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Genomic Analysis of two NDM-1 *Providencia stuartii* Strains Recovered from a Single Patient

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Abstract

In the last years, an increasing number of untreatable infections caused by drug-resistant microbes have impacted the health care system. Worldwide, infections caused by carbapenem-resistant (CR) Gram-negative bacilli have dramatically increased. Among the CR-Gram-negative bacilli, those producing carbapenemases, such as NDM-1, are the main concern. Different *Enterobacterales* harboring NDM-1 have been reported lately. *Providencia stuartii*, a member of the *Morganellaceae* family, is ubiquitous in the environment, but is also known to cause nosocomial infections. Here we describe the genomic analysis of two NDM-1- producing *P. stuartii* strains recovered from the same patient as well as other carbapenem resistant strains recovered from the same hospital. As a result of the genomic analysis thirteen resistance genes, including three to β-lactams ($bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$), four to aminoglycosides (*aphA6, aac(3)-IId, aac(2')-Ia*, *aac(6')-Ib-cr5*), one to sulfonamides (*sul1*), two to chloramphenicol (*catB3, catA3*), one to rifampicin, one to bleomycin (*ble*), and one to tetracycline (*tet(B)*) were found. Moreover, a variety of mobile genetic elements, such as insertion sequences, plasmids and phage- related sequences, were found within *P. stuartii* genomes. The spread of carbapenem-resistant isolates remains a significant clinical and public health concern. Therefore, we considered that the detection of CR isolates is an essential step in addressing this problem.

Introduction

Carbapenem antibiotics are generally considered to be the last option in the treatment of patients with severe bacterial infections. The recent increase in the rates of carbapenem-resistant *Enterobacterales* (CRE) among healthcare-associated *Enterobacterales* is a major concern [1–3]. Under those circumstances, CRE infections are associated with high mortality due to limited treatment options [1, 3]. In addition, according to the CDC report Antibiotic Resistance Threats in the United States 2019, CRE was also found in the urgent threat category. Three groups of carbapenemases (KPC, NDM, and OXA-48) are currently considered to be the major β-lactamases with epidemiological and clinical significance [4, 5]. *Providencia* species are members of the *Morganellaceae* family, among them, *P. stuartii* and *P. rettgeri* are the most common causes of infections in hospitalized patients, mainly urinary tract infections [6, 7]. Moreover, cases of peritonitis, endocarditis, meningitis, bacteremia due to *P. stuartii* has been also reported [6, 8–11].

P. stuartii is intrinsically resistant to colistin and tigecycline, but carbapenem-resistant *P. stuartii* (CRPS) are

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increasingly reported, mainly due to $bla_{\text{NDM-1}}$ [6, 12, 13]. Recently, Molnar et al. described the presence of 77 CRPS isolates, recovered from different hospitals in Romania, of which 67 were NDM-1 positive [14]. In addition, CRPS VIM and KPC positive strains were also described in the literature [15–19].

In the present study, we described the clinical data and the whole genome sequence (WGS) analysis of two NDM-1 CRPS strains from a 55-year-old patient with a history of chronic obstructive pulmonary disease, hypertension and diabetes. Moreover, we described the molecular characterization of two more CR isolates (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) from the same patient together with others CR isolates (*P. stuartii*, *P. rettgeri*, *Proteus mirabilis* and *K. pneumoniae*) recovered from the same hospital.

Materials and Methods

Clinical Case

In April of 2018, the patient was admitted to the intensive care unit (ICU) due to cranial brain trauma requiring craniectomy with evacuation of the intracerebral hematoma plus surgical clipping of the sylvian aneurysm and placement of an external ventricular shunt. In June, the patient presented febrile registers associated with hypoventilation and fatigue, therefore blood cultures, catheter-drawn blood cultures and urine cultures were performed. *K. pneumoniae* (Kb846) grew in the two blood culture bottles and in the blood-culture drawn from the catheter. In addition, the growth of *Providencia stuartii* (Ps848) was obtained in one of two bottles of blood culture. In the urine culture, *Pseudomonas aeruginosa* (PaC850) and *P. stuartii* (Ps850) were isolated (Table 1). The established treatment was with fosfomycin, meropenem and amikacin

Table 1 General features of the clinical strains included in the study

Strain	Specie	Patient	Source	Resistance profile*	Cabapenemases		
					Rapid Blue-Carba test	Metallo-β-lactamases	KPC-MBL Kit
Kb846	*K. pneumoniae*	1	Blood culture, catheter	AMP; SAM; TZP; CEP; CTX; CAZ; IMP; MEM; AMK; GEN; NAL; CIP; NIT; COL; TMS	Positive	Negative	Positive
Ps848	*P. stuartii*	1	Blood culture	AMP; SAM; TZP; CEP; CTX; CAZ; IMP; MEM; GEN; NAL; CIP; NIT; COL; TMS	Positive	Positive	Positive
PaC850	*P. aeruginosa*	1	Urine culture	AMP; SAM; CEP; CTX; CAZ; IMP; NAL; CIP; NIT; FEP	Positive	Positive	Positive
Ps850	*P. stuartii*	1	Urine culture	AMP; SAM; TZP; CEP; CTX; CAZ; IMP; MEM; GEN; NAL; CIP; NIT; COL; FEP	Positive	Positive	Positive
Ps722	*P. stuartii*	2	Abscess culture	AMP; SAM; CEP; GEN; CTX; CAZ; IMP; MEM; NAL; NIT; COL; CIP	(Indeterminate)	Positive	Positive
Pr783	*P. rettgeri*	3	Surgical toilette	AMP; SAM; TZP; CEP; CTX; CAZ; IMP; MEM; NAL; NIT; COL; FEP; TMS	(Indeterminate)	Positive	Positive
Pm856	*P. mirabilis*	4	Urine culture	AMP; SAM; TZP; CEP; CTX; CAZ; IMP; MEM; NAL; NIT; COL; FEP; TMS; GEN; CIP	Positive	Positive	Positive
Kb443	*K. pneumoniae*	5	Tracheal aspirate	AMP; SAM; TZP; CEP; CTX; CAZ; IMP; MEM; NAL; AMK; TMS; GEN; CIP	Positive	Positive	Positive

AMP ampicillin, *SAM* ampicillin-sulbactam, *TZP* piperacillin-tazobactam, *CEP* cefalotine, *CTX* cefotaxime, *CAZ* ceftazidime, *IMP* imipenem, *MEM* meropenem, *AMK* amikacin, *GEN* gentamycin, *NAL* nalidixic acid, *CIP* ciprofloxacine, *NIT* nitrofurantoin, *COL* colistin, *TMS* trimethoprim-sulfamethoxazole, *FEP* cephepime

*Resistance profile only; Indeterminate: a color change was obtained after 2 h of incubation

for 14 days. During treatment the patient evolved with increased blood sodium attributable to fosfomycin, so it was changed to tigecycline. With the exception of an oxygen requirement following a tracheostomy, the patient´s condition remained stable, steadily improving favorably without febrile records (Table 2).

Other Clinical CR Strains

At the same time, four other CR isolates were recovered. Two isolates were *P. stuartii* (Ps722) and *P. rettgeri* (Pr783), which were obtained from the surgery service. The other two isolates were a *Proteus mirabilis* isolate (Pm856) obtained from a urine culture of a dialyzed patient, and a *K. pneumoniae* isolate (Kb443) from a tracheal aspirate of a pediatric patient, were recovered (Table 1).

Antimicrobial Susceptibility Assay

The recovered isolates were characterized at species level by conventional biochemical tests and by the VITEK 2 System (bioMérieux). The susceptibility assay was performed using the VITEK 2 (AST-279 panel). The minimum inhibitory concentration (MIC) results were interpreted using the Clinical and Laboratory Standards Institute [20]. For the rapid detection of carbapenemases, a colorimetric method (Rapid Blue-Carba Test) was also performed [21]. A color change obtained after the reading limit time (120 min) was recorded as an indeterminate result. To study the presence of metallo-β-lactamases (MBL), a double-disc diffusion test using meropenem and imipenem (10 µg, OXOID, UK) was performed on Mueller Hinton agar using an 1900/750 µg EDTA/SMA disc (Laboratorios Britania, Argentina). Additionally, to detect the presence of serine or oxacillinase carbapenemases, the screening method using the KPC-MBL Kit Confirm ID Pack (Rosco Taastrup, Denmark) containing meropenem (10 µg), meropenem 10 µg + dipicolinic acid (Metallo-β-Lactamase inhibitor); meropenem 10 µg + cloxacillin (AmpC inhibitor); meropenem 10 µg + phenylboronic acid (KPC and AmpC inhibitor) was carried out [22].

Table 2 General features of the Ps848 and Ps850 genomes

Features	Whole genome	
	Ps848	Ps850
Total number of base pairs	4,404,416	4,403,514
G + C content (%)	41.2%	41.2%
Number of possible ORFs	4,379	4,382
Number of insertion sequences	8	8
Number of resistance genes	13	13

General Molecular Techniques

To perform the molecular characterization of the isolates recovered, total DNA extraction was carried out using Wizard® Genomic DNA Purification Kit according to manufacturer instructions (Promega, Madison, WI). PCR reactions using GoTaq® enzyme (Promega, Madison, WI) were carried out for the most common carbapenemase genes (bla_{IMP}, bla_{VIM}, bla_{KPC} and bla_{NDM}), as well as, the presence of mobile elements related with them. Plasmid extractions were carried using two different techniques, the QIAprep Spin Miniprep Kit following manufacturer instructions (Qiagen Germantown, MD, USA) and a manual plasmid extraction design for megaplasmids [23, 24].

Whole-genome Sequence and Sequences Analysis of Ps848 and Ps850 Clinical Isolates

Genomic DNA was extracted using a DNeasy Blood and Tissue kit from Qiagen (Qiagen Germantown, MD, USA). Whole-genome shotgun sequencing was performed using Illumina NextSeq550. De novo assembly was performed with SPAdes Assembler (version.3.1.0) [25] using a pre-assembly approach with Velvet [26]. The RAST (Rapid Annotation using Subsystem Technology) server was used to predict and annotate open reading frames [27], and BLAST (v.2.0) software was utilized to confirm the predictions. The Whole Genome Shotgun project has been deposited at GenBank under the accession JABLTB000000000 and JABLTC000000000 for Ps848 and Ps850, respectively. ARG-ANNOT [28] and The Resistance Gene Identifier (CARD-RGI) [29] were used to identify antibiotic resistance genes. The ISfinder and PHASTER ‘s softwares were used to identify insertion sequences and phages and prophages, respectively [30, 31]. The RaxML software was used to predict the phylogenetic relation among the strains [32].

Results

Antibiotic Susceptibility and Carbapenemase Identification in Carbapenem-Resistant Gram-Negative Strains

Antimicrobial susceptibility testing revealed that Ps848, Ps850 and Ps783 exhibited an extreme-drug resistant phenotype, where Ps850 and Ps783 were susceptible to amikacin and trimethoprim/sulfamethoxazole, and Ps848 was only susceptible to amikacin. Among Kb isolates, Kb443 was only susceptible to colistin and nitrofurantoin, while

Kb846 was resistant to all of the antibiotic tested. PaC850 was multidrug resistant with susceptibility to amikacin, gentamicin and colistin. Moreover, Pm856 and Pr783 were both susceptible to amikacin and Pr783 was also susceptible to ciprofloxacin (Table 1).

The four isolates obtained from the same patient showed a positive result for the Rapid Blue-Carba test: a weak positive result (light green color) for Ps848, Ps850 and PaC850 isolates between 60 and 90 min of incubation and a strong positive result for isolate Kb846 at 30 min of incubation were obtained. Moreover, a positive result was also obtained for the isolates Pm856 and Kb443. Also, an indeterminate result was obtained for the isolates Ps722 y Pr783 In addition, synergy between carbapenems and EDTA was observed for Ps848, Ps850, PaC850, Pr783, Ps722, Pm856 and Kb443 isolates. Additionally, the KPC-MBL Kit Confirm ID Pack showed the presence of MBL-type carbapenemase in Ps848, Ps850, PaC850, Pr783, Ps722, Pm856, Kb846 and Kb443 isolates. A halo difference of ≥ 5 mm in the case of meropenem + dipicolinic acid with respect to the meropenem 10 µg disc was observed. Instead, for isolate Kb846, a halo difference of ≥ 5 mm in the case of meropenem + phenylboronic acid with respect to the meropenem 10 µg disc was observed (Table 1).

WGS of Ps848 and Ps850: Genome Features, Antibiotic Resistance Determinant, and Mobile Genetic Elements

The draft genome of Ps848 consists of 4,404,416 bp. The RAST server predicted 4379 protein-coding genes with a corresponding G + C content of 41.2%, whereas the draft genome of Ps850 consists of 4403,514 bp and the RAST server predicted 4382 protein-coding genes with a corresponding G + C content of 41.2% (Table 1).

Phylogenetic analysis was performed using RaxML and showed that both strains belong to the same clone (Fig. 1).

Using the ARG-ANNOT and CARD-RGI software's the presence of 13 resistance genes within the genomes were observed. Among them, we found genes encoding for β-lactamases ($bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$), aminoglycoside modifying enzymes (*aphA6, aac(3)-IId, aac(2')-Ia* and *aac(6')-Ib-cr5*), sulfonamide-resistant dihydropteroate synthase (*sul1*), chloramphenicol acetyltransferase (*catB3, catA3*), rifampin ADP-ribosyltransferase (*arr3*), bleomycin resistance protein (*ble*) and tetracycline resistance protein (*tet(B)*).

Analysis of the mobilome and horizontal gene transfer related features showed a remarkable similarity between the content of both genomes. Both strains carried genetic traits that evidenced the presence of several mobile genetic elements (MGEs): a CR-bearing plasmid, 8 different ISs (IS*Sf1*, IS*Kpn13*, IS*1326*, IS*Aba14*, IS*15*, IS*Cfr1* and two copies of IS*Aba125*) and several phage-related regions (two intact prophages, eight incomplete prophages and one questionable prophage). The sequences corresponding to the plasmid found in both Ps strains were highly similar to the *Proteus cibarius* plasmid p152 (AN CP047288) and the *P. mirabilis* pPm50 (AN MG516911). In-depth backbone analysis revealed that plasmid shares several traits with *P. rettgeri* plasmids p16-01,619–1 (AN KX832929) and pPrY2001 (AN NC_022589), which are pNDM-BJ01-like elements and thus far they have not been classified in an incompatibility group. *P. stuartii* Ps848 and Ps850 strains also encoded the same type I-F CRISPR-Cas system, which consisted of a *cas* operon surrounded by 2 CRISPR arrays of 7 and 11 identical repeat and spacer sequences Four additional CRISPR arrays with high confidence were found at different loci (Supplementary Table S1).

Analysis of $bla_{\text{NDM-1}}$ genetic context and integrons found in Ps848 and Ps850.

A depth analysis was carried out to characterize the genetic context of $bla_{\text{NDM-1}}$ gene, which was found to be plasmid located. The genetic context flanking the upstream and downstream (5′ and 3′ end) region of the $bla_{\text{NDM-1}}$ gene was the same in both stains. The 5′ region was composed of the insertion sequence IS*Aba125* and *aphA6* gene (Fig. 2a). Whereas the downstream (3′ end) region was composed of a bleomycin resistance gene (*ble*) and a phosphoribosylanthranilate isomerase (*trpF*). Moreover, another IS*Aba125* was 7,019 bp away (from the 3′ end of the $bla_{\text{NDM-1}}$ until the 5′ end of IS*Aba125*) (Fig. 2a).

The sequence analysis of the class 1 integron revealed the presence of four gene cassettes (*aac(6')-Ib-cr5*, $bla_{\text{OXA-1}}$, *catB3,* and *arr3*) within the variable region 1 (VR-1) (Fig. 2b). The 3′-CS conserved region containing *qacEΔ1/sul1* genes was also present. In addition, the same class 1 integron array was present in PaC850. However, a different class 1 integron, containing *aac(6')I-b* in the variable region, was found in Kp846. Ps848 and Ps850 strains also harbored a class 2 integron. The presence of Tn7 transposon related genes *tnsA, tnsB, tnsC* and *tnsD* were also found.

Regarding to the others the others CR isolates found in the same hospital, Pm856, Kb443, Ps722, Pr783 exposed the presence of $bla_{\text{NDM-1}}$. $bla_{\text{TEM-1}}$ gene was only found in Pm856 and Kb443. Moreover, a class 1 integron harboring the $bla_{\text{VIM-2}}$ gene was found in Ps722, Pm856 and Kb443.

Discussion

In this work, we present the molecular characterization of two carbapenemase-producing *P. stuartii* and two more CR isolates (*P. aeruginosa* and *K. pneumoniae*) recovered from

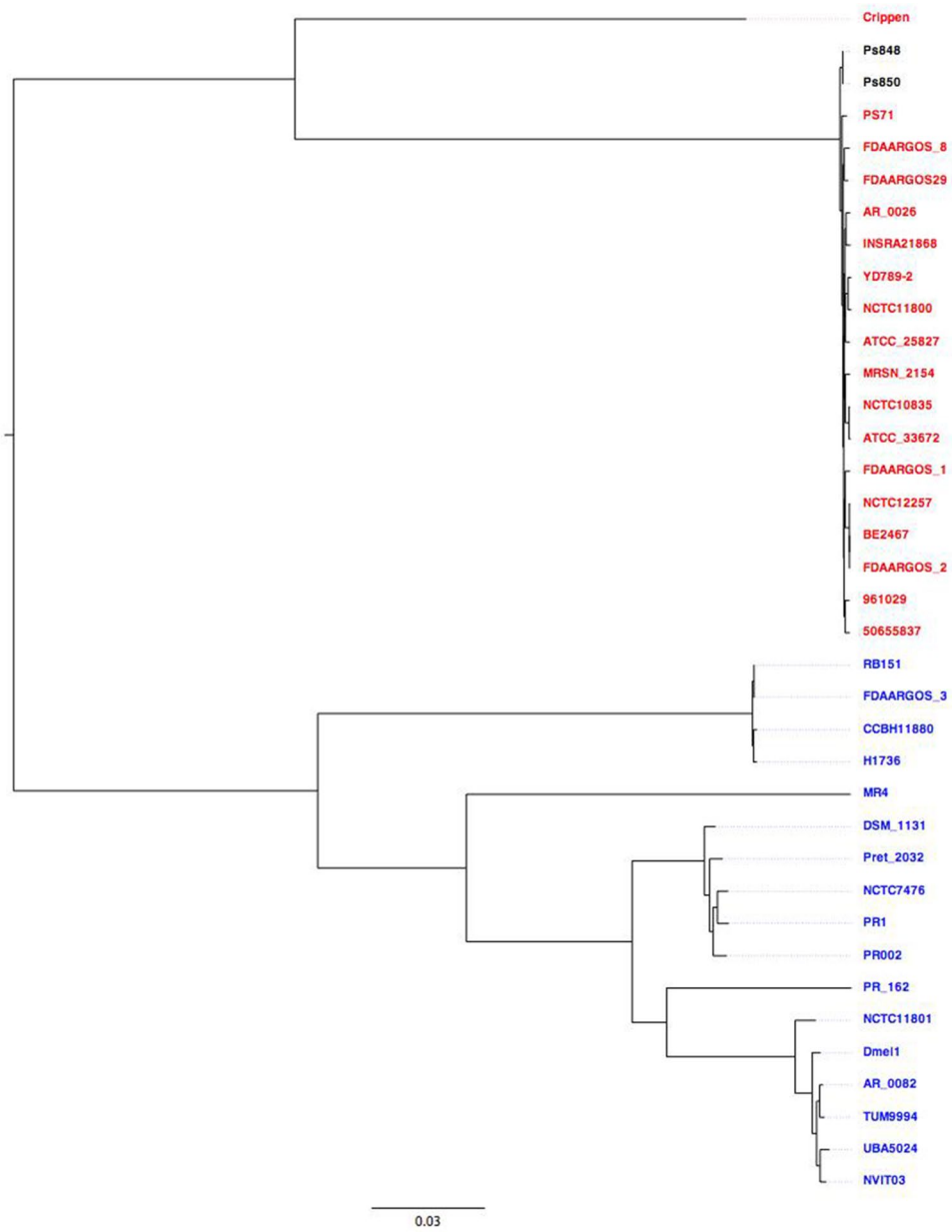

Fig. 1 Core-genome phylogeny of *P. stuartii* and *P. rettgerii*. Twenty *P. stuartii* genomes and eighteen *P. rettgeri* genomes was represented in the phylogenetic tree. *P. stuartii* and *P. rettgeri* available in GenBank database is indicate in red and blue, respectively. The isolates sequenced are indicate in black

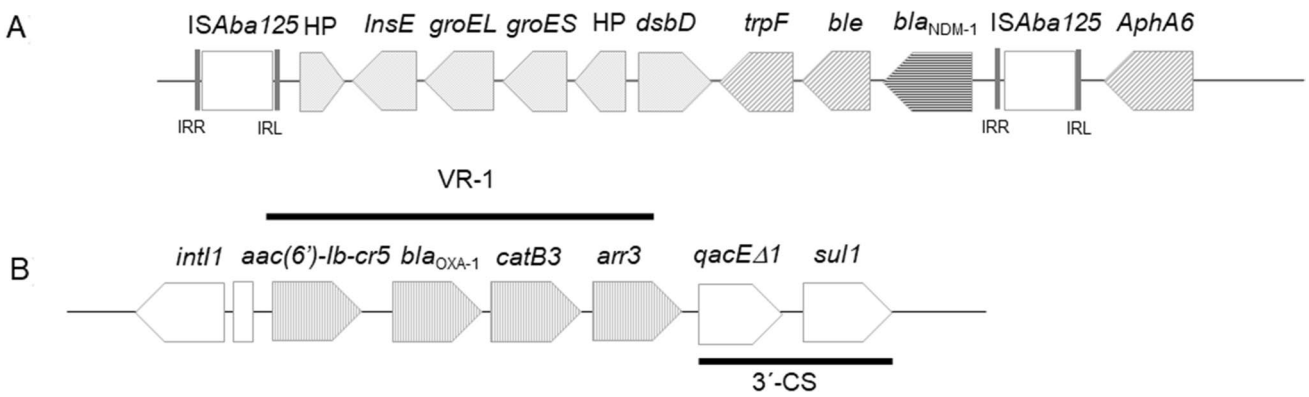

Fig. 2 **a** Genetic context of bla_{NDM-1} gene found in Ps848 and Ps850. **b** Schematic representation of the class 1 integron found in Ps848 and Ps850

a single patient. We also performed the molecular characterization of other CR isolates, recovered from the same period of time in the hospital (*P. stuartii*, *P. rettgeri*, *P. mirabilis* and *K. pneumoniae*). The presence of more than one CR *Enterobacterales* harboring plasmid- mediated NDM-1 in a single patient has been described in the literature [33, 34]. Recently, Abdallah et al. also described a case of hospital-acquired pneumonia due to CR *P. stuartii* and *K. pneumoniae* in a single patient [35]. Among *Providencia 's* members, *P. stuartii* and *P. rettgeri* are the most common causes of infections in hospitalized patients, and are mainly associated with urinary tract infections. It has been also described in nosocomial outbreaks caused by multidrug resistant strains of *P. stuartii* [36–39]. In addition, *P. stuartii* clinical isolates harboring plasmids encoding different beta-lactamases have also been studied [12, 13, 40]. The WGS analysis of Ps850 and Ps848 exposed the presence of 13 resistance genes ($bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$, *aphA6, aac(3)-IId, aac(2′)-Ia* and *aac(6′)-Ib-cr5*, *sul1, catB3, catA3*, *arr3*, *ble* and *tet(B)*). These results are in accordance with previous reports that showed the presence of MDR *P. stuartii* clinical isolates [41–43]. Moreover, the presence of *P. stuartii* clinical isolates harboring $bla_{\text{NDM-1}}$ gene was also described in the literature [12, 13, 35]. Among the other CR isolates analyzed Pm856, Kb443, Ps722 and Pr783 were also NDM-1 positive. Furthermore, in the literature the presence of *P. mirabilis*, *K. pneumoniae* and *P. rettgeri* harboring $bla_{\text{NDM-1}}$ gene was also reported [44–46]. Similarly, the $bla_{\text{NDM-1}}$ gene with the same 3′ end region found in Ps850 and Ps848 was previously reported present in plasmids in *E. coli* 3426 (AN LS992188) and *C. freundii* CRE3 (AN MK101346). While the same context (upstream and downstream) was previously reported in *A. nosocomialis* IEC38057 (AN MK053934) and *A. baumannii* (AN LC032101).

In addition, a variety of MGEs were also identified in Ps850 and Ps848 genomes, such as a resistance plasmid, ISs, several phage-related regions and a CRISPR-Cas type I-F. The analysis of the plasmids identified in Ps850 and Ps848, showed that they share several traits with *P. rettgeri* plasmids p16-01,619–1 and pPrY2001. As a common feature, they all encode the beta-lactamase NDM-1. Martinez et al., proposed that all *P. rettgeri* NDM positive strains harbor a putative conjugative transfer mechanism named Tra-Pre, which is not present in pNDM-BJ01-like plasmids from *Acinetobacter* spp [47]. We hypothesized that this novel plasmid backbone could have emerged as the consequence of different recombination events (homologous and non-homologous) between coexisting plasmids in the clinical setting. Furthermore, a class 1 integron was also identified in both genomes. The same class 1 array was reported in *K. pneumoniae* WCHKP020030 (AN CP028791) and integrons with regions similar to VR-1 were also described in *C. freundii* AA535 and AA593 (AN MH594477 and MH594478, respectively). Only a few cases of a *P. stuartii* harboring class 1 integrons have been reported [16, 48]. In addition, the presence of the class 2 integron and the Tn*7* transposon associated genes were also found in the analyzed genomes. Barlow et al., previously described the presence of the *intI2* gene in *P. stuartii* isolates not associated with the typical class 2 integron array. They also suggested that the *intI2* gene present in their isolates could be part of a novel super-integron structure; however, further analysis needs to be done to confirm [49]. The presence of *P. stuartii* harboring a class 2 integron was scarcely described in the literature [49, 50]. Regarding the other CR isolates PaC850, Kp846, Ps722, Pm856 and Kb443 harbored also class 1 integron. The spread of carbapenemase-producing isolates remains a significant clinical and public health concern. Therefore, we considered that the detection of carbapenemase production is an essential first step in combating this problem.

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Compliance with Ethical Standards

Conflict of interest The authors have declared that they have no conflict of interest.

Ethical Standards All procedures performed in this study were in accordance with the ethical standards of the Hospital Eva Peron, Buenos Aires, Argentina, the 1964 Helsinki Declaration, its later amendments and the National Law on the protection of personal data No 25.326.

Informed Consent Informed consent was obtained from the patient involved in the study.

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