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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Genotypic characterization and clonal relatedness of metallo-β-lactamase-producing non-fermentative gram negative bacteria in the first 5 years of their circulation in Paraguay (2011-2015)

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Abstract

Pseudomonas aeruginosa and species of *Acinetobacter calcoaceticus-baumanii* complex are multiresistant intrahospital opportunistic pathogens, able to acquire carbapenemases and produce outbreaks with high morbidity and mortality. *Pseudomonas putida* has also emerged with similar characteristics. The aim of this research was to characterize the Metallo-β-lactamases (MBLs) detected by surveillance in Paraguay in the first 5 years of their circulation in hospitals. The coexistence of KPC and OXA-type carbapenemases was also investigated. 70 MBL-producing strains from inpatients were detected from clinical samples and rectal swab from 11 hospitals. The strains were identified by manual, automated, and molecular methods. Antimicrobial susceptibility was studied by Kirby-Bauer and automated methods, while colistin susceptibility was determined by broth macrodilution. MBLs were investigated by synergy with EDTA against carbapenems and PCR, and their variants by sequencing. KPC and OXA-carbapenemases were investigated by PCR. Clonality was studied by pulsed-field gel electrophoresis (PFGE). The results demonstrated the circulation of $bla_{\text{VIM-2}}$ (60%), $bla_{\text{NDM-1}}$ (36%), and $bla_{\text{IMP-18}}$ (4%). The MBL-producing species were *P. putida* (45.7%), *P. aeruginosa* (17.2%), *A. baumannii* (24.3%), *A. pittii* (5.7%), *A. nosocomialis*, (4.3%) *A. haemolyticus* (1.4%), and *A. bereziniae* (1.4%). PFGE analysis showed one dominant clone for *A. baumannii*, a predominant clone for half of the strains of *P. aeruginosa*, and a polyclonal spread for *P. putida*. In the first 5 years of circulation in Paraguay, MBLs were disseminated as unique variants per genotype, appeared only in *Pseudomonas* spp. and *Acinetobacter* spp., probably through horizontal transmission between species and vertical by some successful clones.

Keywords MBL · *Pseudomonas aeruginosa* · *Pseudomonas putida* · *Acinetobacter* spp. · PFGE

Introduction

Gram-negative bacteria resistant to carbapenems, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, are emerging causes of health care-associated infections and of global public health concerns [1]. *Pseudomonas putida* has also emerged as a multiresistant nosocomial opportunistic pathogen [2–4]. The most widespread mechanism of carbapenem resistance is the production of carbapenemases [5]. These enzymes can be classified according to their molecular characteristics into classes A, B, and D. The classes A (ex. KPC) and D (the OXAs) carbapenemases include enzymes that hydrolyze their substrates forming an acyl-enzyme through a serine of the active site, while the class B β-lactamases are metalloenzymes or metallo-β-lactamases (MBLs) that use at least one zinc ion from the active site to facilitate the hydrolysis of beta-lactam. The most important types of MBLs due to epidemiological spread and clinical relevance are IMP, VIM [6], and NDM [7]. SPM is characteristic of *P. aeruginosa* in Brazil [8].

The surveillance of MBLs dissemination in Paraguay began in 2009, with 21 laboratories members of the survaillance of the Antimicrobial Resistance Network dependent

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on the Central Laboratory of Public Health—Ministry of Public Health and Social Welfare. The aim of this study was to characterize the MBLs detected among *Pseudomonas* spp. and *A. calcoaceticus-baumannii* complex isolates in Paraguay in the first 5 years of their circulation in hospitals. The characterization involved identification of species and clonality of MBL-producing strains, determination of genotype and subtype of MBL, and coexistence of KPC and OXA-type carbapenemases in the strains.

Materials and methods

From November 2009 to December 2015, clinical carbapenem-resistant isolates of *Pseudomonas* spp. and *A. calcoaceticus-baumannii* complex collected from hospitalized patients from 11 Paraguayan hospitals were sent to the Central Laboratory of Public Health in Paraguay and submitted to phenotypic detection of MBL by inhibition with EDTA [9]. Only one isolate for each patient was included for further studies, except in one case where two isolates with different resistance phenotypes, recovered from tracheal secretion within 4 months of difference were included.

Species identification was performed by classical and automated methods (Vitek 2 Compact–Biomerieux, France). For *A. calcoaceticus-baumannii* complex, in addition to this a multiplex PCR was performed to detect the presence of $bla_{\text{OXA-51}}$ according to the protocol described in the literature, which allows the detection of $bla_{\text{OXA-23}}$; $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ [10]. The $bla_{\text{OXA-51}}$ negative strains were subjected to species-specific PCR for *Acinetobacter pittii* and *Acinetobacter nosocomialis* according to published protocol [11]. Isolates of *Acinetobacter* spp. that were negative for $bla_{\text{OXA-51}}$ and species-specific PCR were subjected to rpoβ amplification PCR [12] and sequencing.

Antimicrobial susceptibility tests were performed by automated method (Vitek 2 Compact-Biomerieux, France). Susceptibility to colistin was determined by broth macrodilution [13]. The results were interpreted according to the CLSI manual [13].

On surveillance, routine genotyping of MBLs was performed by PCR for bla_{IMP}, $bla_{\text{VIM,}}$ and bla_{NDM} according to the protocol of the regional reference laboratory [14], however, for this work, the genotypes were verified according to multiplex PCR [15]. The bla_{SPM}—for *P. aeruginosa* strains—was investigated according to the published protocol [16]. The detection of the Serino-carbapenemase gene bla_{KPC} was performed for *P. aeruginosa* isolates [17].

To determine the variant of each MBL genotype, PCR and subsequent sequencing were made according to the published protocol for IMP and VIM [18]. To determine NDM variants, primers designed by the Laboratório de Pesquisa em Infecção Hospitalar (LAPIH) from the Oswaldo Cruz Institute-Fiocruz, Brazil [19] were used (Table 1).

Macrogen Inc.-Korea performed sequencing. Using the Genbank database through the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) the sequences were analyzed, and further studied by multiple alignments with BioEdit software comparing them with reference sequences available from NCBI.

To perform PFGE, the PulseNet protocol was used [20]. Restriction enzymes (Invitrogen by Thermo Fisher Scientific) and PFGE running conditions for *P. aeruginosa*, *P. putida* and *Acinetobacter* spp. (protocols not included in PulseNet Network) were taken from the scientific literature [21, 22] with adjusted running times, 18.8 h for *Acinetobacter* spp. and 18.7 h for *Pseudomonas* spp. *Salmonella* serovar Braenderup H9812 (restricted with *Xba* I) was used as the standard strain. The images of gels were analyzed (Gel Doc EZ Imager-BioRad) using Gel Compare II Software (Applied Maths) to obtain the relationship between patterns. We determine clusters using the Unweighted Pair Group Mean (UPGMA) method, with 90% Dice's similarity coefficients among patterns to define each clonal group.

Results

Out of 1289 clinical carbapenem-resistant isolates of *Pseudomonas* spp. and *A. calcoaceticus-baumannii* complex studied between 2009 and 2015, 70 strains were MBL positive (5.4%). The first MBL-positive strain appeared in 2011 and the prevalence was 5.8% in 2011, 2.4% in 2012, 3.2% in 2013, 5.9% in 2014, and 12.8% in 2015 (see Fig. 1).

The bla_{VIM} ($n = 60\%$) was the most frequently MBL gene detected, followed by bla_{NDM} ($n = 36\%$) and bla_{IMP} ($n = 4\%$). No bla_{SPM} or bla_{KPC} genes were detected. Only the $bla_{\text{OXA-51}}$ was detected in 65% of *Acinetobacter* spp. isolates (17 strains), since in *A. pittii* (4 strains), *A. nosocomialis* (3 strains), *A. bereziniae* (1 strain), and *A. haemolyticus* (1 strain) OXA-type carbapenemases were not detected.

Regarding geographical origin, all the positive strains came from the Capital and Central Department of Paraguay. Concerning the source, 67% came from several clinical samples, and 33% from rectal swabs. bla_{NDM} was detected mainly in rectal swabs (64%), bla_{VIM} was mainly associated with clinical samples (83.3%) and the only three bla_{IMP} were isolated from blood culture, cerebrospinal fluid, and pleural fluid, respectively.

About the association between MBL genotype and bacterial species, bla_{VIM} was detected in *P. aeruginosa*

Table 1 Primers used in this work

	Primer	Gene	Sequence 5′→3′	Amplicon size (pb)	Ref
A. pittii	Apit-F	*rpoβ*	TGGGCAGTTACCAGATTGACCTA	147	[11]
	Apit-R		AACCAGCAGCTTCCATTTGACG		
A. nosocomialis	Anos-F	*rpoβ*	GCCGCTCGTGAACGTGTAATC	394	[11]
	Anos-R		CATCGTGTGGCATATCTTCAAC		
Amplification of *rpoB*	Ac696- F	*rpoβ*	TAYCGYAAAGAYTTGAAAGAAG	350	[12]
	Ac1093-R		CMACACCYTTGTTMCCRTGA		
MBL genotyping	VIM-F	bla_{VIM}	AGTGGTGAGTATCCGACAG	261	[15]
	VIM-R		ATGAAAGTGCGTGGAGAC		
	IMP-UF	bla_{IMP}	GGYGTTTWTGTTCATACWTCKTTYGA	404	[15]
	IMP-UR		GGYARCCAAACCACTASGTTATCT		
	NDM-F	bla_{NDM}	AGCACACTTCCTATCTCGAC	512	[15]
	NDM-R		GGCGTAGTGCTCAGTGTC		
	SPM-F	bla_{SPM}	AGACCGCGATTTCTATTCTT	505	[16]
	SPM-R		AGTTCCTTCGGCTTTATCAT		
OXA genotyping	OXA-51 F	bla_{OXA-51}	TAATGCTTTGATCGGCCTTG	353	[10]
	OXA-51 R		TGGATTGCACTTCATCTTGG		
	OXA-23 F	bla_{OXA-23}	GATCGGATTGGAGAACCAGA	501	[10]
	OXA-23 R		ATTTCTGACCGCATTTCCAT		
	OXA-24 F	bla_{OXA-24}	GGTTAGTTGGCCCCCTTAAA	246	[10]
	OXA-24 R		AGTTGAGCGAAAAGGGGATT		
	OXA 58-F	bla_{OXA-58}	AAGTATTGGGGCTTGTGCTG	599	[10]
	OXA 58-R		CCCCTCTGCGCTCTACATAC		
KPC screening	KPC-F	bla_{KPC}	AACAAGGAATATCGTTGATG	916	[17]
	KPC-R		AGATGATTTTCAGAGCCTTA		
MBL variants	VIM1-F	bla_{VIM-1}	TGTTAAAAGTTATTAGTAGTTTATTG	801	[18]
	VIM1-R		CTACTCGGCGACTGAGC		
	VIM2-F	bla_{VIM-2}	ATGTTCAAACTTTTGAGTAAG	801	[18]
	VIM2-R		CTACTCAACGACTGAGCG		
	IMP1-F	bla_{IMP-1}	ATGAGCAAGTTATCTGTATTC	741	[18]
	IMP1-R		TTAGTTGCTTGGTTTTGATGG		
	IMP2-F	bla_{IMP-2}	ATGAAGAAATTATTTGTTTTATG	741	[18]
	IMP2-R		TTAGTTACTTGGCTGTGATG		
	NDM-F	bla_{NDM}	CGAAGCTGAGCACCGCATTA	764	[19]
	NDM-R		TCAGCGCAGCTTGTCGGC		

($n=12$) and *P. putida* ($n=30$). bla_{IMP} was detected in two *P. putida* strains and one *A. pittii* strain, while bla_{NDM} appeared only in species of *Acinetobacter* spp.: *A. baumannii* ($n=17$), *A. nosocomialis* ($n=3$), *A. pittii* ($n=3$), *A. haemolyticus* ($n=1$) and *A. bereziniae* ($n=1$). Figure 2 shows the proportion of detection of each genotype with respect to the carrier species and the time. bla_{VIM} was the most prevalent genotype until 2014, mainly associated with *P. putida*.

Regarding the MBL variant genes, all bla_{NDM} sequences aligned 100% with the reference sequence bla_{NDM-1} (GenBank FN396876.1), bla_{VIM}, 38/42 strains aligned 99 to 100% with the reference sequence bla_{VIM-2} (GenBank FN396876.1) (it was not possible to determine the bla_{VIM} allelic variant from four isolates, because they were not viable at the time of sequencing). Concerning to bla_{IMP}, the sequence of one *P. putida* isolate and *A. pittii* corresponded to bla_{IMP-18}. The bla_{IMP} allelic variant of one *P. putida* was not sequenced due to lack of viability too.

Concerning resistance profile (antibiotype), it was variable, and Table 2 shows the summary. The definition of resistance classification was according to Magiorakos et al. [23]. Only colistin maintained 100% susceptibility

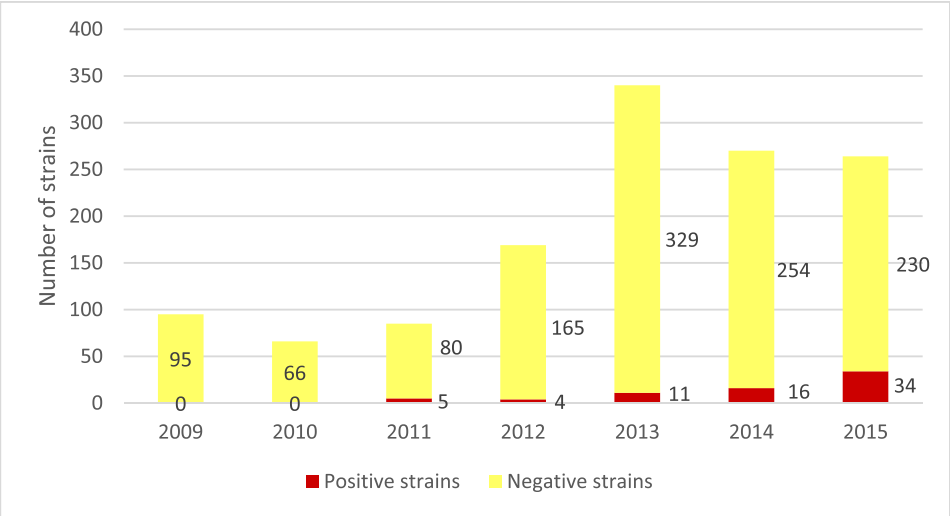

Fig. 1 Number of MBL-positive isolates out of the total strains studied per year

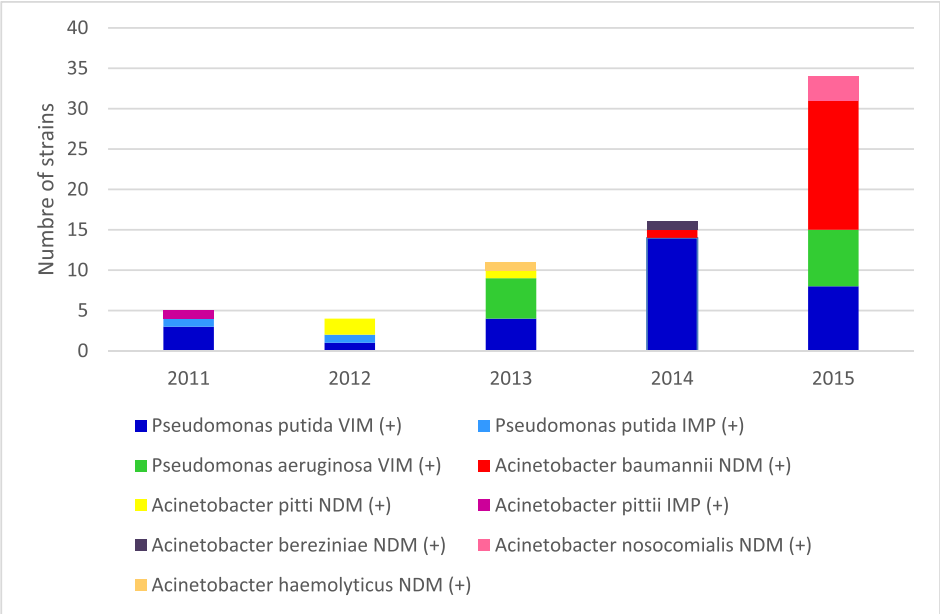

Fig. 2 Number of MBL-positive isolates per year of each bacterial species according to MBL genotype

in all isolates (MIC range ≤ 0.5 to 1 µg/mL, MIC50 and MIC90 = 0.5 µg/mL) (Fig. 3). Regarding *P. aeruginosa*, less than 50% were also susceptible to amikacin (5 strains), aztreonam (4 strains), and/or ciprofloxacin (3 strains). Concerning to *P. putida*, most of the isolates remained susceptible to amikacin (97%). All *Acinetobacter* spp. remained susceptible to tigecycline; some strains were also susceptible to amikacin (46%), gentamicin (42%), ciprofloxacin (38.5%), trimethoprim-sulfamethoxazole (35%), and ampicillin-sulbactam (11.6%). *P. putida* has a higher proportion of XDR (78%) and VIM antibiotypes II and III coincide with some of the IMP. Several of the isolates share the same pattern. The antibiotype mentioned in Table 2 is the same as the dendrograms that appear later (Figs. 4, 5, 6, 7, and 8).

About molecular typing with PFGE, VIM-2-producing *P. aeruginosa* (Fig. 4) revealed six different patterns or pulsotypes and four antibiotypes. Six isolates (50%) belong to dominant pulsotype C, including four subtypes. These strains were isolated in 2013 and 2015, came from four different hospitals and had the same antibiotype. The strains were isolated from both clinical samples and rectal swabs. Despite presenting different antibiotypes, the two isolates from the same patient (Pae 62 and Pae 69 isolates) belonged to the same pulsotype (pattern A). Figure 4 shows the dendrogram and complementary data of the strains studied.

Table 2 Antimicrobial resistance characteristics of the MBL-positive strains

Species	Genotype	Nr. of strains	Resistance pattern (not-sensitive)	Sensitive pattern	AT	RP
P. aeruginosa	VIM					
		7	CAZ, IPM, MPM, PIT, CPM, ATM, GEN, CIP,AMI	COL	I	XDR
		2	CAZ, IPM, MPM, PIT, CPM, GEN, CIP	ATM,AMI,COL	II	MDR
		1	CAZ, IPM, MPM, PIT, CPM, ATM, GEN	CIP,AMI,COL	III	MDR
		2	CAZ, IPM, MPM, PIT, CPM, GEN	ATM,CIP,AMI,COL	IV	MDR
P. putida	VIM	1	CAZ, IPM, MPM, PIT, CPM, ATM, GEN, CIP,AMI	COL	I	XDR
	VIM	13	CAZ, IPM, MPM, PIT, CPM, ATM, GEN, CIP	AMI,COL	II	XDR
	IMP	1	CAZ, IPM, MPM, PIT, CPM, ATM, GEN, CIP	AMI,COL	II	XDR
	VIM	9	CAZ, IPM, MPM, PIT, CPM, ATM, CIP	GEN,AMI,COL	III	XDR
	IMP	1	CAZ, IPM, MPM, PIT, CPM, ATM, CIP	GEN,AMI,COL	III	XDR
	VIM	4	CAZ, IPM, MPM, PIT, CPM, ATM, GEN	CIP,AMI,COL	IV	MDR
	VIM	2	CAZ, IPM, MPM, PIT, CPM, GEN, CIP	ATM, AMI,COL	V	MDR
	VIM	1	CAZ, IPM, MPM, PIT, CPM, GEN	ATM,CIP,AMI,COL	VI	MDR
Acinetobacter spp.	NDM	13 [Ab]	CAZ,IPM, MPM, PIT, CPM, GEN, AMI, CIP, SUT,AMS	COL,TGC	I	XDR
		2 [Ab]	CAZ,IPM, MPM, PIT, CPM, GEN, CIP, SUT, AMS	COL,AMI,TGC	II	MDR
		1 [An]	CAZ,IPM, MPM, PIT, CPM, SUT, AMS	COL,GEN,AMI,CIP, TGC	III	MDR
		1 [Ab]	CAZ,IPM, MPM, PIT, CPM, CIP, SUT	COL,GEN,AMI,AMS,TGC	IV	MDR
		7 [Ap, An, Ab, Abe] *	CAZ,IPM, MPM, PIT, CPM	COL,GEN,AMI,CIP, SUT,TGC	V	MDR
		1 [Ah]	CAZ,IPM, MPM, PIT, CPM, AMI	COL,GEN,CIP,SUT, AMS,TGC	VI	MDR
	IMP	1 [Ap]	CAZ,IPM, PIT, CPM, AMS	MPM,COL,GEN,AMI,CIP,SUT, AMS,TGC	VII	MDR

[Ab]*A. baumannii*, [An]*A. nosocomialis*, [Abe]*A. bereziniae*, [Ap]*A. pittii*, [Ah]*A. haemolyticus*, *7: Ap $n=3$, An $n=2$, Ab $n=1$, Abe $n=1$. *AT* antibiotype, *RP* resistance profile, *CAZ* ceftazidime, *CPM* cefepime, *IPM* imipenem, *MPM* meropenem, *PIT* piperacillin/tazobactam, *ATM* aztreonam, *GEN* gentamicin, *AMI* amikacin, *CIP* ciprofloxacin, *COL* colistin, *SUT* trimethoprim/sulphametoxazole, *AMS* ampicillin/sulbactam, *TGC* tigecycline

PFGE analysis for *P. putida* (Fig. 5) showed 25 different pulsotypes in the VIM-producing strains with only six antibiotypes, and no large clonal group was observed. However, there are isolates from different hospitals belonging to the same pulsotype. The IMP-producing strains belonged to different clones than VIM-producing isolates but share some of

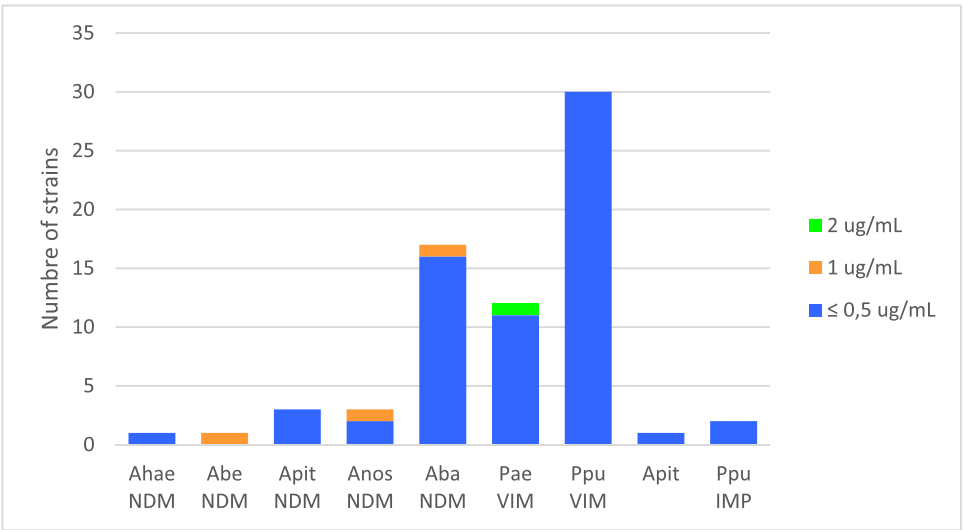

Fig. 3 Minimal inhibitory concentrations of colistin. Abbreviations: Ahae: *A. haemolyticus*, Abe: *A. bereziniae*, Apit: *Acinetobacter pittii*, Anos: *A. nosocomialis*, Aba: *A. baumannii,* Pae: *P. aeruginosa,* Pput: *P. putida*

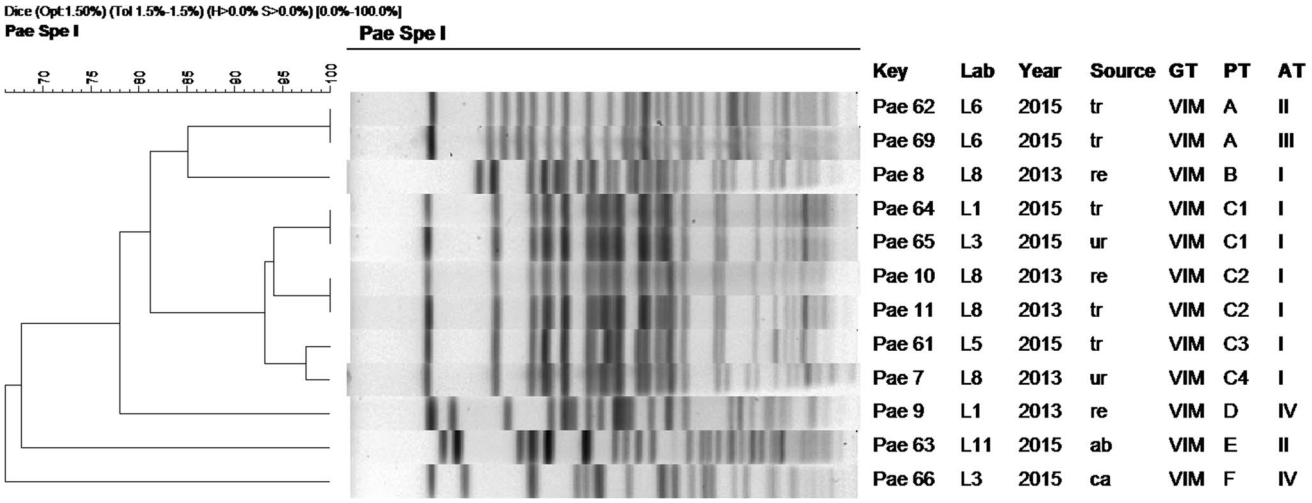

Fig. 4 Dendrogram of molecular typing of *P. aeruginosa* isolates. Abbreviations: tr: traqueal, re: rectal swab, ur: urine, ab: abdominal fluid, ca: catheter, Lab: origin (hospital laboratory) GT: genotype, PT: pulsotype, AT: antibiotype

the antibiotypes. The strains came from both clinical samples and rectal swabs.

The dendrogram for NDM-producing *A. baumannii* isolates (Fig. 6) revealed five different pulsotypes (A-E) and three antibiotypes (I, II, and V). Pulsotype A including seven subtypes (A1-A7) was found in 13 strains, 12 of them from the same hospital (L1), and all except one with the same antibiotype. The first NDM-producing *A. baumannii* isolate detected in 2014 recovered from a different hospital (L6) also belonged to the pulsotype A. The four remaining strains presented different patterns. In this group of strains, 12/17 came from rectal swabs.

Molecular typing with PFGE for *A. pittii* (Fig. 7) revealed three different pulsotypes (A–C) for two antibiotypes, one of them also present in *A. baumannii* (V, see Fig. 6). The first two NDM-producing *A. pittii* isolates, recovered from the same hospital, belonged to different pulsotypes (pulsotype A and C), already investigated in a previous publication [24]. Pulsotype A has with two closely related strains from the same hospital, isolated in different years, and the B and C pulsotypes are observed in one strain each. All NDM-positive strains showed the same antibiotypes, and they came from the same hospital. The IMP positive strain shows its own pulsotypes and antibiotype.

A. nosocomialis strains revealed two different pulsotypes (Fig. 8) and two antibiotypes, one of them (V) also present in *A. baumannii* and *A. pittii*. Two strains were grouped as A1 and A2 subtypes, while the third strain have a different pulsotype. All strains were isolated from the rectal swab.

Two strains were not included in the previous dendrograms, since they were typified as *A. haemolyticus* (antibiotype VI present in this single strain) and *A. bereziniae* (antibiotype V also present in *A. baumannii* and *A. pittii*).

Discussion

There are few studies published about intrahospital carbapenemases in Paraguayan non-fermenting gram-negative rods using molecular methods. One of them is from Pasteran et al. [24] which includes our strains *A. piitii* 1 and *A. pitti* 2 (see Fig. 7). Pasteran et al. identified those two strains as *A. pittii* by MALDI-TOF and were not clonally related by PFGE. These findings are consistent with our results. The study published by Rodriguez et al. [25] including *A. baumannii* paraguayan strains among others, found only OXA-23 carbapenemase and no MBL. None of these strains is part of the present work. In 2021, Melgarejo et al. [26] studying fermenting and non-fermenting gram-negative bacilli in Paraguay, found strains of *A. baumannii* with OXA 51, OXA 23, NDM, and NDM + OXA 58, and *P. aeruginosa* with NDM, but all those strains were isolated in 2021. Our work shows the beginning and the first years of MBLs dissemination in non-fermenting gram-negative bacilli and their behavior over time. Since then, MBL-producing strains have not ceased to be detected and as expected, the isolates increased gradually. It is possible to affirm that in Paraguay the MBL began to appear in BGNNF to spread years later in fermenting gram-negative bacilli also since the first publication in an Enterobacterales is from the year 2016 [27].

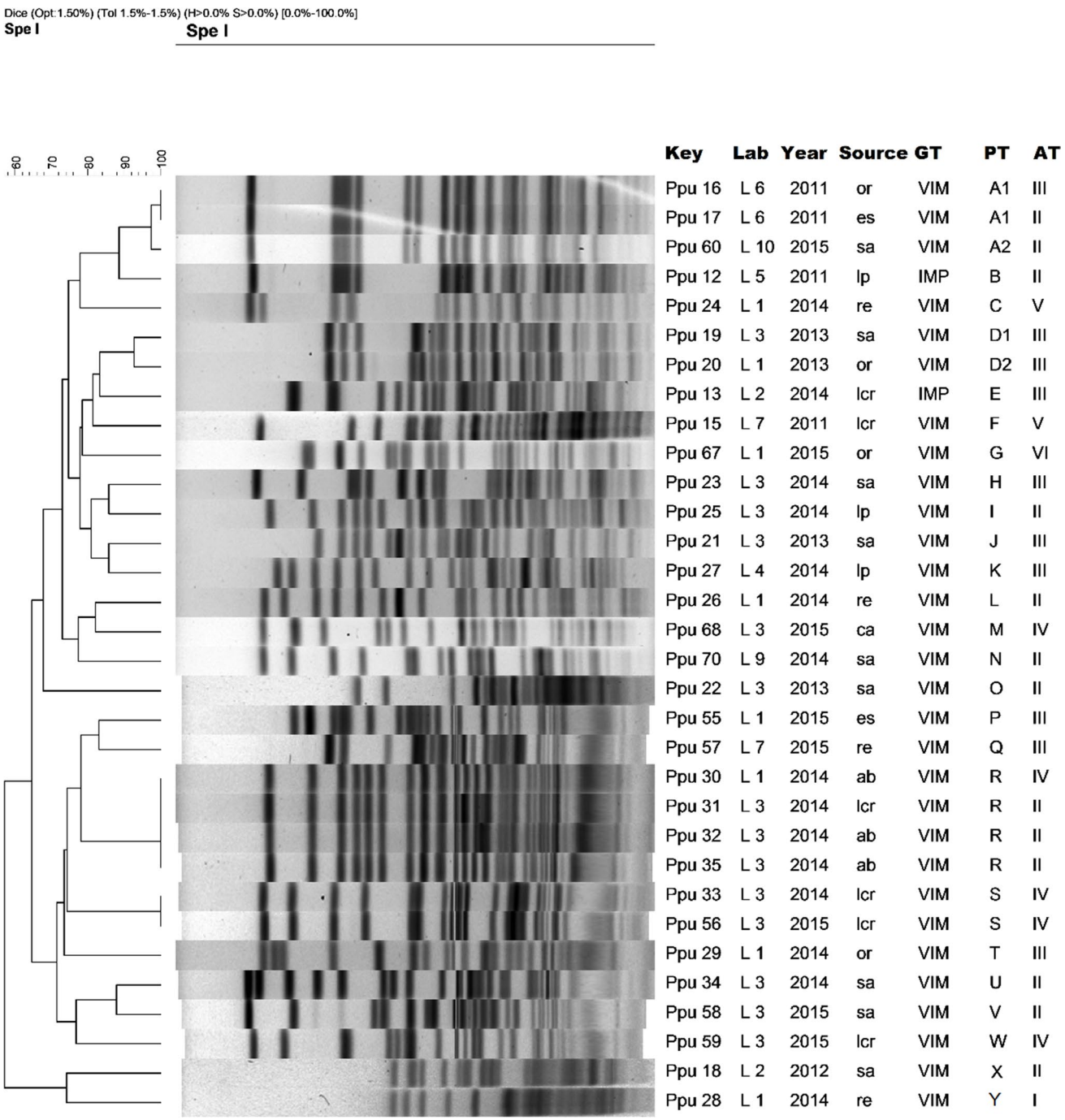

Fig. 5 Dendrogram of molecular typing of *P. putida* strains. Abbreviations: ur: urine, sp: sputum, bl: blood, pl: pleural fluid, re: rectal swab, tr: traqueal, cf: cerebrospinal fluid, ca: catheter, ab: abdominal fluid, GT: genotype, PT: pulsotype, AT: antibiotype

Regarding the MBL-producing species, *P. putida*, *P. aeruginosa* and *A. baumannii*, were the most prevalent in this investigation, as in another studies [8]. As expected, *A. baumannii* turned out to be the most prevalent of its genus (65%), followed by *A. pittii* and *A. nosocomialis*. This last two species, as well as *A. haemolyticus* and *A. bereziniae*, less prevalent, have been sporadically described as species involved in hospital infections [28, 29]. The most frequent association between bacterial species and genotype was observed with *P. putida* and VIM (43%), followed by *A. baumannii* associated with NDM (24%). It is interesting to note that NDM was detected in *Acinetobacter* spp. from

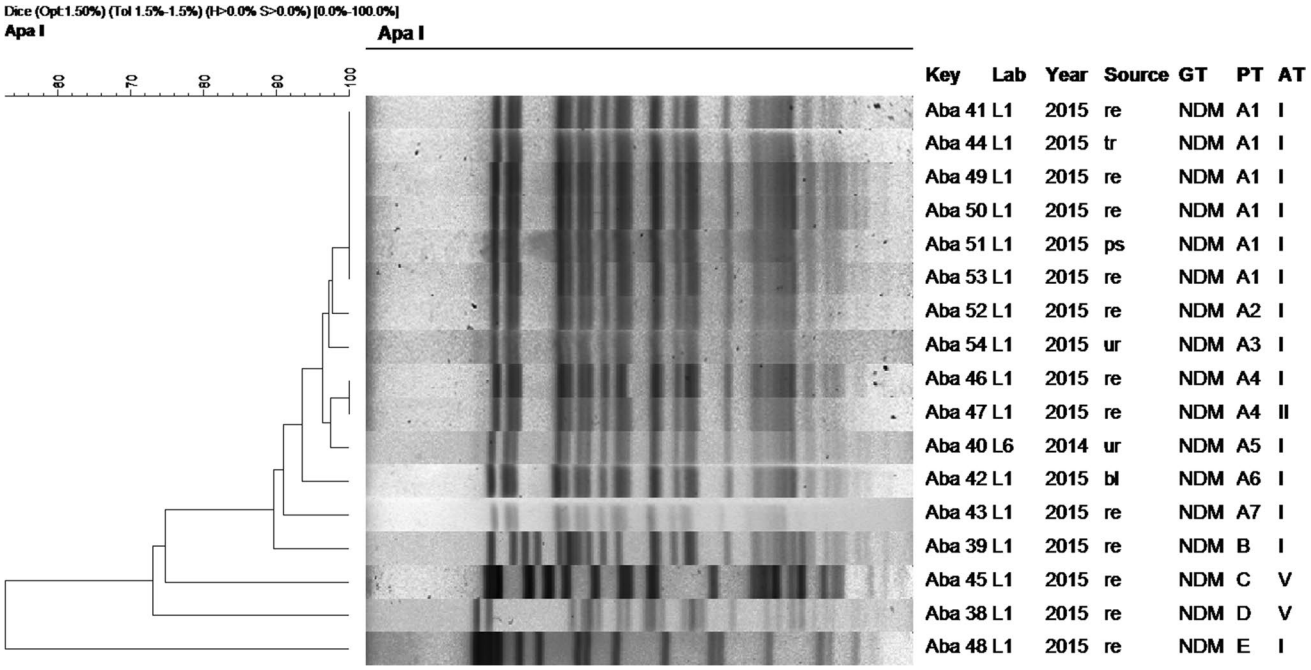

Fig. 6 Dendrogram of molecular typing of *A. baumannii* isolates. Abbreviations: ur: urine, re: rectal swab, ps: ps: secretion, tr: traqueal, bl: blood, GT: genotype, PT: pulsotype, AT: antibiotype

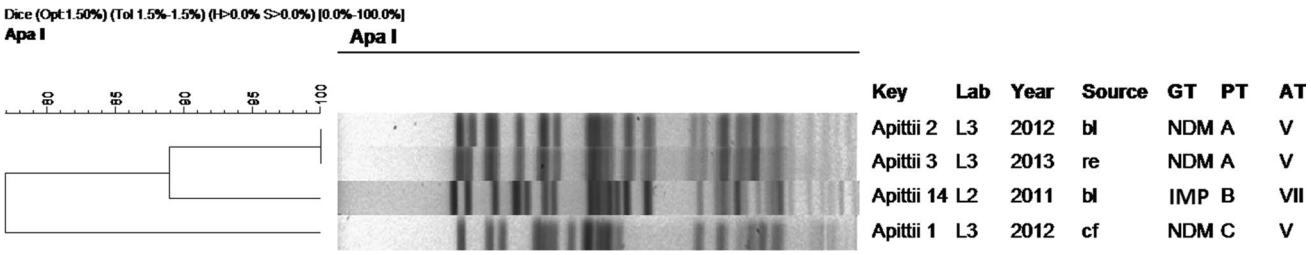

Fig. 7 Dendrogram of molecular typing of *A. pittii* strains. Abbreviations: bl: blood, re: rectal swab, cf: cerebrospinal fluid, GT: genotype, PT: pulsotype, AT: antibiotype. Obs: The strains *A. pittii* 1 and *A. pittii* 2 were studied in a previous publication [17]

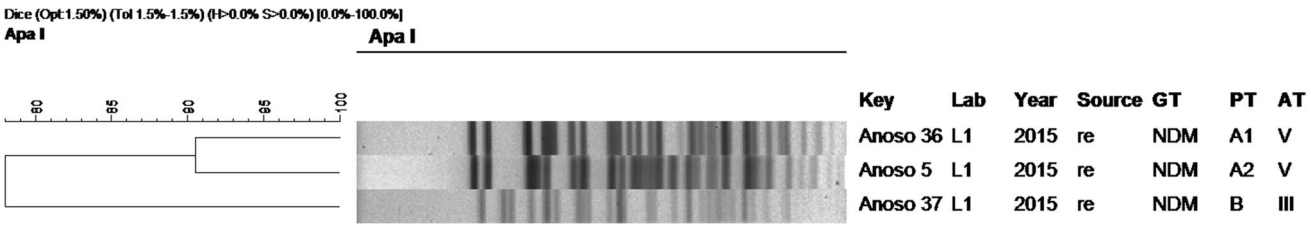

Fig. 8 Dendrogram of molecular typing of *A. nosocomialis* isolates. Abreviations: re: rectal swab, GT: genotype, PT: pulsotype, AT: antibiotype

2011 to 2014 in "non-*baumannii*" species (*A. pittii*, *A. haemolyticus*, *A. nosocomialis*, *A. bereziniae*). However, appeared in *A. baumannii* in 2015 with practically absolute predominance.

Although IMP, VIM, and NDM genotypes are the most frequent MBLs in the world, [7, 30], the frequency is variable, characteristic of each country, region, or hospital, consequently, studies published, show a variable

prevalence [31, 32]. Before the description of NDM, the most frequently detected MBLs in the world were IMP and VIM type, being VIM the most predominant [5, 30]. In our study, the distribution percentage found was 60% VIM, 36% NDM, and 4% IMP, but the prevalence varied over to the years. In fact, the VIM-producing, the most frequently isolated until 2014, was surpassed in 2015 by NDM associated to *A. baumannii* evidencing the great dissemination capacity of the mechanism in this genomespecies. In 2015 by the way, NDM was considered to becoming the most commonly and distributed carbapenemase worldwide [30].

Regarding the allelic variants described, VIM-1 and VIM-2 are the most frequent, reported in Europe [7, 33] and Asia [7]. In Latin American countries VIM-2 has been mostly detected [7] in Chile and Venezuela [34], Argentina [35], Brazil [36], Uruguay [37], and other countries, especially in *P. aeruginosa* followed by *P. fluorescens*. In this study, we demonstrate the circulation of VIM-2, associated with *P. putida* and *P. aeruginosa*. The allelic variant NDM-1, found in the present study, has been reported around the world, including Latin American countries such as Colombia, where it was detected in *A. baumannii* and *A. nosocomialis* [38], NDM-1 was also confirmed in *A. baumannii* [39] and in *A. pittii* [40] in Brazil. The IMP has been described with a lower prevalence than others. IMP-1 was the first variant described, followed later by IMP-2 [41], subsequently more variants were described. In this study, the IMP-18 subtype was detected in a lower prevalence. This variant has been detected in *P. aeruginosa* in Brazil [42].

In this work, only OXA-51, intrinsic to *A. baumannii*, was detected. None other OXA were found associated with MBL. In fact, the coexistence of OXA-type carbapenemase with NDM is unusual but has been documented [43].

Multiple clonalities of *P. putida* has been reported [44]. Some studies reported several *P. putida* clones with the same VIM-2 transposon in plasmids, together with a high proportion of MBL-producing strains compared to *P. aeruginosa*, suggesting that *P. putida* is a reservoir of these elements MDR transferable [44]. This situation could have been observed in this study, where in 2014 *P. aeruginosa* was not isolated and 43% of the VIM-2-producing strains were *P. putida* versus 17% of *P. aeruginosa*.

European reports suggest intrahospital transmission of VIM associated with *P. aeruginosa* [45]. Publications from Argentina [35], Spain [45], Colombia [46], and Brazil [47] show polyclonal propagation as well as spread by successful clones. In our study, we observed intra and out-off-hospital spread of the predominant clonal group. An outbreak would have started in one hospital, lasted 2 years and spread at two other hospitals.

Clonal diversity as well as dissemination of successful clonal groups of *A. baumannii* has been demonstrated in several studies [48, 49]. All this has been verified in the present work. NDM-producing *A. baumannii* presented a group of strains with a common clonal origin (13/17 isolates), all epidemiologically related, since 12 were from the same hospital (L1) and from the same year (2015). This could describe an outbreak in a hospital (L1) that would have originated in another hospital (L6), which had only one closely related isolate (95% similarity).

With this study, we ascertain the intra and interhospital dissemination of MBL-type carbapenemases, through polyclonal and successful clones capable of generating outbreaks, evidenced with the pulsotype A of *A. baumannii*, pulsotypes C of *P. aeruginosa* and R of *P. putida*. We also observed evidence of dissemination by mobile genetic elements due the presence of the same MBL variant in different species like VIM-2 in both *P. aeruginosa* and *P. putida*, IMP-18 in both *P. putida* and *A. pitti*, and NDM-1 in different *Acinetobacter* species. The presence of the same genotype variant and the same antibiotype in clonally unrelated strains suggests the spread of resistance by mobile genetic elements. It will be very interesting to compare this work with the subsequent evolution of carbapenemases in non-fermenting gram-negative rods in Paraguay, and the study of mobile genetic elements in more recent times.

Obviously, it is necessary to maintain and strengthen the surveillance of MBLs, as well as in-hospital containment measures to avoid the dissemination of these resistance mechanisms and others that could be successfully transferred through mobile genetic elements sometimes associated with successful clones, both within the same hospital, as between hospitals in Paraguay. The transfer of patients from one hospital to another or the fact that in Paraguay health personnel work at the same time in different hospitals, could be part of the problem of the spread of colonizing bacteria of intrahospital origin.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval Ethical approval was received from the Institutional Ethics Committee (IEC) of the Central Laboratory of Public Health, (approval no. CEI / LCSP 41/030314).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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