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Multiple clones of metallo-β-lactamase-producing *Acinetobacter ursingii* in a children hospital from Argentina

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ABSTRACT

Acinetobacter spp. are opportunistic pathogens being *A. baumannii* the most frequently identified in nosocomial settings. *A. ursingii* was mainly described as causing bacteremia and outbreaks in neonatal intensive care units. Ten *A. ursingii* isolates were recovered from rectal swab screening for carbapenemase-producing bacteria between June 2013 and December 2015 from a children hospital in Argentina. All ten isolates were metallo-β-lactamase-producing, nine were positive for bla_{IMP-1} and one for bla_{NDM-1}. IMP-positive isolates were also positive for bla_{OXA-58} gene. All isolates were susceptible to ciprofloxacin, colistin and minocycline, and nine were susceptible to ampicillin-sulbactam and gentamicin. Two *A. ursingii* displayed high level of resistance to aztreonam associated with $bla_{CTX-M-15}$ in one isolate, and bla_{VEB-1} in the other. Eight *Sma*I-PFGE patterns were recognized. We evaluated the usefulness of *Acinetobacter* MLST-Pasteur scheme, to analyse *A. ursingii* isolates, however the *rpo*B gene was not amplified. A new set of primers were designed for specific amplification and sequencing, allowing the analysis of *rpo*B gene for this species. New alleles and the sequence types 748, 749, 750, 751, 993, 1186, 1187, and 1189 were included at the *Acinetobacter* MLST-Pasteur database. Those isolates showing related PFGE patterns were assigned to the same ST. To the best of our knowledge, this is the first report of MBL-producing *A. ursingii* in Argentina. The inclusion of *A. ursingii* species to the *Acinetobacter* MLST-Pasteur scheme allows deeper molecular characterization and a better understanding about the epidemiology of this germen.

1. Introduction

Acinetobacter genus comprises a broad group of biochemically and physiologically versatile bacteria inhabiting different natural ecosystems. A total of 62 different genomospecies were currently described (http://www.bacterio.net/acinetobacter.html; September 2018). Reliable phenotypic identification of *Acinetobacter* species is a challenge, being genotypic methods a helpful tool for the precise identification, including sequencing of the 16S rRNA (*rrs*) gene and housekeeping genes such as *gyr*B and *rpo*B(Dortet et al., 2006). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-ToF MS) showed to be a good method for identification of *Acinetobacter* species(Hsueh et al., 2014).

Acinetobacter spp. are opportunistic pathogens being *A. baumannii* the most frequently identified nosocomial pathogen in the genus(Peleg et al., 2012). Others species occasionally cause infections in humans including *A. nosocomialis*, *A. pittii*, and less frequently *A. ursingii* and *A. haemolyticus*(Turton et al., 2010). *A. ursingii* was found to be a causative agent of bacteremia in susceptible hosts, but also causing other kind of infections and outbreaks in neonatal intensive care units(Chiu et al., 2015; Dortet et al., 2006; Máder et al., 2010; Turton et al., 2010).

Acquired carbapenem resistance in *Acinetobacter* spp. is mainly driven by class D β-lactamases, while metallo-β-lactamases (MBL) are increasingly being detected in this genus (Alkasaby and El Sayed Zaki, 2017; Cornaglia et al., 2011; Evans and Amyes, 2014). MBL production in *A. ursingii* is rare and only four isolates were reported in Japan and

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Netherlands (Endo et al., 2012; Sieswerda et al., 2017). We describe here the characterization of ten MBL-producing clinical isolates of *A. ursingii* from a children hospital in Argentina. We also adapted and evaluated the usefulness of *Acinetobacter* multilocus sequence-typing (MLST), Pasteur scheme, to analyse *A. ursingii* isolates.

2. Materials and methods

Ten *A. ursingii* clinical isolates were recovered from rectal swab screening for carbapenemase-producing bacteria, one per patient, between June 2013 and December 2015 in a children hospital from Argentina. The isolates are submitted to the National Reference Laboratory (NRL) for further characterization. Bacterial identification was performed using the MALDI-ToF MS Bruker Biotyper 3.0 system (Bruker Daltonics, Germany).

Susceptibility testing was performed by agar dilution and interpreted according to CLSI guidelines for *Acinetobacter* spp.(Clinical and Laboratory Standards Institute (CLSI)., 2018). Carbapenemase activity was evaluated by Blue-Carba test(Pasteran et al., 2015), and MBL-screening was performed using double disc synergy test between imipenem or meropenem and EDTA discs. Carbapenems and combination with EDTA (0.4 mM) further confirmed by microdilution assay as recommended(Lee et al., 2003). Aztreonam minimal inhibitory concentration (MIC) was evaluated as a screening for extended-spectrum β-lactamase (ESBL) production.

PCR was performed under standard conditions to detect ESBLs and carbapenemases genes including: $bla_{\text{CTX-M}}$, bla_{VEB}, bla_{PER}, bla_{IMP}, bla_{NDM}, bla_{VIM}, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, and $bla_{\text{OXA-143}}$. DNA sequence was performed using the BigDyeTM Terminator methodology (Applied Biosystems/Perkin Elmer, Foster City, CA) and analysed in an ABI 3500 Genetic Analyzer (Applied Biosystems).

Genetic relationship was evaluated by pulsed-field gel electrophoresis (PFGE) using *Sma*I restriction enzyme. DNA fragments were separated in a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, CA, US) using 5 s and 35 s as initial and final switching times, respectively, during 24 h.

MLST was performed using the Pasteur scheme as described at http://pubmlst.org/abaumannii/ with the following modification: a set of primers for amplification/sequencing of *rpo*B gene were designed, rpoBAur-F 5′- GGTGAAATGACAGAGAACCA-3′, and rpoBAur-R 5′-GAGTCTTCGTAGTTATAACC-3′, yielding a 1076 bp amplicon. MLST sequences were uploaded to *Acinetobacter baumannii* PubMLST and new allelic and sequence type were kindly assigned. Ninety two concatenated sequences of *A. baumannii* (19), *A. pittii* (16), *A. nosocomialis* (13), *Acinetobacter calcoaceticus* (12), *Acinetobacter seifertii* (11), *A. ursingii* (9) *Acinetobacter* genomospecies 13BJ (6), *Acinetobacter* genomospecies 15 (2), *A. soli* (2), *Acinetobacter bereziniae* (1) and *Acinetobacter junnii* (1) deposited at public databases (PubMLST, Genbank) were included for the phylogenetic analysis. Phylogenetic analysis was inferred using MEGA6 by maximum likelihood method based on the Jukes-Cantor model and a bootstrap of 1000 replicates (Tamura et al., 2013).

3. Results

Ten *A. ursingii* isolates were recovered from rectal screening from 6 months to 16 years-old patients from a children hospital during the period June 2013 to December 2015. Fecal carriage is weekly evaluated in patients from intensive care unit, and immunocompromised. Those derived from other institutions for neonatal therapy or cardiology units, are also screened. In these patients the rate of fecal carriage of MBL-producing gram-negative bacilli during the period 2013–2015 was 3.72% (46/1236). Patients were hospitalized several days or months apart from each other, six of them were female (Table 1). Five children were at two units (U9 and U10) for immunocompromised patients, and the remaining five in a unit (U4) for patient with liver-related diseases.

Table 1
Epidemiological data, susceptibility profiles, antimicrobial resistance genes and molecular typing of *A. ursingii* isolates.

Strain no.	Age	Sex	Date of isolation	Ward	Susceptibility profile (MIC)								β-Lactamases	PFGE type	MLST (ST)
					IMP	I + E	MEM	SAM	AMK	GEN	CIP	MIN	COL			
M15845	16 y	F	06–05-13	U4	≥ 32 (R)	≤0,12	16 (R)	0,5 (S)	64 (R)	2 (S)	0,12 (S)	0,06 (S)	0,25 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	A	748
M15846	NA	F	07–17-13	U10	4 (I)	≤0,12	8 (R)	0,25 (S)	32 (I)	0,5 (S)	0,12 (S)	0,06 (S)	0,5 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	C	751
M15976	3 y	M	10–01-13	U4	8 (R)	≤0,12	16 (R)	0,5 (S)	32 (I)	1 (S)	0,25 (S)	0,06 (S)	0,5 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	D	749
M17068	5 y	M	11–08-13	U9	≥ 32 (R)	≤0,12	≥ 32 (R)	4 (S)	64 (R)	2 (S)	0,12 (S)	0,06 (S)	0,5 (S)	$bla_{\text{IMP}-1}$,$bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-58}}$	E	750
M17739	11 y	F	09–10-14	U9	4 (I)	≤0,12	4 (I)	0,25 (S)	64 (R)	2 (S)	0,12 (S)	0,12 (S)	0,5 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	F	1186
M19113	16 y	M	02–26-15	U9	≥ 32 (R)	≤0,12	≥ 32 (R)	1 (S)	64 (R)	2 (S)	0,12 (S)	0,12 (S)	0,25 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	A	748
M19244	14 y	F	04–30-15	U4	8 (R)	≤0,12	16 (R)	0,25 (S)	32 (I)	1 (S)	0,25 (S)	0,12 (S)	0,5 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	B	993
M19235	16 y	F	05–07-15	U4	4 (I)	≤0,12	4 (I)	0,25 (S)	32 (I)	0,5 (S)	0,12 (S)	0,06 (S)	0,25 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	G	1187
M19540	6 m	F	08–20-15	U4	4 (I)	≤0,12	4 (I)	0,25 (S)	32 (I)	1 (S)	0,12 (S)	0,06 (S)	0,5 (S)	$bla_{\text{IMP}-1}$, $bla_{\text{OXA-58}}$	B	993
M19845	10 y	M	12–16-15	U9	≥ 32 (R)	≤0,12	16 (R)	64 (R)	32 (I)	8 (I)	0,5 (S)	0,06 (S)	0,5 (S)	$bla_{\text{NDM}-1}$,$bla_{\text{VEB-1a}}$	H	1189

Abbreviation: resistant, R; susceptible, S; intermediate, I; female, F; male M; imipenem, IMP; imipenem plus EDTA, I + E; meropenem, MEM; ampicillin-sulbactam, SAM; amikacin, AMK; gentamicin, GEN; ciprofloxacin, CIP; minocycline, MIN; colistin, COL; not available, NA. Liver Unit, Unit 4 (U4); Immunocompromised Units, Units 9 and 10 (U9, U10).

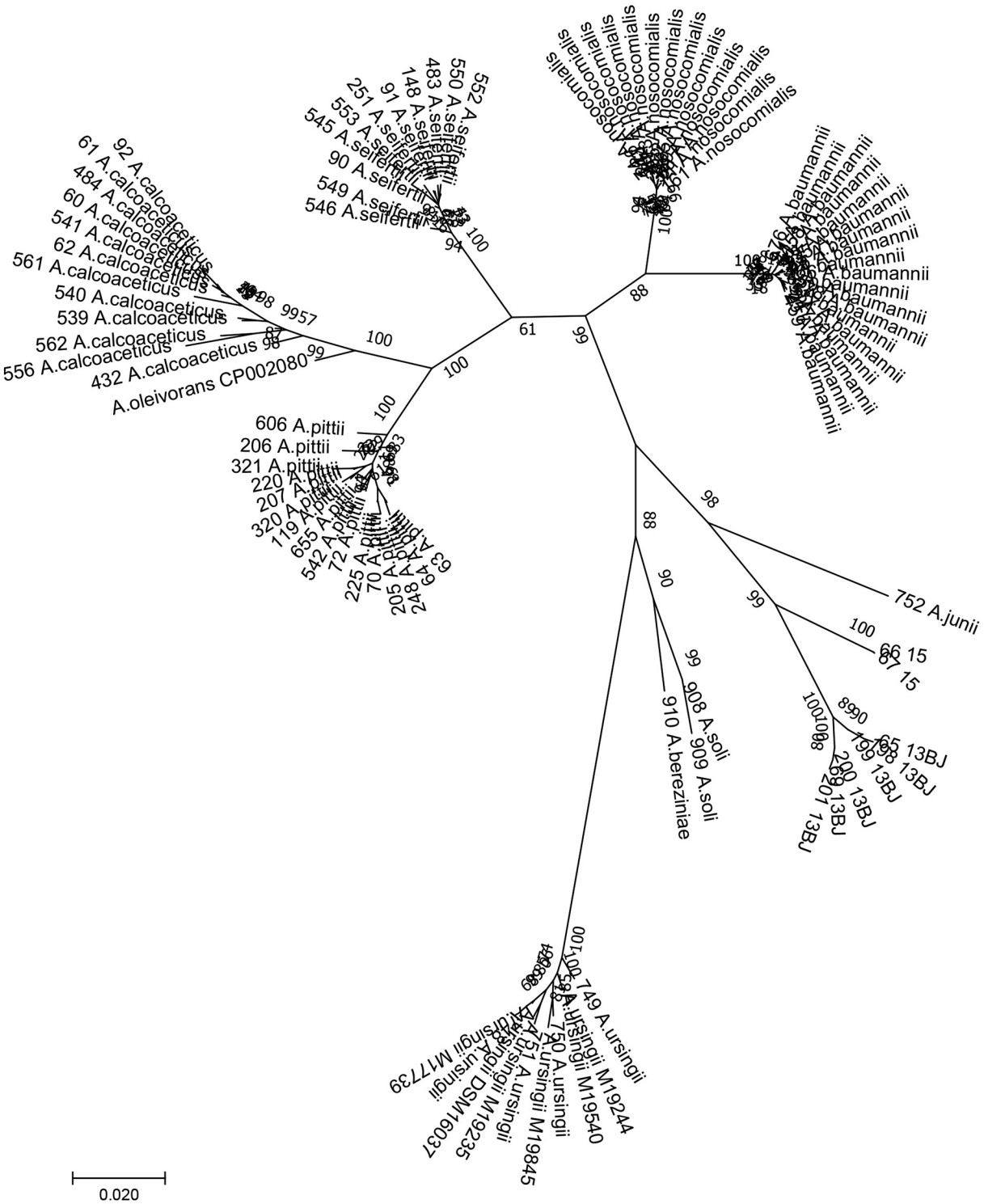

Fig. 1. Phylogenetic analysis of 92 concateneted sequences (2976 bp) of eleven *Acinetobacter* species.

The isolates were identified as *A. ursingii* by MALDI-ToF MS yielding scores > 2.0.

Positive results for Blue-Carba test was observed in < 1 h for all strains. MIC to imipenem and meropenem were categorized as intermediate or resistant in all isolates, 3 or more two-fold dilution decrease was observed when imipenem was combined with EDTA (Table 1). All isolates were susceptible to ciprofloxacin, colistin and minocycline. All but one were susceptible to ampicillin-sulbactam and gentamicin. Two isolates displayed high level of resistance to aztreonam (≥ 256 μg/ml) due to the presence of ESBL, *A. ursingii* M17068 was positive for $bla_{\text{CTX-M-15}}$ gene, while isolate M19845 harbors $bla_{\text{VEB-1}}$ (Table 1).

Nine *A. ursingii* isolates were positive for $bla_{\text{IMP-1}}$, while the remaining M19845 harbors $bla_{\text{NDM-1}}$. IMP was in a class 1 integron with a $bla_{\text{IMP-1}}$-*aac6-II-aadA4* cassettes array. The $bla_{\text{NDM-1}}$ was located in a IS*Aba125*-$bla_{\text{NDM-1}}$-ble_{MBL}-*trpF-tat* structure. All nine IMP-positive isolates were also positive for $bla_{\text{OXA-58}}$ gene. All ten isolates were negative for $bla_{\text{OXA-23}}$,$bla_{\text{OXA-24}}$ and $bla_{\text{OXA-143}}$ genes.

Eight PFGE patterns were defined among the ten *A. ursingii* isolates

(Table 1). Isolates M19244 and M19540 showing related PFGE patterns were recovered four months apart, while M15845 and M19113 also related between them were isolated almost 21 months apart. These results suggested the presence of multiple clones but also the ability of this specie to survive for long periods of time in the hospital setting.

Considering that the *A. baumannii* MLST-Pasteur scheme is including other non-*baumannii* species, we evaluated the ability of the protocol to characterize the *A. ursingii* isolates. Six out of seven genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*) were amplified and sequenced using the standard procedure. The *rpoB* gene could not be amplified using different PCR conditions. Therefore, a new set of primers (rpoBAur-F/rpoBAur-R), yielding a 1076 bp fragment, were designed for specific amplification and sequencing. All new gene alleles and eight new sequence types (748, 749, 750, 751, 993, 1186, 1187, and 1189) were uploaded to the *A. baumannii* MLST-Pasteur database (Table 1). Those isolates genetically related by PFGE, clones A and B, showed the same sequence type, ST748 and ST993 respectively (Table 1).

The phylogenetic analysis of a 2976 bp concatenated sequences, including 94 STs from 12 *Acinetobacter* species, grouped the *A. ursingii* sequences in a compact cluster (Fig. 1). A neat discrimination for other species was also observed, however for some of them, like *A. soli* and *A. bereziniae*, it was not so clear, may be due to the limited number of available sequences for these specie. Comparison of the phylogenies obtained using each gene individually showed strong congruence for *A. ursingii* specie among the seven genes. (data not shown).

4. Discussion

A. baumannii is the most clinically relevant *Acinetobacter* species, causing nosocomial infections and exhibiting resistance to multiple drugs, including carbapenems. An increased number of reports of nosocomial and community infections caused by *Acinetobacter* non-*baumannii* species, including *A. ursingii* were observed. Different type of *A. ursingii* infections were reported although bacteremia has been the most frequent(Chiu et al., 2015; Máder et al., 2010; Salzer et al., 2016; Turton et al., 2010). Moreover two outbreaks in neonatal wards affecting 5 and 3 patients, respectively, were reported(Kilic et al., 2008; Máder et al., 2010). In general *A. ursingii* is susceptible to most clinically relevant drugs(Chiu et al., 2015; Dortet et al., 2006; Horii et al., 2011; Máder et al., 2010), nevertheless recently two reports of carbapenemase-producing *A. ursingii*, bla_{IMP-1} (1) or bla_{IMP-4} (3) genes, were published(Endo et al., 2012; Sieswerda et al., 2017). We are describing the finding of ten MBL-producing *A. ursingii* isolates recovered from rectal swab carbapenemase-producer screening, nine were positive for IMP-1 while the remaining was NDM-1-positve. A high level of resistance to aztreonam observed in two strains was associated with $bla_{CTX-M-15}$ and bla_{VEB-1} genes. These ESBLs genes were occasionally described in *Acinetobacter* species, but not in *A. ursingii*(Chatterjee et al., 2016; Pasterán et al., 2006). The nine bla_{IMP-1}*A. ursingii* described herein, remain susceptible, at least in vitro, to ampicillin-sulbactam. Sulbactam is a β-lactamase inhibitor with specific intrinsic bactericidal activity against multidrug resistant *Acinetobacter* spp. strains, including carbapenem resistant *A. baumannii* (Michalopoulos and Falagas, 2010).

An increasing number of *A. ursingii* clinical isolates causing different kind of infections has been observed(Chiu et al., 2015; Horii et al., 2011; Kilic et al., 2008; Máder et al., 2010; Sieswerda et al., 2017). PFGE has shown excellent results for the analysis of the genetic relationship between the isolates in epidemiologic studies at a local level. However, MLST is also a highly discriminative method for typing microorganisms which permit global epidemiologic analysis and comparison of the results obtained among different laboratories around the world. Therefore, based on the need of a MLST scheme for epidemiological studies of *A. ursingii*, we designed a set of primers to amplify and sequence the *rpoB* gene. This allows the inclusion of *A. ursingii* species to the Pasteur-MLST scheme for future epidemiological studies, even more these primers also enable the amplification and sequencing of *A. junnii* species (Personal communication). Additionally the MLST-Pasteur scheme has shown a high potential to discriminate diverse genomo species of *Acinetobacter* genus.

Resistance to carbapenems mediated by MBL or OXA carbapenemases was described in clinical isolates of different species of *Acinetobacter*, which emphasizes the importance of accurate epidemiological investigation of non-*A. baumannii* species, including *A. ursingii*. To the best of our knowledge, this is the first report of MBL-producing *A. ursingii* outside Japan and Netherlands. The finding of strains with a heterogeneous genetic background could suggest horizontal mobilization of MBL indicating that *A. ursingii* may act as an unsuspected reservoir of carbapenemases and highlights the relevance of epidemiological surveillance of nonclassic pathogens.

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Conflict of interest

None to declare.

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