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ORIGINAL ARTICLE

Full characterization of an IncR plasmid harboring *qnrS1* recovered from a VIM-11-producing *Pseudomonas aeruginosa*

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Metallo-carbapenemase;
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Abstract Metallo-β-lactamases (MBL) producing *Pseudomonas aeruginosa* isolates have been well characterized. Quinolones are commonly used in the treatment of carbapenem-resistant *P. aeruginosa* infections; however, data about PMQR in this species are scarce. The objective of this study was to report the simultaneous presence of *qnrS* and bla_{VIM-11} in *P. aeruginosa*, and to characterize the *qnrS*-harboring plasmid.

Thirty-eight carbapenem-resistant *P. aeruginosa* isolates were recovered from a hospital in Buenos Aires during 2012. Screening for MBL was assessed by the double disk synergy test using EDTA and carbapenem discs. Plasmid DNA extraction was performed by a method using phenol-chloroform. PCR followed by sequencing was carried out to determine each MBL and PMQR allele. PCR-*Bse*GI-RFLP was performed to detect *aac-(6′)-Ib-cr*. The *gyrA*-QRDR was sequenced in those PMQR-harboring isolates. Plasmid incompatibility groups and addiction systems were characterized by PCR. The PMQR-carrying plasmid was sequenced using Illumina technology, annotated using RAST and manually curated.

Eleven/38 isolates were VIM producers (bla_{VIM-2} and bla_{VIM-11}) while 1/38 harbored bla_{IMP-13}. One isolate harbored bla_{VIM-11} and the PMQR *qnrS1*; however, both markers were located in different plasmids.

The *qnrS1*-harboring plasmid (pP6qnrS1) was 117 945 bp in size, presented 154 CDS and corresponded to the IncR group. In addition to *qnrS1*, it harbored several aminoglycoside resistance markers. Although pP6qnrS1 was non-conjugative, it presented an *oriT* which made it possible for this plasmid to be transferable.

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This is the first report on *P. aeruginosa* carrying both bla_{VIM-11} and *qnrS1*, plus the first detection of an IncR plasmid in Argentina.

PALABRAS CLAVE
Metalo carbapenemasa;
PMQR;
Plásmido *qnrS1*;
Pseudomonas aeruginosa

Caracterización completa de un plásmido portador de *qnrS1* perteneciente al grupo de incompatibilidad IncR, recuperado de un aislamiento de *Pseudomonas aeruginosa* productor de VIM-11

Resumen Las quinolonas son comúnmente utilizadas en el tratamiento de las infecciones producidas por *Pseudomonas aeruginosa* resistentes a carbapenems (PARC); aun así, la información sobre la resistencia a quinolonas mediada por plásmidos (PMQR) en esta especie es escasa. El objetivo de este trabajo fue reportar la presencia simultánea de los genes *qnrS* y bla_{VIM-11} en PARC y caracterizar el plásmido portador de *qnrS*.

Durante 2012 se recuperaron 38 PARC en un hospital de Buenos Aires. El tamizaje para detectar producción de metalo-beta-lactamasas (MBL) se llevó a cabo mediante sinergia de doble disco utilizando EDTA y carbapenems. El ADN plasmídico fue extraído utilizando fenol-cloroformo. Para determinar los alelos de los genes implicados en la síntesis de MBL y de PMQR, se llevó a cabo PCR-secuenciación. Para la detección de *aac-(6′)-Ib-cr* se realizó PCR-*BseGI*-RFLP. En aquellos aislamientos portadores de PMQR se secuenció el gen *gyrA*. Los grupos de incompatibilidad y sistemas de adicción fueron caracterizados por PCR. El plásmido portador de PMQR fue secuenciado completamente y curado manualmente.

De 38 aislamientos, 11 fueron productores de VIM (bla_{VIM-2} y bla_{VIM-11}), mientras que uno contenía bla_{IMP-13}. Si bien un aislamiento fue portador de bla_{VIM-11} y de *qnrS1*, dichos marcadores se encontraban en distintos plásmidos. El plásmido portador de *qnrS1* (pP6qnrS1) presentó un tamaño de 117.945 pb y 154 secuencias codificantes (CDS); este correspondió al grupo de incompatibilidad IncR. Además de *qnrS1*, el plásmido portaba diversos marcadores de resistencia a aminoglucósidos. Aun cuando pP6qnrS1 no resultó conjugativo, presentó un oriT, de modo que posiblemente sea transferible.

Este es el primer informe acerca de PARC portadora de bla_{VIM-11} y de *qnrS1* en simultáneo, además, es la primera descripción de un plásmido IncR en Argentina.

Introduction

Pseudomonas aeruginosa is a ubiquitous non-fermenting Gram-negative rod, considered an opportunistic pathogen which can cause infections mainly in immunocompromised patients. Reports on this matter include hospital-acquired pneumonia, bacteremia, site infections in extensive burned areas, otitis and urinary tract infections[2].

P. aeruginosa displays natural resistance to a wide set of antimicrobial agents including most β-lactams, thus narrowing the therapeutic options to a small group of antibiotics such as ceftazidime, cefepime, monobactams, carbapenems, fluoroquinolones, colistin and to a lesser extent, aminoglycosides. However, over the past years, resistance levels have increased by the acquisition of resistance mechanisms, rendering therapeutic alternatives useless. Mutations of the molecular target have become the main cause of quinolone resistance while impermeability-type mutants are mainly involved in resistance to aminoglycosides and carbapenems[10]. Even though inaccessibility to the molecular target is the main cause of carbapenem resistance in *P. aeruginosa*, metallo-β-lactamases (MBL) are a major determinant of transferable resistance. In Argentina MBL account for 65% of the enzymatic resistance to these antibiotics. In our region, bla_{VIM} and bla_{IMP} have been reported to be the most relevant MBL coding genes in *P. aeruginosa*, bla_{VIM-2} and bla_{IMP-13} being the most frequent variants of each gene[16,18].

Plasmid-Mediated Quinolone Resistance (PMQR) genes confer diminished susceptibility to quinolones, facilitating the selection of higher-level resistance due to target mutation[11]. PMQR include genes coding for active efflux pumps (namely OqxAB and QepA), antibiotic modifying enzymes (*aac-(6′)-Ib-cr*) and a set of genes coding for target protective proteins belonging to the pentapeptide repeat family, generically named Qnr. The latter are the widest set of PMQR genes including 7 families (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qnrVC* and *qnrE*) and their allelic variants[11].

Broad host range resistance plasmids belonging to IncU and IncW groups have been reported in *P. aeruginosa* using

PCR-based methods. However, in the case of plasmids native to the *Pseudomonas* genus, conjugation-based assays are more appropriate. Few documented cases of *P. aeruginosa* plasmids carrying *qnr* are available worldwide[1,3,13,19]. The aim of this study was to report the simultaneous presence of *qnrS and* bla_{VIM-11} in *P. aeruginosa* for the first time, and to fully characterize the *qnrS*-harboring plasmid.

Materials and methods

Isolates and antimicrobial susceptibility

A total of 38 carbapenem resistant *P. aeruginosa* isolates were recovered from a hospital in Buenos Aires city during 2012. Antimicrobial susceptibility tests were performed by the disk diffusion method according to CLSI guidelines, and those suggested by *Sociedad Argentina de Bacteriología, Micología y Parasitología clínicas (SADEBAC) – AAM*[6,17].

Detection and characterization of MBL-coding genes.

In order to detect the possible presence of MBLs, double disk synergy tests using imipenem (IPM), meropenem (MEM) and EDTA (1 μmol) were carried out[17]. Plasmid DNA extraction was performed according to Kado & Liu. Screening for MBL genes was conducted by multiplex PCR according to Ellington et al.[8] KPC-coding genes were investigated according to Bradford et al. in all isolates[4]. Several electroporation protocols and conjugation assays, using both *E. coli* DH5α and *P. aeruginosa* PAO-1 as recipient cells, were attempted[9].

Simplex PCR reactions using bla_{VIM} and bla_{IMP} specific primers (Table 1) were used for further sequencing (Macrogen, Korea) and amplicon sequences were compared with the database using the NCBI BLASTn tool (www.blast.ncbi.nlm.nih.gov – 15/6/2019). The presence of MBL-coding genes in class 1 integrons was inferred combining 3′CS and 5′CS primers with either bla_{IMP} or bla_{VIM} forward and reverse primers (Table 1).

Detection and characterization of Plasmid-Mediated Quinolone Resistance mechanisms

PMQR markers were investigated using primers targeting the most common determinants (Table 1) and plasmid DNA as template. Additionally, the presence of *aac-(6′)-Ib-cr* was sought by PCR followed by digestion with *BseG*I. Amplicon sequences were identified using the NCBI BLASTn tool.

In those quinolone resistant isolates which were also positive for PMQR, the presence of mutations in the Quinolone-Resistance-Determining Region (QRDR) of *gyrA* was investigated on total DNA by PCR (Table 1) and sequencing (Macrogen, Korea). The QRDR sequence was compared with the same region of *gyrA* in PAO-1 (NC002516.2) using the BLASTn tool.

Plasmid characterization

Plasmid DNA harboring PMQR was extracted by a modification of the phenol-chloroform method proposed by Kado & Liu. Both, the PCR Based Replicon Typing scheme (PBRT) proposed by Carattoli et al.[5] and addiction system detection proposed by Mnif et al.[14] were performed.

Whole plasmid DNA was sequenced using Illumina Miseq technology. Fragment libraries were constructed by shotgun (NEB Ultra II) followed by 250 bp paired-end sequencing; the reads were assembled using plasmid SPAdes V3.9 with default settings to include only contigs of more than 500 nucleotides. Genes were annotated using the RAST online tool and PROKKA software, contigs were thereafter manually curated and assembled. The complete plasmid sequence was compared with GenBank and transfer origin (*oriT*) databases (http://dnatools.eu/MOB/plasmid.html – 15/4/2019).

Table 1 Primers used in this study.

Primer name	Primer sequence (5′–3′)	Amplicon size (bp)
VIM START-F	ATGTTCAAACTTTTGAGTAAGT	801
VIM END-R	GCTACTCAACGACTGAGCG	
IMP-AF	GAAGGCGTTTATGTTCATACTT	510
IMP-AR	GTTTGCCTTACCATATTTGGA	
5′CS	GGCATCCAAGCAGCAAG	–
3′CS	AAGCAGACTTGACCTGA	–
qnrAF	ATTTCTCACGCCAGGATTTG	516
qnraR	GATCGGCAAAGGTTAGGTCA	
qnrSF	GCAAGTTCATTGAACAGGGT	428
qnrSR	TCTAAACCGTCGAGTTCGGCG	
qnrBF	GGMATHGAAAATCGCCACTG	264
qnrBR	TTTGCYGYYCGCCAGTCGAA	
qnrBCF	GTTRGCGAAAAAATTRACAG	626
qnrBCR	CCMATHAYMGCGATRCCAAG	
qnrCF	GGGTTGTACATTTATTGAATCG	330
qnrCR	CACCTACCCATTTATTTTCA	
qnrDF	CGAGATCAATTTACGGGGAATA	582
qnrDR	AACAAGCTGAAGCGCCTG	
qnrVCm-F	GAGYTKTATGGTTTAGAYCCTCG	71
qnrVCm-R	TGTTCYTGYTGCCACGARCA	
aac(6′)-IbF	CGATCTCATATCGTCGAGTGTT	476
aac(6′)-IbR	TTAGGCATCACTGCGTGTTC	
gyrAF-Pae	GAGCTGAAACAGTCCTATCTCGACT	112
gyrAR-Pae	TCATGGCATAAAGCACACGG	

Results

Antimicrobial susceptibility. Detection and characterization of resistance markers

Twelve of the 38 (31%) carbapenem resistant *P. aeruginosa* isolates rendered positive in the double disk synergy test, suggesting the presence of an MBL. Ten of 12 isolates were also resistant to both ciprofloxacin and levofloxacin, and 8/12 to aminoglycosides (Table 2).

Using the MBL-multiplex PCR approach, bla_{VIM} was detected in 11/12 isolates while bla_{IMP} was detected in the remaining one. No KPC coding genes were detected in any of the 38 isolates. PCR amplification using specific primers and further sequencing identified bla_{VIM-2} (5), bla_{VIM-11} (6), and bla_{IMP-13} (1). Despite many attempts, none of the plasmids harboring bla_{VIM} could be transferred. Both bla_{VIM} and

Table 2 Antimicrobial resistance profile of the tested isolates.

Isolate	*qnrB*	*qnrA*	*qnrS*	*qnrC*	*qnrD*	*qnrVC*	*aac-(6′)-Ib*	*oqxA*	*oqxB*	*qepA*	MBL	Zone inhibition diametre (mm)			
												LEV	CIP	AKN	TOB
6	-	-	-	-	-	-	+	-	-	-	VIM-11	6	9	22	23
33	-	-	-	-	-	-	+	-	-	-	VIM-2	6	6	22	12
86	-	-	-	-	-	-	+	-	-	-	IMP-13	26	34	23	24
184	-	-	-	-	-	-	+	-	-	-	VIM-2	6	6	19	6
4824	-	-	-	-	-	-	+	-	-	-	VIM-2	6	6	29	19
33592	-	-	-	-	-	-	-	-	-	-	VIM-11	6	10	9	6
373501	-	-	-	-	-	-	+	-	-	-	VIM-11	6	6	9	6
74337	-	-	-	-	-	-	-	-	-	-	VIM-11	20	24	16*	14*
62102	-	-	-	-	-	-	+	-	-	-	VIM-2	6	10	9	6
4055214	-	-	-	-	-	-	-	-	-	-	VIM-2	6	11	21	8
C6	-	-	+	-	-	-	+	-	-	-	VIM-11	9	13	11	6
C6-3	-	-	-	-	-	-	+	-	-	-	VIM-11	6	11	9	6

None of the *aac-(6′)-Ib* detected corresponded to the quinolone modifying variant. Lighter shades of gray indicate susceptibility while darker shades indicate resistance.
*: Correspond to intermediate category.

bla_{IMP} were located as the only gene in the variable region of a class 1 integron.

One isolate, named *Pae*C6, rendered positive PMQR PCR results, the amplicon was further identified as *qnrS1*. *Pae*C6, also harbored bla_{VIM-11}. No other PMQR determinant could be detected in any isolate. Even though *aac-(6′)-Ib* was detected in 10 isolates, none of them harbored the quinolone-modifying variant.

The *gyrA* QRDR of PaeC6 exhibited 2 substitutions, Thr83Ile and Ile89Leu, when compared to the same region in PAO-1.

Plasmid characterization

All the plasmids were non-typeable by the PBRT scheme.

The plasmid harboring *qnrS1* (pP6qnrS1) could not be transferred by electroporation. This plasmid was fully sequenced (A.N.: MH061383) and had a size of 117 945 bp with 154 CDS and an average G+C content of 51.55%. The replicon was contained in a region that spanned from the base 55069 to 64995, within this fragment the *repB*, *parA* and *parB* genes showed 100% identity with replicons of IncR plasmids either when using PlasmidFinder database or GenBank. This region also contained the partitioning site *ParS* and the UV reparation system *umuCD*. Near the 5′ end of this 9.9 kb region, a resolvase coding gene (*resD*) was found, which could be potentially involved in the multimer resolution system of this plasmid. The replication regulator *korC* was located downstream the replicon region.

The addiction systems investigated by PCR according to Mnif et al. rendered negative results. However, in the plasmid sequence, the VapCB toxin-antitoxin system which belongs to the VagCD superfamily was detected at position 9500.

A *tra* region was found; nevertheless *traS* was missing and some of the remaining *tra* genes were either truncated or incomplete, thus explaining the failure to render transconjugants. An *oriT* sequence was detected between positions 100 478 and 100 873, displaying 100% identity with the same region of 3 different plasmids (NC004998, NC009837 and LT985322).

IS*Kpn19* was detected upstream of the replicon region, followed by *qnrS1* and IS*26*. Furthermore, plasmid maintenance genes such as *klcA*, *sopA*, *sopB* were located following IS*26* (Fig. 1). Additional aminoglycoside resistance genes were found along the plasmid such as *aac-(3)-IId*, *aph-(6)-Id* and *aph-(3″)-Ib*; however bla_{VIM-11} was not identified in this platform. Although the replicon of pP6qnrS1 corresponded to an IncR plasmid of *P. aeruginosa*, the overall backbone showed the strongest homology with that of pHNEP28_cfr from *E. coli* (AN: KT845955.1) (Fig. 2).

Discussion

Even though MBL production is not the prevalent carbapenem resistance mechanism in *P. aeruginosa*, their potential dissemination places it as the most clinically concerning. Reports on *P. aeruginosa* plasmids harboring MBL coding genes are common worldwide. The high plasticity of this species to acquire plasmid-borne resistance markers allows the horizontal genomic pool of *P. aeruginosa* to become a reservoir of a growing number of antibiotic resistance genes. The bla_{VIM} and bla_{IMP} alleles detected in this study are in accordance with those previously reported in Argentina[12]; nevertheless, to the best of our knowledge there are no previous reports of isolates carrying simultaneously MBL and PMQR determinants in *P. aeruginosa* in this country.

Although a wide set of plasmids harboring *qnrS1* have been described in *Enterobacteriales* (IncColE, IncFI, IncHI, IncI1, IncL/M, IncN, IncX and IncR), the literature on PMQR in *P. aeruginosa* is scarce. There are descriptions of *aac-(6′)-Ib-cr*, *qnrA1*, *qnrD*, *qnrS1* and *qnrVC1*; however only the last two markers were associated to β-lactamases, bla_{TEM-1} and bla_{VIM-2}, respectively[3,13]. Although studies were carried out to detect PMQR in *P. aeruginosa* collected since 1990, the recent date of these reports may suggest that there is an

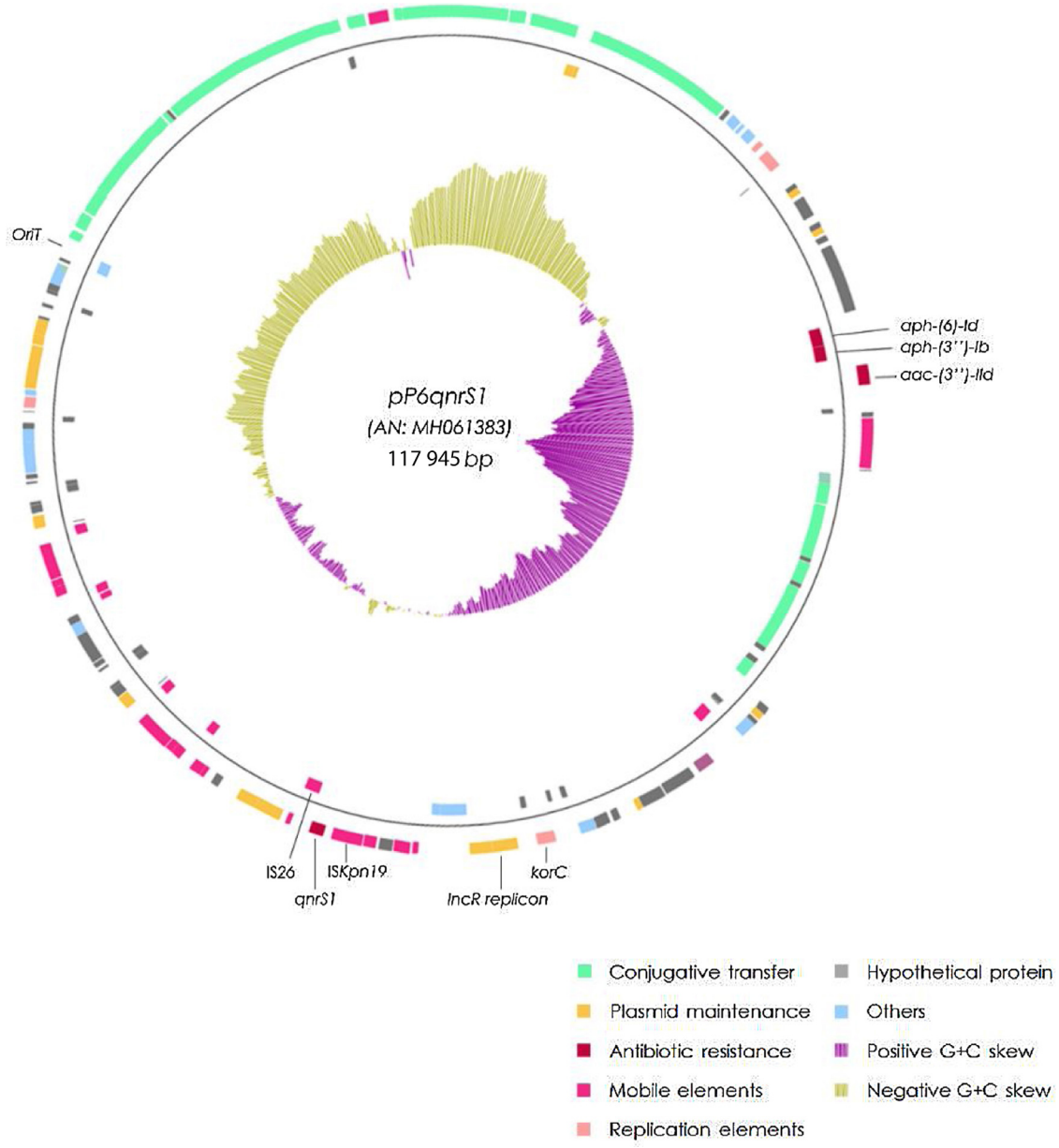

Figure 1 Plasmid harboring *qnrS1* recovered from *P. aeruginosa* P6 (pP6qnrS1) AN: MH061383.

ongoing increase in the mobile genetic pool in *P. aeruginosa* which could provide multiple resistance to the most clinically used antibiotics[15].

The fluoroquinolone resistance observed in *Pae*C6 was allegedly the product of a pair of mutations in *gyrA*, one of which is known to generate the mentioned phenotype (Thr83Ile) while the other has not been described to date[20]. This should not, nevertheless, draw the attention out of the PMQR determinants found in this isolate since the presence of such markers is known to be a first step toward the selection of quinolone-resistant bacteria.

Just like the plasmids belonging to the IncR incompatibility group, pP6qnrS1 holds a conserved ''replicon backbone'' in which the genes necessary for the replication are located. In contrast, while most of the plasmids belonging to this incompatibility group show a set of genes related to plasmid maintenance immediately upstream of the replicon, the plasmid here described was found to harbor a PMQR marker, *qnrS1*, flanked by IS*Kpn19* and IS*26* in this position.

In accordance with previous reports on IncR plasmids, pP6qnrS1 carries multiple IS and mobile elements, but also several resistance markers, mainly conferring resistance to aminoglycosides[7]. All IncR plasmids reported to date, including pP6qnrS1, are known to be non-transferable by conjugation, due either to a complete absence of some of the *tra* genes or to the presence of an abnormal truncated *tra* operon[7].

IncR plasmid deposits are scarce in databases, none of them being from Argentina. Considering that plasmids belonging to this complex are known to be non-conjugative, the possible role of the emerging IncR complex in the spread

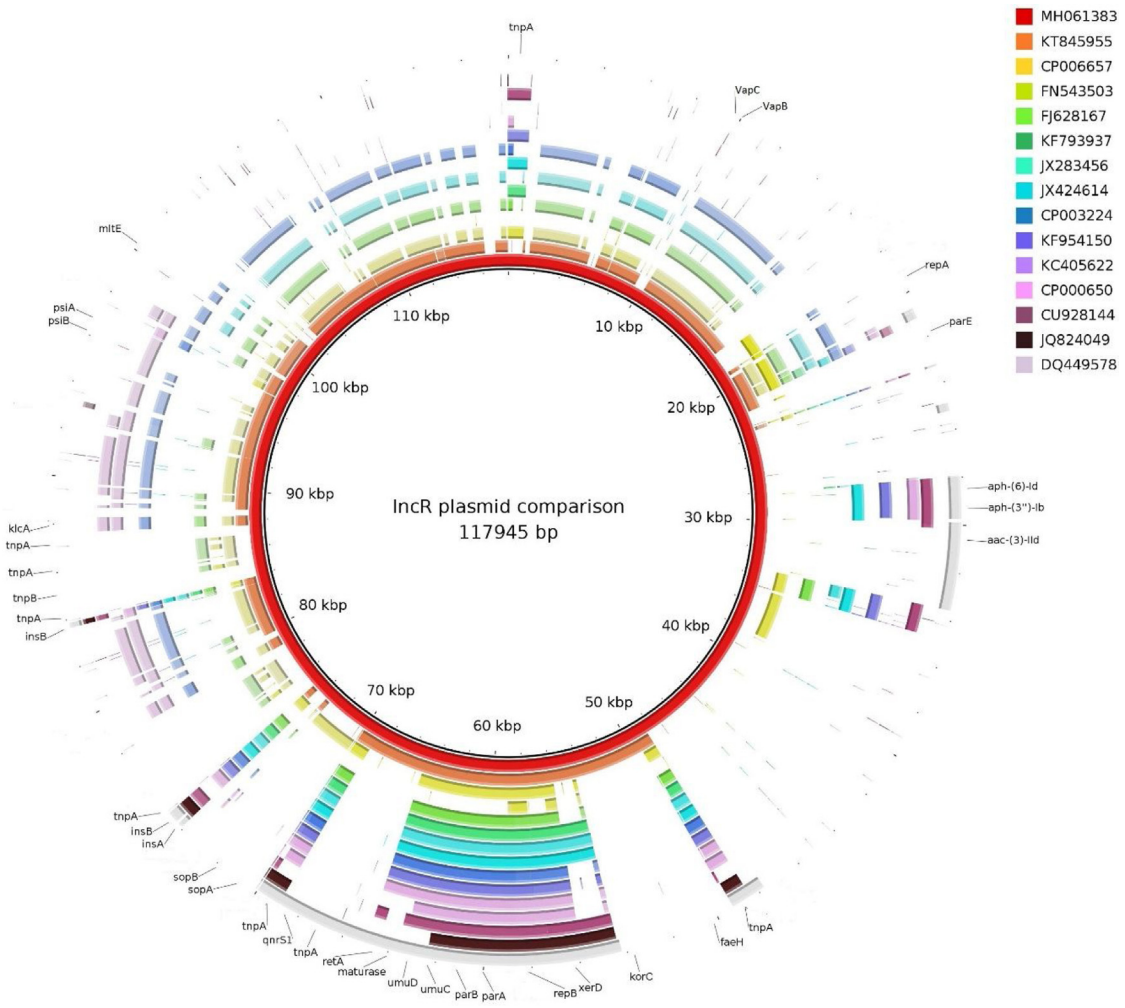

Figure 2 Comparison of IncR plasmids available in public databases.

of multidrug resistances is difficult to evaluate. However, the presence of an *OriT* in plasmid pP6qnrS1, makes it possible for this plasmid to be mobilizable.

Conflict of interest

The authors declare that they have no conflicts of interest.

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