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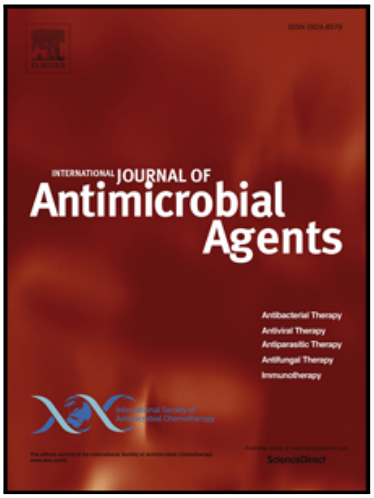

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Highlights

- Eleven *bla*$_{PER-2}$-harboring plasmids from *Enterobacterales* were fully sequenced
- The *bla*$_{PER-2}$ gene was encoded on a variety of plasmid types (IncA, C, FIB, HI1B, N2)
- IS*Pa12*-composite transposons are suggested to be involved in *bla*$_{PER-2}$ origin recruitment and dissemination
- A novel IS*Pa12*-composite transposon Tn*7390* carrying *bla*$_{PER-2}$ gene was identified
- IS*Kox2*-like elements seem to play a role in *bla*$_{PER-2}$ gene in dissemination

Diversity of genetic platforms harboring the *bla*$_{PER-2}$ gene in Enterobacterales and insights into the role of IS*Pa12* in its mobilization and dissemination.

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Abstract

The production of PER-like extended-spectrum β-lactamases has recently been associated with reduced susceptibility to the last resort drugs aztreonam/avibactam and cefiderocol. PER-2 have been mainly confined to Argentina and neighboring countries. Until now, only three plasmids harboring *bla*$_{PER-2}$ genes have been characterized but very little is known about the involvement of different

plasmid groups in its dissemination. This study analyzed the diversity of genetic platforms associated with *bla*$_{PER-2}$ genes from a collection of PER-producing Enterobacterales by describing both the close environment as well as the plasmid backbones. Full sequences of eleven plasmids were obtained by short (Illumina) and long read (Oxford Nanopore or PacBio) sequencing technologies. *De novo* assemblies, annotation and sequence analysis were performed by Unicycler, Prokka and BLAST. Plasmids analysis revealed that *bla*$_{PER-2}$ gene is encoded on plasmids of different incompatibility groups (A, C, FIB, HI1B, N2) suggesting that this gene may have been disseminated through a variety of plasmids. Analysis and comparison with the few public available nucleotide sequences describing *bla*$_{PER-2}$ genetic environment, including those from the environmental species *Pararheinheimera* spp. (considered as the progenitor of *bla*$_{PER}$ genes), suggests a role of IS*Pa12* in *bla*$_{PER-2}$ gene mobilization from the chromosome of *Pararheinheimera* spp. Also, the *bla*$_{PER-2}$ gene was carried by a novel IS*Pa12*-composite transposon Tn*7390.* In addition, its association with IS*Kox2*-like elements in the close genetic environment in all analyzed plasmids suggests a role of this IS in further dissemination of *bla*$_{PER-2}$ genes.

Keywords: PER-2, ESBL, plasmid-mediated resistance, transposons

1. Introduction

The PER extended spectrum β-lactamases (ESBL) family counts for at least sixteen different variants up to date (http://www.bldb.eu/BLDB.php?prot=A#PER) [1]. PER enzymes share distinctive biochemical and structural features, such as high catalytic efficiency on all oxyimino-cephalosporins (e.g. cefotaxime, ceftazidime) and a differential folding of the Ω-loop due to the presence of a trans bond between residues 166-167 [2]. Among them, PER-1 and PER-2 are the most frequently reported enzymes [3]. Despite their hydrolytic efficiency, plasmids encoding PER-2 have been mainly confined

to Argentina [3,4] or neighboring countries [5–7], and have been only sporadically reported outside South America [8,9].

Avibactam shows lower inhibition constants on PER-2 as compared to other class A β-lactamases [10]; therefore, production of PER enzymes has been associated with reduced susceptibility to aztreonam/avibactam, and also to cefiderocol, another last resort drug [9,11]. The origin of the PER family is associated with different (and for sure, some yet not described) environmental species of the recently proposed *Pararheinheimera* genus, excised from *Rheinheimera* [12].

In a previous study, we have described the complete sequence of an IncA plasmid (pCf587) harboring bla_{PER-2} gene from a *Citrobacter freundii* isolate recovered almost 20 years ago in Argentina [4], one of the few reports describing a complete bla_{PER-2}-harboring plasmid ([13] and Genbank # CP046506) and of its genetic environment [14,15]. To evaluate the diversity of genetic platforms associated with bla_{PER-2}, eleven bla_{PER-2}-harboring plasmids from Enterobacterales isolated between 1997-2012 from hospitalized patients in different cities of Argentina were fully sequenced and the genetic environments of bla_{PER-2} genes compared, to assess the genetic events likely at the origin of initial gene mobilization and further dissemination in clinical isolates.

2. Materials and methods

2.1. Bacterial isolates

Eleven strains were randomly selected from a collection of PER-2-producing Enterobacterales recovered between 1997 and 2012 in Argentina (Table 1). Purified plasmid DNA [16] was electroporated into *Escherichia coli* Top10 (Invitrogen). The transformant cells were plated on Lysogeny Broth (LB) agar plates containing 100 µg/ml ampicillin, and presence of bla_{PER-2} gene was confirmed by conventional PCR [17]. Also, minimal inhibitor concentrations (MICs) on parental strains and transformant clones were determined by the agar dilution method according to CLSI guidelines (Table S1) [18].

2.2 Whole genome sequencing (WGS)

Genomic DNA was extracted from the *E. coli* transformant cells using the PureLink™ genomic DNA Mini Kit (Thermofischer, Les Ulis France) following manufacturer's instructions. Dual-indexed sequencing libraries were constructed using NEBNext® library preparation kit and the Multiplex Oligos for Illumina® (NEB, Boston, USA). Libraries were pooled and 100 pM were sequenced on the Illumina Next 500 (2x150 bp) (Illumina, California, USA). In order to obtain large DNA fragments for long read sequencing, genomic DNAs were extracted using Qiagen Genomic-tips 20/G (Qiagen, Les Ulis, France) and sequenced either by a MinION sequencer (Oxford Nanopore Technologies, UK) using a R9.4 flow cell and the 1D native barcoding genomic DNA kit (SQK-LSK109), or by PacBio sequencing technology (Macrogen, Seoul, South Korea). Hybrid *de novo* assemblies were obtained with Unicycler v.0.4.8.0 [19] using both generated libraries. Gene prediction and annotation were performed by using Prokka v.1.14.6 [20], and manually curated with BLAST online. Identification of acquired antibiotic resistance genes and plasmid incompatibility group were conducted using Web based software available at the Center of Genomic Epidemiology (https://www.genomicepidemiology.org/): ResFinder 4.1 and PlasmidFinder 2.1, respectively. .

3. Results and Discussion

The overall features of parental strains and *E. coli* derived clones expressing bla_{PER-2} genes are listed in Table 1.

MICs reflected the expected behavior towards β-lactams and their combination with β-lactamase inhibitors according to the antibiotic resistance genes detected in each plasmid (Table S1). No categorization is provided for transformants. Of notice, resistance to ceftazidime/avibactam is not uniformly detected in all clinical isolates and may depend on how ceftazidime resistance is defined in CLSI/EUCAST recommendations, on enzymatic activity (kinetics and expression levels) and the presence of other potential resistance markers. Even if PER-2 increases by itself ceftazidime/avibactam

and aztreonam/avibactam MICs values, the effect is lower compared to other PER variants in isogenic bacterial backgrounds [11].

The *bla*$_{PER-2}$-harboring plasmids varied in size (range 45 to 304 kb) and belonged to different incompatibility groups, namely A (1), C (4), FIB (1), FIB/HI1B (1), N2 (4). Many of them encode several other antimicrobial resistance genes (ARGs) (Table 1). The previously described "IS*Pa12*/*bla*$_{PER-2}$/*gst*-like/Δ*abct*" gene arrangement [4,17] was observed in all cases, either with a complete or partial IS*Pa12* (Fig. 1). In addition, one or two copies of an IS*Kox2*-like element is usually flanking these structures. Schematic representations of the sequenced plasmids are shown in Fig. S1.

3.1 The IncA plasmid

The IncA plasmid pKpnLUIS presented 100% coverage and 99% sequence identity with the previously reported IncA pCf587 plasmid (MG053108) where *bla*$_{PER-2}$ gene is embedded in the same resistance island (RI) (Fig. 1).

3.2 IncC plasmids, the novel Tn*7390* and the role of IS*Pa12* and IS*Kox2*-like elements in *bla*$_{PER-2}$ gene recruitment and dissemination

Plasmids pKpn3863 and pEc5706 belong to an IncC type 2 [21]. They shared 97% coverage with 100% identity between them, diverging by a particular RI (Fig. S1). Several locations close to or within the *rhs*2 gene (encoding for a large RHS protein with an hypothetical toxin activity) of IncC type 2 plasmids are the site for insertion of different RIs [21]. For pKpn3863 and pEc5706, the same novel composite transposon Tn*7390* (13,249 bp) [22] carrying the *bla*$_{PER-2}$ gene is present in this area but inserted in a novel location compared to those previously reported. This novel location lies between the sites 5 and 6 (see Figure 3 in [21]) that corresponds to positions 117,230-117,237 in the IncC type 2 pR55 reference plasmid (JQ010984). The novel Tn*7390* is flanked by two inversely oriented copies of IS*Pa12;* one of them is located next to the *bla*$_{PER-2}$ gene (IS*Pa12*/*bla*$_{PER-2}$/*gst*-like/Δ*abct*) as previously described, and the other is 4,875 bp upstream an IS*Kox2*-like element, next to the Δ*abct* gene (this gene encodes for a putative truncated ABC-transporter). Direct repeats were found adjacent to the transposon

boundaries suggesting its mobilization occurred by a conventional transposition event. The region between the IS*Kox2*-like element and the right-hand IS*Pa12* (Fig. 1) is similar to that previously reported in IncA pCf587 [4] and IncA pKpnLUIS, except that is in a 1,173-bp region (probably derived from a deletion in pCf587 and pKpnLUIS), and identical to that found in plasmids pEc8791 and pKpnC6 (discussed later), although, in these four plasmids (pCf587, pKpnLUIS, pEc8791 and pKpnC6), this region is present downstream the bla_{PER-2}-associated IS*Pa12*, denoting a rearrangement of the "IS*Pa12*/bla_{PER-2}/*gst*-like/Δ*abct*" environment (Fig. 1).

According to PlasmidFinder, pEc8791 and pKpnC6 *rep*A loci share 98% identity with the *rep*A locus probe from pNDM-KN IncC plasmid (JN157804); the encoded replication initiator protein RepA displays three and four amino acids substitutions, respectively. Compared to the IncC plasmid backbone [21], pEc8791 has an inversion of the region between the conjugative transfer system *traN* and the H-NS-like *acr2* genes; the large *orf* between the conjugative transfer system *traA* and the thiol:disulfide interchange *dsbC* genes predicts a 1,847-amino acids protein having 97% identity with that corresponding to orf1847 present in type 2 IncC plasmids. Also, the *rhs* gene is replaced by a RI carrying the bla_{PER-2} gene (Fig. S1) in which the region between an IS*Kox2*-like and the bla_{PER-2}-associated IS*Pa12* is equivalent to that in Tn*7390* as previously mentioned (Fig. 1). Upstream the Δ*abct* gene a ΔIS*Kox2*-like is also present.

On the other hand, pKpnC6 shows a large deletion comprising the region between the DNA cytosine methyltransferase *dcm2* and the integrase *int* genes, with the loss of several *tra* genes (among others), although a RI carrying the bla_{PER-2} gene is still present as in the other plasmids (Fig. S1). The genetic arrangement between an IS*Kox2*-like and the bla_{PER-2}-associated IS*Pa12* is equivalent to that previously discussed for pEc8791 and Tn*7390*. Interestingly, upstream the Δ*abct* gene, two extra copies of IS*Kox2*-like elements are present (Fig. 1); one, located next to the Δ*abct* gene, and the other at 16,812 bp upstream this last IS. The region between these two IS*Kox2*-like elements includes a copy of ΔIS*Pa12*, the remaining part of Δ*abct*, and two other genes encoding for hypothetical proteins having 92 and

93% identity with those present in *Rheinheimera mangrovi* sp. nov. LHK132 chromosome (CP034683) [23] (as the closest relative in the genus), among others. Also, the *bla*$_{PER-2}$ gene shares 92% nucleotide sequence identity with the *bla*$_{PER}$ gene from LHK132, and the *in silico* reconstructed *abct* gene of pKpnC6 shows 95% identity with the homologous gene in LHK132. This observation suggests disruption of the *abct* gene by an IS*Kox2*-like element and plasmid-mediated recruitment and acquisition of *bla*$_{PER}$ gene from some *Pararheinheimera* spp. (Fig.1 and S2), likely involving IS*Pa12*. A similar gene arrangement is found in IncFIB pKpn33002 and IncFIB/HI1B pKpnJMC plasmids (described later), but in equivalent orientation with the *abct* gene environment present in LHK132, indicating a subsequent gene rearrangement in pKpnC6 RI. Furthermore, the *bla*$_{PER-2}$ gene environment present in *Acinetobacter radioresistens* A154 strain (NZ_PXJD01000035.1) [15] discloses a complete *abct* gene and the two same genes encoding for hypothetical proteins as those found in pKpnC6, pKpn33002 and pKpnJMC in a composite transposon made of two IS*Pa12* copies different from Tn*7390*. This finding strongly suggests a first recruitment from the environmental species in an IS*Pa12*-composite transposon followed by interruption of the *abct* gene by the IS*Kox2*-like (in some plasmids) and its further role in *bla*$_{PER-2}$ gene dissemination.

In the previously reported IncC pCf164_LMB-1 plasmid from *C. freundii* strain 164 (MH475146) [13], the *bla*$_{PER-2}$ gene is present in another composite transposon as compared with Tn*7390,* or that found in *A. radioresistens* A154, which also consists of two IS*Pa12* copies. In this transposon, the *abct* gene is complete and the two genes encoding for hypothetical proteins described above are absent. Also, the right-hand IS*Pa12* is interrupted by an IS*30* family transposase. Moreover, the gene arrangement in contig 34 in the *Shewanella* sp. Shew256 PER-2 producing strain (NZ_NAJR01000034) [14] is similar to this composite transposon (Fig. 1).

3.3 IncFIB and HI1B plasmids

The pKpn33002 plasmid was classified as belonging to IncFIB group; the *rep*FIB locus has 99% nucleotide identity to the probe from pNDM-Mar plasmid (JN420336) (no amino acid substitutions in

the translated protein). In pKpnJMC plasmid two replicons were identified, IncFIB-type and HI1B-type. In both plasmids the *bla*$_{PER-2}$ gene is present in RIs where the *abct* genes are split in two by an IS*Kox2*-like and the genes coding for hypothetical protein homologous to those in LHK132 and a ΔIS*Pa12* next to them are present (Fig. 1). Also, the *bla*$_{PER-2}$-associated IS*Pa12* is interrupted by an IS*26* and pKpnJMC has a second copy of the *bla*$_{PER-2}$ gene within the RI.

3.4 IncN2 plasmids

The four plasmids pEcI23, pKpnN11, pKpnC22 and pEclC18 belong to IncN2 group and share a similar relatively small backbone (Fig. S1). In all cases, an also small RI is present at the same backbone location as described in other IncN2 plasmids [24] carrying the *bla*$_{PER-2}$ gene. In these RIs, a ΔIS*Pa12* located next to the *bla*$_{PER-2}$ gene and a IS*Kox2*-like element are found upstream the Δ*abct* gene as seen before in all studied plasmids.

4. Conclusion

In spite of the small size of the plasmids collection analyzed in this study, the *bla*$_{PER-2}$ gene was found to be associated with plasmids of different incompatibility groups, irrespectively of their high (IncF, C, N), or low prevalence (IncA) among Enterobacterales [21,25] as part of different IS*Pa12*-composite transposons or RIs, denoting its capability for mobilization between plasmids. Despite its high hydrolytic efficiency, PER-2 has a low prevalence in South America, and even lower prevalence worldwide [2].

This study provides insights into a 15-years period history of *bla*$_{PER-2}$ gene mobilization from its original source and its horizontal transfer suggesting a role of IS*Pa12* in the recruitment from its origin, as well as in horizontal mobilization mediated by different IS*Pa12*-composite transposons. Additionally, the association of the *bla*$_{PER-2}$ gene environment with IS*Kox2*-like suggests its possible role in the dissemination among different RI and plasmid types. Interestingly, IS*Kox2*-like elements were also associated with the *bla*$_{NDM-1}$ carbapenemase gene mobilization [26,27]. Recent studies reported an

association between production of PER variants with metallo-β-lactamases, i.e. co-expression of PER-1 or PER-7 with NDM-1 in *Providencia rettgeri* and *Acinetobacter baumannii*, among others [28,29]. PER and NDM enzymes have been associated with reduced susceptibility to cefiderocol [11]; and PER enzymes also to reduced susceptibility to aztreonam/avibactam [9,11]. Both cefiderocol and aztreonam/avibactam are last resort drugs for infections due to multidrug-resistant Gram-negative bacilli. Thus, the association of bla_{PER} variants with clinically relevant carbapenemase genes in the same genetic platform may play a role in future co-selection by antibiotics and in dissemination.

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Competing Interests

No conflict of interest.

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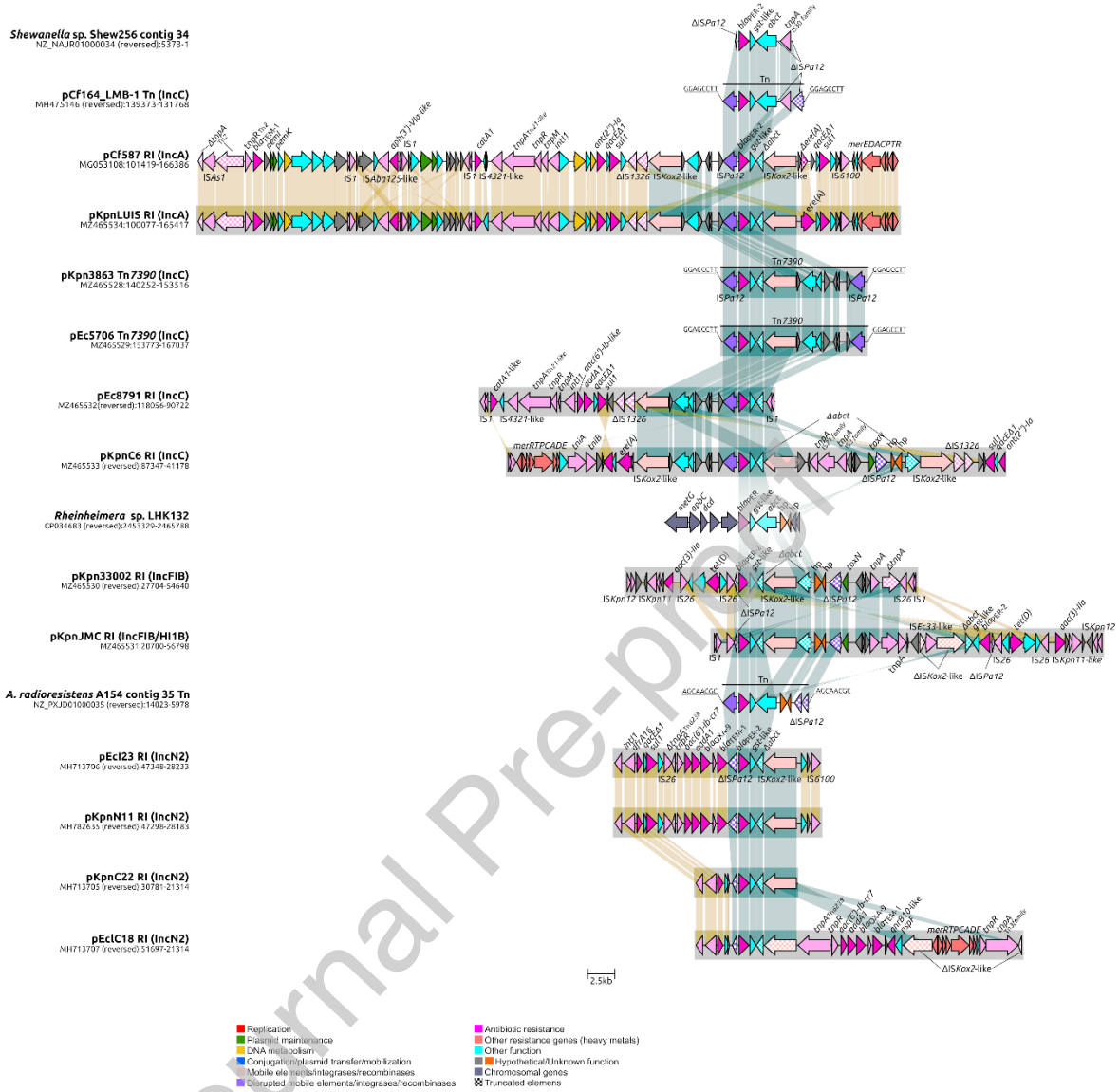

FIG 1. Comparative schematic representation of *bla*$_{PER-2}$ containing resistant islands or transposons. The figure was created using Clinker v0.0.26. Each CDS is color-coded by its predicted function as shown in the figure. Shading denotes regions of homology. Direct repeats from transposons are written in the figure. Grey boxes indicate entries from this study.

Table 1. Description of the PER-2-producing source strains, derived *E. coli* transformant cells, and *bla*$_{PER-2}$ harboring plasmids.

City	Source strain name	Isolation year	Sample type	Transformant name[a]	*bla*$_{PER-2}$ harboring plasmid					
					Name	Incompatibility group[b]	Size (kb)	Other antibiotic resistance genes	Additional resistance	GenBank accession number
Buenos Aires	*K. pneumoniae* LUIS	1997	Urine	TF LUIS	pKpnLUIS	A	191.5	*bla*$_{TEM-1}$, *ant(2'')-Ia*, *aph(3')-VIa*-like, *ere(A), catA1, sul1*	mercury	MZ465534
Buenos Aires	*K. pneumoniae* JMC	1997	Blood	TF JMC	pKpnJMC	FIB*, HI1B*	304.0	*bla*$_{TEM-1}$, *bla*$_{SCO-1}$, *bla*$_{OXA-9}$, *aac(6')-Ib*-like, *aac(3)-IIe*, *aadA1*, *tet(B)*, *tet(D)*, *catA1*, *dfrA8.*	nickel, chromate, tellurium.	MZ465531
Buenos Aires	*K. pneumoniae* 33002	1999	Urine	TF 33002	pKpn33002	FIB*	240.0	*bla*$_{SCO-1}$, *bla*$_{SHV-2}$, *aph(3')-Ia*, *aac(3')-IIe*, *tet(D)*, Δ*catA1*, *dfrA8*.	nickel	MZ465530
Buenos Aires	*E. coli* 8791	2000	Urine	TF 8791	pEc8791	C*	161.7	*aadA1*, *aac(6')-Ib*-like, *catA1*, *sul1*, *sul2.*	-	MZ465532
Buenos Aires	*K. pneumoniae* 3863	2004	Broncho alveolar lavage	TF 3863	pKpn3863	C	175.6	*bla*$_{CTX-M-2}$, *bla*$_{OXA-2}$, *aac(6')-Ib*-like, *aph(6)-Id*, *aph(3'')-Ib*, *tet(A)*, *mph(E)*, *msr(E)*, *floR*, *sul1*, *sul2.*	mercury	MZ465528
Buenos Aires	*E. coli* 5706	2004	Urine	TF 5706	pEc5706	C	189.1	*bla*$_{OXA-2}$, *aac(6')-Ib*-like, *aph(3'')-Ib*, *aph(6)-Id*, *tet(A)*, *erm(B)*, *mph(E)*, *mrs(E)*, *floR*, *sul1*, *sul2*, *dfrA16.*	mercury	MZ465529
Santa Fe	*K. pneumoniae* C6	2012	Urine	TF C6	pKpnC6	C*	117.2	*bla*$_{TEM-1}$, *bla*$_{OXA-9}$, *aac(6')-Ib-cr7*, *aadA1*, *ant(2'')-Ia*, *ere(A) sul1*.	mercury	MZ465533
Santa Fe	*E. coli* I23	2012	Feces	TF I23	pEcI23	N2	62.4	*bla*$_{TEM-1}$, *bla*$_{OXA-9}$, *aadA1*, *aac(6')-Ib-cr7*, *sul1*, *dfrA16*.	-	MH713706

Santa Fe	*K. pneumoniae* N11	2012	Urine	TF N11	pKpnN11	N2	62.4	*bla*$_{TEM-1}$, *bla*$_{OXA-9}$, *aadA1*, *aac(6')-Ib-cr7*, *sul1*, *dfrA16.*	-	MH782635
Santa Fe	*K. pneumoniae* C22	2012	Blood	TF C22	pKpnC22	N2	45.9	Δ*sul1*, *dfrA16.*	-	MH713705
Santa Fe	*E. cloacae* C18	2012	Blood	TF C18	pEclC18	N2	66.8	*bla*$_{TEM-1}$, *bla*$_{OXA-9}$, *aadA1,* *aac(6')-Ib-cr7*, Δ*sul1*, *dfrA16*, *qnrB10*-like.	mercury	MH713707

[a]Recipient strain, *Escherichia coli* TOP 10. [b]Asterisks indicate that the closest replicon probe sequence used by the PlasmidFinder tool do not match at 100% nucleotide identity.