PRELIMINARY COMMUNICATION

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Future MICROBIOLOGY

Extending the reservoir of bla_{IMP-5}: the emerging pathogen *Acinetobacter bereziniae*

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Aim: *Acinetobacter bereziniae* clinical relevance is starting to be recognized; however, very few descriptions of its carbapenem resistance currently exist. Here we characterize two carbapenem-resistant *A. bereziniae* isolates. **Materials & methods:** Isolates were obtained from environmental and clinical samples. Carbapenemases were searched by phenotypic, biochemical and PCR assays. Clonality was studied by ApaI-PFGE and genetic location for carbapenemase genes were assessed by I-CeuI and S1 hybridizations. **Results:** Isolates were not clonally related but both produced the 'exclusively Portuguese' IMP-5, with the clinical isolate also producing an OXA-58. The carbapenemase genes were plasmid located. **Conclusion:** Our results emphasize the role of non-*baumannii Acinetobacter* species as important reservoirs of clinically relevant resistance genes that could also contribute to their emergence as nosocomial pathogens.

KEYWORDS
- *Acinetobacter bereziniae*
- carbapenem resistance
- IMP-5 • integron
- metallo-β-lactamase
- oxacillinase

Metallo-β-lactamases (MBLs) currently represent an important and emerging mechanism of acquired-carbapenem resistance in Gram-negative bacteria, responsible for difficult-to-treat nosocomial infections, and are consequently associated with both high morbidity and mortality rates. Enzymes belonging to VIM and IMP families are among the most prevailing MBLs, rapidly growing and conferring the greatest clinical threat. Thus far, the IMP-5 MBL has only been described in Portugal, initially in an *Acinetobacter baumannii* clinical isolate belonging to the sequence type (ST) 120 and posteriorly in *Pseudomonas aeruginosa* clinical isolates [1–3]. The bla_{IMP-5} gene was described as part of the class 1 integron In*76*, located on a Tn*402*-like transposon, with two miniature inverted-repeat transposable elements (MITE)-like flanking the 5′CS to 3′CS regions [1,2]. This structure, also associated with other resistance determinants, such as bla_{PER-1} in *Acinetobacter johnsonii*, represents a mobilizable unit which might be responsible for intra- and interspecies transfer, via transposition or homologous recombination [2,3]. Notwithstanding, the reservoir of the 'exclusively Portuguese' bla_{IMP-5} gene is yet to be known.

Within the last decades, *Acinetobacter* species have, surprisingly and successively, evolved from traditionally harmless organisms into important nosocomial pathogens exhibiting high levels of antibiotic resistance, including extreme drug resistance [4,5]. *Acinetobacter bereziniae* (formerly

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Acinetobacter genomospecies 10) is presently considered one of the non-*baumannii Acinetobacter* emerging pathogenic species [6]. Descriptions of this species include vegetable samples [7], fecal carriage among healthy humans [8] and different human clinical specimens, suggesting its wide distribution [9]. More recently, it has also been associated with chronic obstructive pulmonary disease, urinary tract infection, pneumonia, sepsis and bacteremia [6,10–11]. Reports on carbapenem resistance in *A. bereziniae* were previously associated with the production of IMP-1 and IMP-19, SIM-1, VIM-2 and NDM-1 MBLs [10–13]. Additionally, two variants of the intrinsic oxacillinase OXA-228-like, OXA-229 and OXA-257, able to confer carbapenem resistance when overexpressed, were recently described in *A. bereziniae* [14,15].

This report aims the characterization of two *A. bereziniae* isolates obtained in the same hospital, from the water of a newborn incubator's humidity chamber and from a blood culture, which exhibited an unusual multidrug resistance phenotype, including resistance to carbapenems.

Materials & methods

Two *A. bereziniae* isolates, obtained at an interval of 4 years in the same University Hospital in Portugal, were studied. *Acinetobacter bereziniae* HGSA93 was collected in 2008 from the water reservoir of a new-born's incubator humidity system, while *A. bereziniae* HGSA593 was obtained in 2012 from a blood culture of a hospitalized patient with chronic renal disease. Isolates were initially identified as *Acinetobacter lwoffii* by the vitek 2 system. Further identification included *rpoB* gene partial sequencing [16] and PCR searching for *Acinetobacter* species-specific oxacillinase, OXA-228-like, using previously described primers [14].

Antibiotic susceptibility testing was performed by disc diffusion method and E-test following CLSI guidelines [17]. Carbapenemase production was assessed by the Blue-Carba test [18]. Furthermore, isolate crude extracts were used to conduct a bioassay with imipenem (with and without EDTA).

Acquired carbapenemase genes (MBL and carbapenem-hydrolyzing class D β-lactamases [CHDL]) were searched by PCR using primers and conditions as previously described [1,19], and confirmed by sequencing. Class 1 integrons were searched with primers directed for 5′CS and 3′CS regions [20] and confirmed by sequencing. Additionally, association of the MBL genes with the Tn*402*-like transposon (GenBank accession number JF810083) was investigated by PCR mapping with a combination of primers for the MBL gene [1], *orf5* [21] and MITEs sequences (MITE_Fw_2: 5′-GATAACCAATCCATTTATGACA-3′; MITE_Rv_2: 5′-TGACTGACCATTAAAGTCTCAA-3′).

The carbapenemase genes location was assessed by hybridization of I-*Ceu*I- and S1-digested genomic DNA with specific probes for the carbapenemases and 16S rRNA genes. Plasmid characterization was performed using the PCR-based replicon typing scheme for *A. baumannii* plasmids [22].

Transfer of carbapenemase genes was attempted by conjugation with rifampicin-resistant mutant of *A. baumannii* ATCC 17978 (kindly provided by Dr Ruth Hall), and by electrotransformation with the plasmid DNA from HGSA593 isolate, using *Acinetobacter baylyi* ADP1 (kindly provided by Dr German Bou), as the recipient strain. Selection was performed on Mueller-Hinton agar plates supplemented with imipenem (0.5 mg/l) and rifampicin (100 mg/l) for conjugation assays and only with imipenem (0.5 mg/l) for electrotransformation. Presumptive transformants were confirmed by searching carbapenemases genes by PCR.

Clonal relatedness for the two isolates was assessed by genomic DNA macrorestriction with

Table 1. Molecular characterization of *A. bereziniae* isolates included in this study.

Isolate	Year	Source	Carbapenemase genes (plasmid size, Kb)	Class 1 integron content (size, Kb)	Antibiotic resistance profile (MIC, mg/l)
HGSA93	2008	Water from a newborn incubator humidity system	bla_{IMP-5} (~310)	bla_{IMP-5} (1.5)	IMP (>32); MEM (>32); ATM (32); FEP (>32); CAZ (>256); PTZ (>256); K
HGSA593	2012	Blood culture (inpatient with chronic renal disease)	bla_{IMP-5} (~200), bla_{OXA-58} (~90)	bla_{IMP-5}- bla_{OXA-4} (2.5), *aacA4* (1.2)	IMP (**>32**); MEM (**>32**; 12); ATM (**32**); FEP (**32**); CAZ (**>256**); PTZ (**>256**); K; CN; TOB

β-lactam MICs are presented; resistance transferred by electrotransformation is bold (when only bla_{OXA-58} was transferred) or bold and underlined (when both bla_{IMP-5} and bla_{OXA-58} were transferred).
ATM: Aztreonam; CAZ: Ceftazidime; CN: Gentamicin; FEP: Cefepime; IMP: Imipenem; K: Kanamycin; MEM: Meropenem; PTZ: Piperacillin-tazobactam; TOB: Tobramycin.

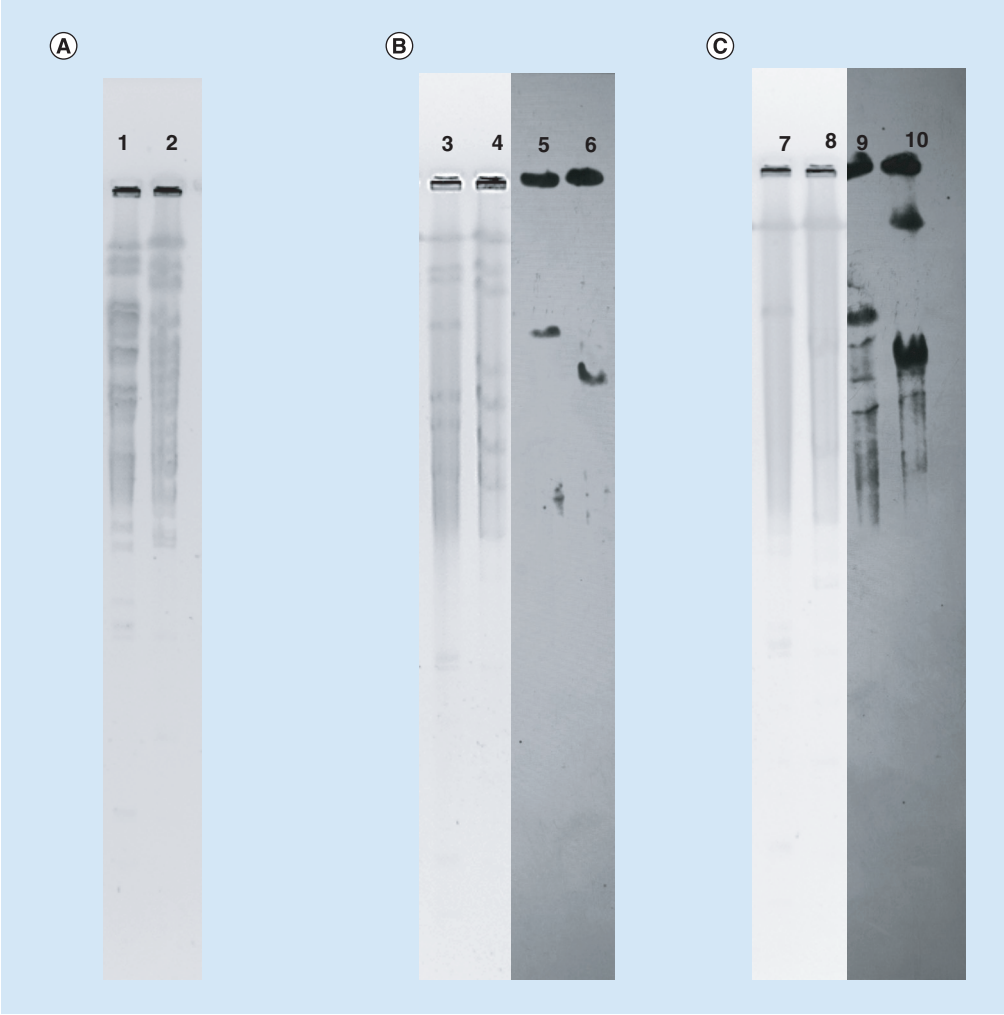

Figure 1. Apa I-, I-Ceu I- and S1-PFGE patterns from *A. bereziniae* isolates. (A) Apa I-PFGE, lane 1 HGSA93, lane 2 HGSA593; **(B)** I-Ceu I- PFGE and respective southern blot, lane 3 HGSA93, lane 4 HGSA593, lane 5 HGSA93 IMP-5 hybridization, lane 6 HGSA593 IMP-5 hybridization; S1-PFGE and respective Southern blot, lane 7 HGSA93, lane 8 HGSA593, lane 9 HGSA93 IMP-5 hybridization, lane 10 HGSA593 IMP-5 hybridization.

*Apa*I followed by pulsed-field gel electrophoresis (PFGE).

Results & discussion

HGSA93 and HGSA593 isolates belonged to *A. bereziniae* species, according to the *rpoB* partial gene sequencing (with both displaying 98% nucleotide identity with *A. bereziniae* type strain CIP 70.12^{T}, GenBank accession number DQ207475.1). The $bla_{OXA-228}$, an intrinsic oxacillinase gene from *A. bereziniae* species, was detected by PCR (100% homology with $bla_{OXA-228}$ gene described in *A. bereziniae* strain CIP 70.12^{T}GenBank accession number JQ422053.1) and presented the same promoter sequences (TTCAAT and TGGTAT for the -35 and -10 sequences, respectively) that have been related with its low expression [14,15].

Both isolates presented an unusual antibiotic resistance profile that included resistance to all tested β-lactams. HGSA93 remained susceptible to aminoglycosides (except kanamycin), while HGSA593 was only susceptible to amikacin **(Table 1)**. Blue-carba test revealed the presence of carbapenemase activity and the bioassay with EDTA strongly suggested the presence of an MBL, further identified as IMP-5 in both isolates by PCR and sequencing. Search for acquired CHDL revealed that HGSA593 also harbored bla_{OXA-58}, which is a carbapenemase that had

been endemic within this hospital among *A. baumannii* clinical isolates and disappeared after 2006 [5]. Sequencing of the bla_{IMP-5}-encoding class 1 integron, from HGSA93, revealed 100% homology with the previously described In*76* [1] and its nucleotide sequence was deposited in the GenBank database (accession number KF732850.1). PCR mapping showed that this genetic platform was also flanked by MITE structures, a fact that might justify bla_{IMP-5} gene mobilization [2,3]. Curiously, sequence analysis indicated the presence of a weak -35 promoter (TTGACA instead of TTGATA) upstream bla_{IMP-5} gene [23], contrary to what was expected by the observed high carbapenem resistance level **(Table 1)**. HGSA593 harbored similar genetic vicinity for bla_{IMP-5}, but the integron content included an extra gene cassette (bla_{OXA-4}) downstream bla_{IMP-5} gene. Moreover, HGSA593 harbored another class 1 integron carrying an *aacA4* gene, which might explain its additional resistance to aminoglycosides. I-*Ceu*I- and S1 hybridizations revealed that bla_{IMP-5} gene was located in a ~310 Kb plasmid for HGSA93 and in a ~200 Kb plasmid for HGSA593 **(Figure 1)** and bla_{OXA-58} was located in a ~90 Kb additional plasmid. These plasmids were untypeable using the current replicon typing scheme [22], which is now commonly reported among non-*baumannii Acinetobacter* species [24]. Conjugation attempts were unsuccessful, but plasmids from HGSA593 were transferred and expressed in *A. baylyi* ADP1, with transformants exhibiting resistance to β-lactams, including carbapenems **(Table 1)**.

Curiously, *Apa*I-PFGE assay revealed that *A. bereziniae* isolates were unrelated **(Figure 1)**, suggesting that this species might easily acquire different plasmids and act as reservoir of clinically relevant resistance genes.

Conclusion

Our data highlight the ability of non-*baumannii Acinetobacter* species to acquire relevant resistance genes which could contribute for their emergence as nosocomial pathogens. Furthermore, cumulative findings suggest that different species of *Acinetobacter* seem to constitute important reservoirs of resistance genes outside clinical settings. In this study, although the first *A. bereziniae* isolate was not associated with any infection episode, its detection in the newborn's incubator humidity chamber, along with the second bloodstream isolate and previous works on this species, should indicate the possibility of 'environmental' species emerge as etiologic agents of nosocomial infections, particularly within vulnerable populations.

Finally, we believe that further studies focused on the local microbial ecology of carbapenemase producers are imperative for the full understanding of the observed endemicity of IMP-5 in our country and for the effective prevention of its dissemination.

Financial & competing interests disclosure

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EXECUTIVE SUMMARY

- We extended the species reservoir of the 'exclusively Portuguese' IMP-5 carbapenemase.
- Environmental *Acinetobacter bereziniae* isolates could act as reservoirs for bla_{IMP-5}.
- Newborn incubator systems could be a source of carbapenemase producing *Acinetobacter* spp.
- We confirm the pathogenic potential of carbapenem-resistant *A. bereziniae*.

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