⁽¹⁰⁾.

Detection of genes encoding oxacillinases

Bacterial deoxyribonucleic acid (DNA) was extracted by heat shock⁽¹²⁾. Two to three medium-sized colonies of each isolate were suspended in 150µL of sterile ultrapure water (MilliQ, São Paulo, Brazil). The suspensions were heated in a boiling water bath for 5 min, followed by cooling in an ice bath for 5 min. The boiling and cooling procedures were repeated once more, followed by centrifugation at 8,000 × g for 10 min. The supernatants containing the DNA were collected and the absorbance at 260 and 280nm was determined by spectrophotometry. An aliquot of each supernatant (100µL) was stored at -20°C.

To confirm the identity of the clinical *A. baumannii* isolates to species level, the extracted DNA was used for PCR amplification of a single 722-bp segment of the citrate synthase gene (*gltA*) using the specific primer pair *gltA*-F (5'-AATTTACAGTGGCACATTAGGTCC-3') and *gltA*-R (5'-GCAGAGATACCAGCAGAGATACACG-3'). PCR reactions were performed in an XP Cyclor device (BIOER Technology, Tokyo, Japan) using the following program: initial denaturation at 94°C for 3 min, 30 cycles at 94°C for 1 min, 62°C for 30s, and 72°C for 1 min, and a final extension at 72°C for 10 min⁽¹³⁾.

Multiplex PCR was used to identify five families of oxacillinase-encoding genes, namely *bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-51-like}, *bla*_{OXA-58-like}, and *bla*_{OXA-143-like}, using the gene-specific primer pairs listed in **Table 1**^{(14) (15)}.

For this purpose, PCR reactions were performed in a final volume of 50µL containing 50 to 500ng of extracted DNA, 1U Platinum[®] Taq DNA Polymerase (Invitrogen, São Paulo, Brazil), 0.2mM dNTPs (GE Healthcare, Little Chalfont, UK), 1× PCR Buffer (Invitrogen), 1.5mM MgCl₂ (Invitrogen), and 20 pmol of each primer (Invitrogen). The thermal cycling program was adapted from Woodford et al.⁽¹³⁾ and consisted of denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 1 min, 52°C for 30s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

The PCR products were subjected to 1% agarose gel electrophoresis, and their size was confirmed under ultraviolet (UV) light (MiniBis-Pro Photodocumentation System; DNR Bio-Image Systems Ltd., Jerusalem, Israel), followed by image scanning.

The following standard oxacillinase-producing *A. baumannii* strains from the culture collection of the Oswaldo Cruz Foundation [*Fundação Oswaldo Cruz* (FIOCRUZ/RJ)] were used as positive controls: strain 7892 (*bla*_{OXA-24-like}), strain 7740 (*bla*_{OXA-58-like}), and strain 7572 (*bla*_{OXA-23-like} and *bla*_{OXA-143-like}).

RESULTS

The 118 clinical isolates of *A. baumannii* obtained during routine diagnostic assessment of patients with suspected infection were collected primarily from bronchoalveolar lavage fluid (n=64) and blood (n=27), accounting for 77.1% of the isolates, and less frequently from wound secretions (n=10),

TABLE 1 - Primer pairs used for the detection of oxacillinase-encoding genes.

Target gene	Sequence (5' to 3')	Product size (bp)	Reference
<i>bla</i> _{OXA-23-like}	GATCGGATTGGAGAACCAGA ATTCTGACCGCATTCCAT	501	15
<i>bla</i> _{OXA-24-like}	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	246	15
<i>bla</i> _{OXA-51-like}	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	353	15
<i>bla</i> _{OXA-58-like}	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	599	15
<i>bla</i> _{OXA-143-like}	TGGCACTTTCAGCAGTTCCT TAATCTTGAGGGGGCCAACC	149	14

bp: base pair.

urine (n=8), other bodily fluids (n=5), sputum (n=2), and catheter tips (n=2). Most (62.7%; n=74) isolates were obtained from patients hospitalized in the ICU, and the remaining isolates (n=44) came from patients housed in other hospital sectors.

Phenotypic assessment

All 118 isolates were resistant to the antimicrobials cefepime, ceftriaxone, ciprofloxacin, imipenem, meropenem, and piperacillin/tazobactam (**Figure 1**). Resistance to amikacin and gentamicin was confirmed in 80.5% (n=95) and 83.9% (n=99) of the isolates, respectively. For ampicillin/sulbactam, 94.1% (n=111) of the isolates exhibited complete resistance and 5.9% (n=7) exhibited intermediate resistance. A single (0.8%) isolate was susceptible to sulfamethoxazole/trimethoprim. In addition, all isolates tested were susceptible to polymyxin B, whereas 88.1% (n=104) and 11.9% (n=14) exhibited complete and intermediate susceptibility to tigecycline, respectively.

The Etest® results revealed that all isolates exhibited minimum inhibitory concentrations (MICs) $\geq 16\mu\text{g/mL}$ (resistant phenotype) for imipenem and meropenem, whereas the MICs for polymyxin B and tigecycline ranged from 0.2-2.0 $\mu\text{g/mL}$ and 0.5-4.0 $\mu\text{g/mL}$, respectively.

Genotypic assessment

Most (n=103; 87.3%) isolates simultaneously carried the *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes, whereas three (2.5%) isolates only harbored the *bla*_{OXA-51-like} gene. Genotyping results were inconclusive for twelve (10.2%) isolates. The isolates in which both the *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes were detected and those harboring only the *bla*_{OXA-51-like} gene had MICs $\geq 16\mu\text{g/mL}$ for imipenem and meropenem. The isolates that exhibited either intermediate or complete susceptibility to tigecycline all carried both the *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes. From the 103 clinical isolates that simultaneously harbored the *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes, 62 (60.2%) were isolated from the ICU,

and the remaining isolates (n=41) originated from other hospital wards.

Evolution of the antimicrobial susceptibility profile

In 2010, nine *A. calcoaceticus-A. baumannii* complex isolates were identified, from which a single isolate exhibited intermediate susceptibility to the carbapenems imipenem and meropenem. In 2011, 22 carbapenem-resistant *Acinetobacter baumannii* (CRAb) isolates were identified, representing 84.6% of the samples. In June of that year, the first nosocomial outbreak of *A. baumannii* was reported; six isolates were confirmed CRAb, four of which were derived from bronchoalveolar lavages of ICU patients. In December 2011, a second outbreak was confirmed, during which five CRAb isolates were identified, three of which were from bronchoalveolar lavages of ICU patients. Notably, up until 2011, all isolates had remained susceptible to tigecycline. Moreover, all CRAb isolates associated with the outbreaks in 2011 (n=11) had both the *bla*_{OXA-51-like} and *bla*_{OXA-23-like} genes, were susceptible to tigecycline and polymyxin B, and did not show any differences in the characteristics evaluated when compared with the other isolates.

In 2012, the number of carbapenem-resistant isolates increased to 64 (96.9%), 40 of which originated from the ICU; 34 of these were collected from bronchoalveolar lavage fluids. In the next year, 53 isolates were confirmed to be resistant, 36 of which were obtained from the ICU, 26 of which were collected from bronchoalveolar lavage fluids. Further, in 2013, eight (15.1%) and three (5.7%) isolates exhibited intermediate and complete resistance to tigecycline, respectively. **Figure 2** shows the evolution of the susceptibility to imipenem, meropenem, and tigecycline.

Figure 3 shows the evolution of the incidence of CRAb isolates between 2011 and 2013 as compared with *Pseudomonas* spp. from the same hospital that are usually associated with nosocomial infections and are resistant to carbapenems.

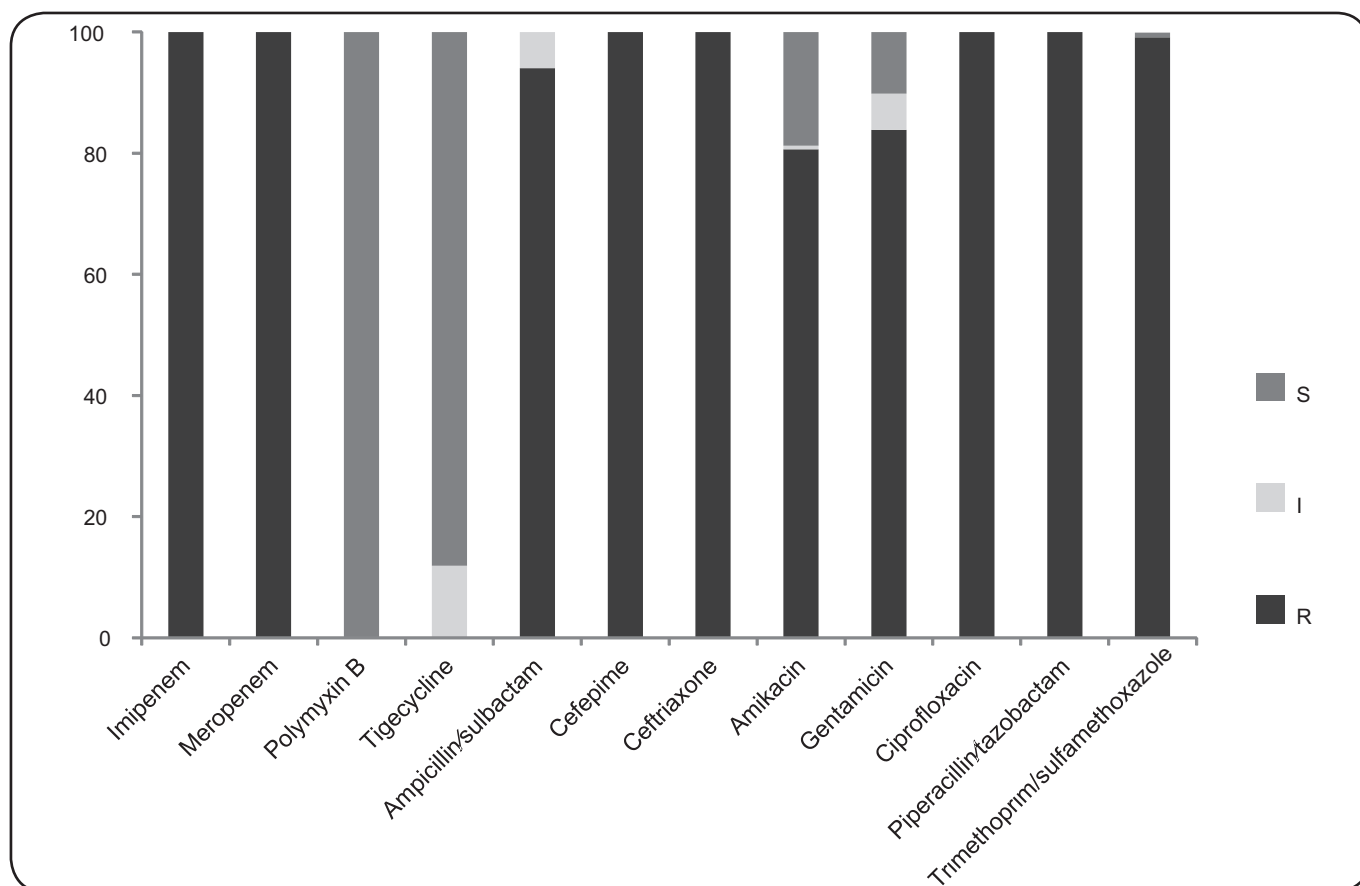


FIGURE 1 - *In vitro* antimicrobial susceptibility profiles for clinical isolates of the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex. S: susceptible; I: intermediate; R: resistant.

DISCUSSION

Our results revealed that the $bla_{OXA-23-like}$ and $bla_{OXA-51-like}$ genes were widely distributed in the clinical isolates analyzed, which might have contributed to the increasing incidence of CRAB strains in the hospital investigated. The predominance of the genotypic profile $bla_{OXA-23-like}/bla_{OXA-51-like}$ among the clinical isolates investigated led us to hypothesize the occurrence of cross infection.

The *A. calcoaceticus*-*A. baumannii* complex has been responsible for nosocomial infection outbreaks in Europe since 1980, mainly in England, France, Germany, Italy, Spain, and the Netherlands⁽¹⁶⁾. In Brazilian hospitals, infections have been reported since 1996, mainly at ICUs⁽¹⁷⁾. Carbapenems became the primary therapeutic options in various hospitals, but the continued use of these antimicrobials has been compromised by the emergence of novel resistance mechanisms⁽¹⁾. The production of oxacillinase enzymes, mainly through the expression of the $bla_{OXA-23-like}$ gene, is the most common resistance mechanism reported in CRAB isolates in diverse countries including Bulgaria, China, Iraq, Afghanistan, and French Polynesia⁽¹⁸⁾.

Previous studies conducted in Brazil revealed an increasing incidence of nosocomial outbreaks caused by multi-drug

resistant (MDR) strains of the genus *Acinetobacter*^{(19) (20)}. In a study performed from January 2006 to September 2007 in eight hospitals in Rio de Janeiro, 110 imipenem-resistant *A. baumannii* strains were isolated, among which 87.3% were producers of OXA-23 carbapenemase⁽¹⁹⁾. Another Brazilian study analyzed the genotypic characteristics of 46 CRAB isolates from eight hospitals in the State of Paraná and reported that all isolates harbored the $bla_{OXA-23-like}$ gene⁽²⁰⁾.

Other studies have revealed that *A. baumannii* strains typically spread through clonal dissemination. A study conducted in Australia in a single hospital during a 10-year period showed that the $bla_{OXA-23-like}$ gene, present in most isolates, was responsible for carbapenem resistance. In addition, the clonal relationship among the isolates was evaluated through pulsed-field gel electrophoresis, which demonstrated that the antimicrobial susceptibility profile does not predict isolate clonality⁽²¹⁾.

Despite the identification of New Delhi metallo- β -lactamase 1 (NDM-1)-producing *A. baumannii* in Londrina in the state of Paraná, the major mechanism of carbapenem resistance in Brazil is through the expression of the $bla_{OXA-23-like}$ gene^{(22) (23)}. In addition, this gene is frequently detected in isolates from Asia and Europe⁽²⁴⁾, and in most cases, it is found concomitantly with the $bla_{OXA-51-like}$ gene^{(2) (14)}. Notably, the $bla_{OXA-23-like}$ and $bla_{OXA-51-like}$

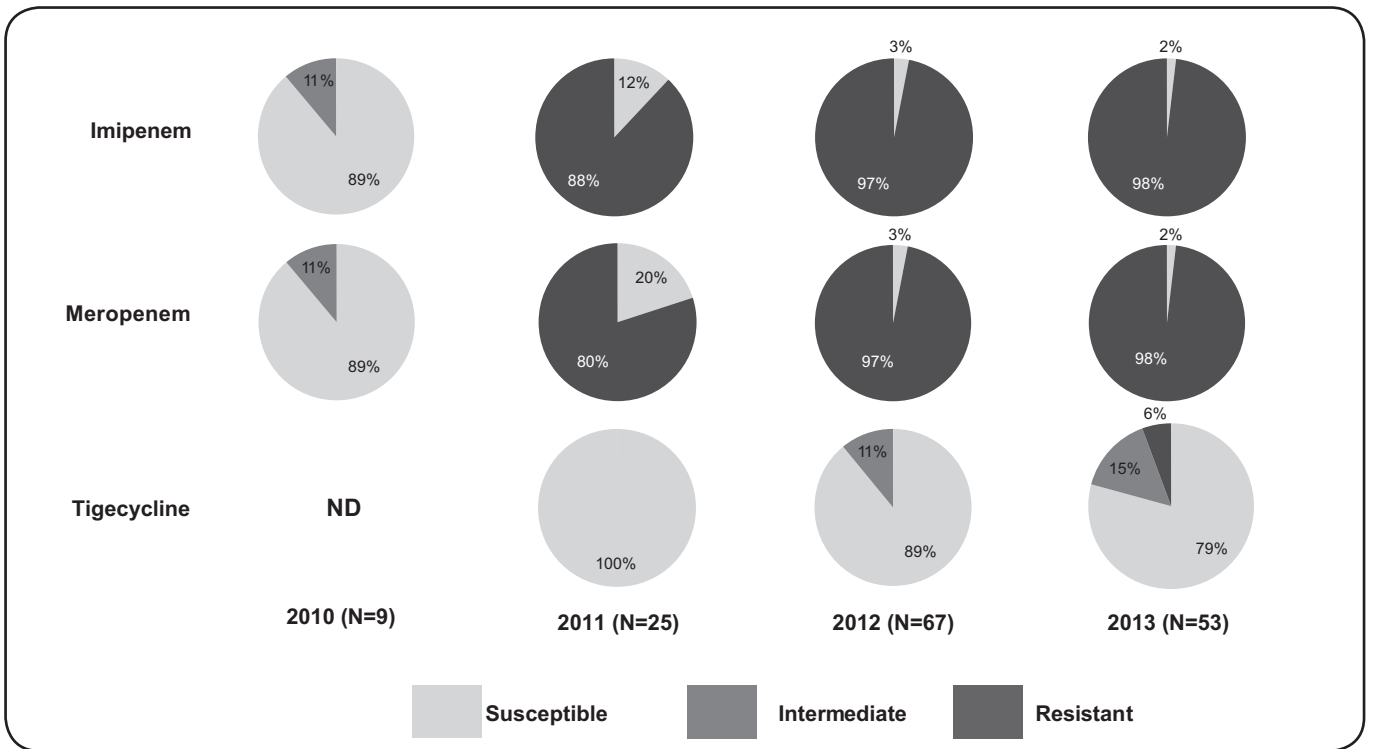


FIGURE 2 - Susceptibility profiles of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex to the antimicrobials imipenem, meropenem, and tigecycline between 2010 and 2013. ND: not done.

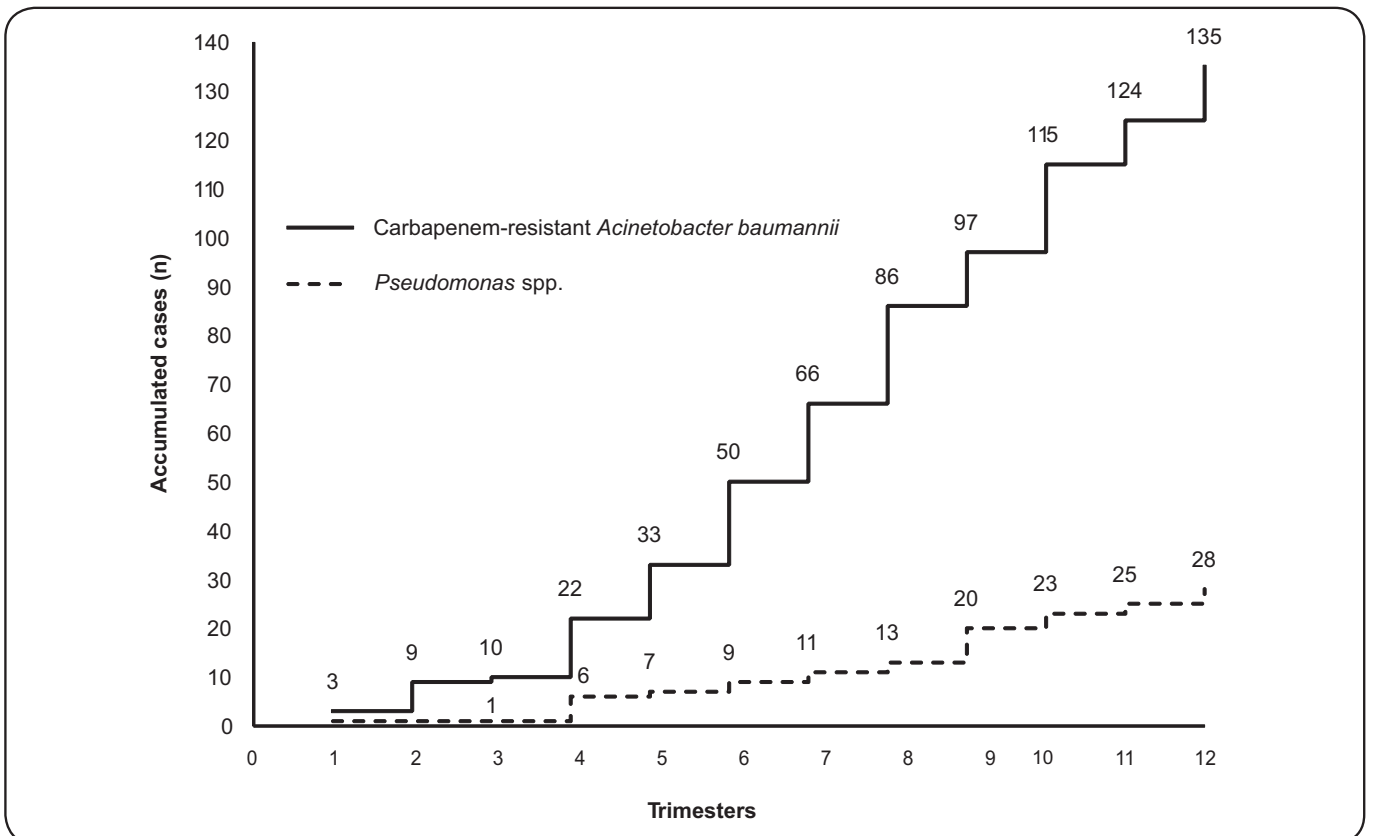


FIGURE 3 - Comparative incidence of carbapenem-resistant *Acinetobacter baumannii* and carbapenem-resistant *Pseudomonas* spp. isolates.

genes may be overexpressed when associated with the insertion sequence ISAb1 because this sequence constitutes a promoter region for both genes⁽²⁵⁾. In the present study, the resistance of the CRAb isolates (n=6) that did not contain any of the *bla*_{OXA} genes investigated may be associated with other, less frequent resistance mechanisms such as altered outer membrane permeability and increased activity of efflux pumps⁽⁵⁾.

Many institutions have observed an increased incidence of healthcare-associated infections by *A. baumannii*. This pathogen has become resistant to all β -lactam antibiotics (including carbapenems), all fluoroquinolones, sulfamethoxazole/trimethoprim, and most, if not all, aminoglycosides⁽²⁶⁾. Therefore, treatment options are limited. Tigecycline, colistin, and polymyxin B can be used as last-resort drugs for the treatment of infections caused by CRAb isolates in hospital settings. However, these antimicrobials have their own limitations and may not be indicated for several clinical conditions. For example, polymyxin B has nephrotoxic and neurotoxic effects⁽²⁾. In addition, decreased susceptibility to these antimicrobials became a major concern. A study performed in Spain revealed that the susceptibility to tigecycline decreased during the course of treatment, with an increase in the MIC to $\geq 2\mu\text{g/mL}$ ⁽²⁷⁾. In our study, all isolates were susceptible to polymyxin B during the period investigated; however, 10.4% and 15.1% of the isolates had intermediate resistance to tigecycline in 2012 and 2013, respectively. Even worse, three (5.7%) isolates were resistant to tigecycline in the last year of data collection.

The overuse of antimicrobials and the neglect of standard routines for hand hygiene have led to the emergence and spread of MDR pathogens in hospital settings, and the adoption of effective measures to prevent nosocomial outbreaks is warranted⁽⁴⁾ (28). The antimicrobial susceptibility profiles evaluated during the period considered in our study indicated a fast increase in circulating CRAb strains, especially when compared to data related to carbapenem-resistant *Pseudomonas* spp. in the same environment. In addition, the identification of an increasing incidence of CRAb strains also showing phenotypic resistance to tigecycline highlights that treatment options are becoming limited. Taken together, our results suggest an extensive dissemination of CRAb isolates in hospital environments and warrant the need for a systematic review of preventive measures and the implementation of effective outbreak control measures.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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