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Abstract

Plasmids are the main vectors of antimicrobial drug resistance and virulence genes, especially in Enterobacteriaceae. Identification and classification of plasmids is essential for analysis of their distribution. The most widely used typing method is PCR-based replicon typing (PBRT). A new classification scheme based on relaxase gene typing has been described recently. We propose a practical application of this method, with the development of a multiplex PCR set targeting relaxase genes found on plasmids most frequently encountered in Enterobacteriaceae. This method, here called “plasmid relaxase gene typing” (PRaseT), was validated with 60 transconjugants and transformants harboring various replicon types. The method was tested with 39 multidrug-resistant clinical isolates including *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica* subsp. *enterica* carrying 1 to 7 replicons as well as with 17 plasmids non-typeable using PBRT; all replicons were tested in parallel with PBRT for comparison.Six multiplex PCRs and one simplex PCR, including 24 pairs of primers, recognized plasmids of groups A/C, B/O, colE, FIA, FIB, FIC, FV, FIIk, HI1, HI2, I1, K, L/M, N, P1α, Q1, U, W, X1, X2, X3 and X4. There was perfect correlation between PRaseT and PBRT results in 31/39 (79.5%) clinical isolates. Moreover, 11/17 (64.7%) plasmids non-typeable by PBRT could be typed by PRaseT. Our set of multiplex PCRs showed high sensitivity and specificity for the classification of resistance plasmids. It has proved complementary to the widely used PBRT and will improve the monitoring of plasmid distribution in every-day practice.

Keywords

Plasmid; Classification; Replicon typing; Relaxase; Multiplex PCR

Introduction

Plasmids are important agents of gene flux and have found to be responsible for the dissemination of multiple antibiotic resistance genes. Identification and classification of plasmids is essential for analysis of their distribution, their genetic relatedness and evolution, as well as for study of horizontal gene transfer. A classification scheme should be based on genetic traits that are universally present and constant. It should be robust and the corresponding experimental procedure should be easy. The basic replicon locus, which is always present on plasmids, has been used historically for classification. Plasmids were initially classified according to their incompatibility, which is directly related to replication. Incompatibility (Inc) was defined as the inability of two plasmids sharing common replication control (same Inc group) to be maintained in the subsequent lineage during conjugation (Datta and Hedges, 1971 and Novick, 1987). This method which requires plasmid transfer to the same host for testing is time-consuming and not practical for large-scale studies. Couturier *et al*. tested a method using hybridization with cloned replication regions as probes but with this method plasmid diversity is underestimated due to cross-hybridization (Couturier et al., 1988). In 2005, Carattoli *et al*. developed a PCR scheme of targeting replicons called PCR-based replicon typing (PBRT) (Carattoli et al., 2005). Eighteen pairs of primers were designed in order to perform 5 multiplex and 3 simplex PCRs recognizing the most frequently encountered plasmid incompatibility groups among Enterobacteriaceae. With this method, 27 Inc groups are currently recognized (Carattoli, 2009). It has been widely used to study plasmid spread and diversity in Enterobacteriaceae. However, PBRT has several drawbacks: (i) plasmids may carry multiple replicons and/or mosaic replicons, and new replicon types may escape classification with this technique; (ii) false-negative results with some Inc groups (e.g. L/M) have been reported (Carattoli et al., 2005) and (iii) PBRT targets multiple sites

such as the replication initiation protein gene (*rep*), the active segregation partitioning system (*par*), replication control systems (iterons, antisense RNA), and recently also the relaxase gene (Carattoli et al., 2005 and Johnson et al., 2012), which may cause confusion. Plasmid multilocus sequence typing were also developed to refine classification of plasmid subgroups (García-Fernández and Carattoli, 2010, García-Fernández et al., 2008, García-Fernández et al., 2011 and Phan et al., 2009).

Bacterial conjugation represents a unique process allowing transfer of plasmid DNA from a donor to a recipient bacterium through cell-to-cell contact. In this process relaxase is a key protein encoded by all transmissible plasmids, i.e. mobilizable and conjugative plasmids involved in horizontal gene transfer (reviewed by Smillie et al., 2010 and Wong et al., 2012). A classification scheme based on the mobilization region of transmissible plasmids has recently been developed (Francia et al., 2004 and Garcillán-Barcia et al., 2009). The scheme classified relaxases in six protein families and 31 subfamilies, depending on their phylogeny; subsequently, 19 degenerate primer pairs targeting the relaxase genes of γ-proteobacterial plasmids were designed (Alvarado et al., 2012). This degenerate primer MOB typing (DPMT) method has been used with success in previous studies (Curiao et al., 2011, Mata et al., 2012, Mata et al., 2010 and Valverde et al., 2009). However, this set of primers was not designed for screening purposes in clinical practice, but rather for experimental purposes in order to discover new relaxases (Alvarado et al., 2012).

The aim of the present study was to design a multiplex PCR method, called "plasmid relaxase gene typing" (PRaseT), including novel oligonucleotide primers targeting relaxase genes of the plasmids most frequently encountered in Enterobacteriaceae in clinical practice. These multiplex PCRs were carried out under maximum consensus thermal cycling conditions, and applied to various plasmids present in clinical isolates from several collections.

Materials and methods

Database search and primer design

An *in silico* analysis was carried out using GenBank BLAST (http://blast.ncbi.nlm.nih.gov/). For each Inc group, the relaxase/helicase gene was used as template; the presence of relaxase-specific multidomains was checked using CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/). Multiple alignments were performed with ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primer pairs covering most sequences in each family were designed using FastPCR software (http://primerdigital.com/fastpcr.html), while minimizing codon degeneracy (Table 1A).

In silico primer assay

Oligonucleotide primers were tested *in silico* for hybridization with plasmids of the Enterobacteriaceae referenced in GenBank. Some primers were refined to cover a maximum of reported sequences.

Bacterial strains

For validation of the PCR assays, experiments were conducted with 60 *Escherichia coli* transconjugants or transformants of Enterobacteriaceae (Table 2). All strains carried replicons of various types that encoded diverse β-lactamases conferring resistance to third-generation cephalosporins or carbapenems. They were part of three collections of, respectively, (i) *E. coli* strains isolated between 1997 and 2002 in various French university hospitals (for further

details see Marcadé et al., 2009 and Branger et al., 2005), (ii) *Klebsiella pneumoniae* strains from various geographical regions collected since the 1980s (D. Decré and G. Arlet, personal collection) and (iii) *Salmonella enterica* subsp. *enterica* strains representing various serovars (collection of the French National Reference Center for *E. coli*, *Shigella*, and *Salmonella*, Institut Pasteur). Assays with transconjugants and transformants for multiplex PCR optimization were carried out in triplicates.

All transconjugants, transformants and clinical strains used in this study were analyzed in parallel with PRaseT and the PBRT method applied previously (Carattoli et al., 2005, García-Fernández et al., 2009, Götz et al., 1996, Osborn et al., 2000 and Villa et al., 2010). The transconjugants used as positive controls in PRaseT reactions are given in Table 2. Other controls used in this study included three strains of the ECOR collection (Ochman and Selander, 1984) (ECOR 6, ECOR 10 and ECOR 19 harboring, respectively, pTPqnrS-1a-like-, pcolE1-like- and IncX2-plasmids), as well as reference plasmids pFBAOT6 (IncU) (Rhodes et al., 2004) and RP4 (IncP1α) (Datta et al., 1971).

After optimization on transconjugants or transformants carrying replicon of various types according to PBRT, we applied the PRaseT method to a panel of 39 clinical strains (21 *E. coli*, 16 *K. pneumoniae* and 2 *S. enterica*) carrying replicons of one to seven different types (Table 3), and 17 transconjugants or transformants that were non-typeable with PBRT (Table 4).

DNA extraction and PCR conditions

InstaGene matrix (Biorad, Marnes la Coquette, France) or lysis by boiling were used for total DNA extraction as previously described (Dallenne et al., 2010). Multiplex PCR was carried out using the Qiagen Multiplex PCR Kit (Qiagen, Courtaboeuf, France). The master mix

contained pre-optimized concentrations of HotStarTaq DNA polymerase and $MgCl_2$, deoxynucleotide triphosphate and PCR buffer. To all multiplex PCRs, solution Q (Qiagen) that facilitates the reaction with difficult-to-amplify templates by modifying DNA melting behavior was added. Total DNA in 5µl of bacterial lysate was subjected to multiplex PCR in a 50 µl volume. The conditions for multiplex PCR were optimized to ensure that all targets were sufficiently amplified for amplicons to be easily visible on 1.5% agarose gels. The optimal primer concentrations are reported in Table 1A. PCR conditions consisted in initial activation at 95°C for 15 min, followed by 30 cycles at 94°C for 30 s, 60°C for 90 s and 72°C for 90 s with a final extension at 72°C for 10 min; for Multiplex IV and V the annealing temperature was elevated to 65°C for 90 sec. Simplex PCR were performed in a 50 µL mix with 2 U of Taq DNA polymerase (Roche Diagnostics), 10× PCR buffer/$MgCl_2$ (Roche Diagnostics), 200 µM of each deoxynucleotide triphosphate (dNTP Mix Eurobio), 0.2 pmol/µL of each primer, 40 µL of sterile water and 2 µL of total DNA extract. PCR conditions consisted in 30 cycles [94°C for 1 min, 55°C for 40 sec, 72°C for 1 min], preceded by one cycle at 94°C for 5 min followed by one cycle at 72°C for 5 min. PCR products were separated at 100 V for 90 minutes in 1.5% agarose gel containing ethidium bromide and visualized using GelDoc (Biorad). PCR products were purified using the Exosap purification kit (illustra ExoStar 1-Step, Dutscher, Brumath, France) and subjected to bidirectional DNA sequencing using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730 XL capillary sequencer. Sequence analysis was carried out using BLAST and GenBank sequences.

Results and discussion

In silico analysis

We mainly focused on plasmid families previously found to be involved in the spread of resistance genes in Enterobacteriaceae (Carattoli, 2013, 2011 and 2009). Since no complete sequence of IncFIII, IncFIV, IncFVI, IncFVII and IncY plasmids have been reported and their sequenced segments do not contain relaxase genes, these plasmids were excluded from this study. So were those of the IncR family which is known not to contain any relaxase gene (Alvarado et al., 2012) and those of the IncJ family and R391-like elements that are part of integrative and conjugative elements (Burrus et al., 2006) the analysis of which is beyond the scope of this study. Also not considered here were some relatively rare groups (e.g. IncI2 plasmids) or poorly resolved groups (e.g. IncQ3 or IncT plasmids) (Alvarado et al., 2012).

The majority of plasmids had a single putative relaxase locus, which is consistent with the review by Smillie *et al.* (Smillie et al., 2010). In contrast, complete GenBank sequences of IncHI1 and IncHI2 plasmids had one to three putative relaxase/helicase loci on each reported plasmid (Supplementary Table S1). CD-Search results for each protein confirmed the presence of relaxase- or helicase-specific multidomains. We finally used five relaxase clades (arbitrarily designed HIα, HIβ, HIγ, HIδ and HIε) present among the various IncHI1 and/or IncHI2 plasmids (Supplementary Table S1). HIβ and HIδ relaxases were found to be encoded only on IncHI1 plasmids and HIγ and HIε relaxases only on IncHI2 plasmids while HIα relaxases were encoded on both IncHI1 and IncHI2 plasmids. We therefore designed three oligonucleotide primers covering the HIα, HIβ and HIγ relaxase, respectively.

In silico analysis led to the design of 24 pairs of primers for six multiplex PCRs (targeting the relaxase genes of plasmids belonging to Inc groups A/C, colE, FIA, FIB, FIC, FII, FIIk, FV, HI1, HI2, I1, L/M, N, P1α, Q1, U, W, X1, X2, X3 and X4) and one simplex PCR (targeting the relaxase genes of plasmids belonging to Inc groups B/O and K) (Table 1A).

Primer evaluation using transconjugants and transformants

In order to assess the sensitivity and specificity of each PCR, primers were tested using a collection of 60 recipient cells, with PBRT as the reference method (Table 2). Each primer pair was validated using all recipient cells, first in a simplex and then a multiplex PCR and target DNA of either single cells or cell mixtures was used. PCR conditions were optimized and all amplicons were sequenced. *E. coli* strain J53 was used as negative control in PCR experiments to test for possible cross-hybridization with chromosomal DNA. No non-specific amplification was observed.

All PRaseT results were consistent with the PBRT results, except for two strains which carried an IncR (*K. pneumoniae* strain S51) or an IncFIB/FII replicon (*E. coli* strain 81), that were undetected by the PRaseT method. For the first strain the result was not unexpected as IncR plasmids do not encode relaxases. For the second strain, the result was more surprising as IncF plasmids are known to be conjugative in most cases (Smillie et al., 2010). As it has been previously reported that Mob regions and mating pair formation (MPF) systems were in general of the same type (Smillie et al., 2010), we designed new primers targeting other conserved genes of the type IV secretion system (T4SS), i.e. *traB* (encoding a secretin-like protein) and *traC* (encoding an ATPase) (Supplementary Table S2). The eighteen PRaseT-positive IncF plasmids reported in Table 2 tested positive for *traB* and *traC* while the PRaseT-negative IncFIB/FII plasmid also tested negative with T4SS typing (data not shown). Interestingly, no transconjugant but only transformant was obtained from the parental strain of the later plasmid. We considered three possibilities: (i) a very divergent IncF relaxase gene that could not hybridize with our primers was present, (ii) the relaxase gene was truncated or (iii) the gene was absent. Complete sequencing of the plasmid will be performed to confirm one of these possibilities.

For IncHI plasmids, three primer sets were mandatory to differentiate IncHI1 from IncHI2. As noted above, in most cases, the identification of relaxases from IncHI1 and IncHI2 plasmids was obtained with positive results for HIβ- and HIγ-primers respectively (e.g. transconjugants S01477 and 102, Table 2). However, when Hiα PCR was the only positive result (e.g. IncHI2-containing Salmonella S09118), we used T4SS-typing as a complement to PRaseT (Supplementary Table S2). The positive result of PCR targeting the T4SS from IncHI2 plasmids confirmed the presence of a HI2 relaxase in S09118.

Evaluation of relaxase gene typing using clinical strains

To further confirm the specificity of the designed primer set, 39 clinical strains, each carrying from one to seven different replicon types, were submitted to PRaseT (Table 3). An example of the results is shown in Fig. 1. For 31 strains (79.5%) there was a perfect correlation between the results obtained with PRaseT and PBRT.

Five strains were positive with PBRT but negative with PRaseT. Among these, two (*E. coli* strains 19 and 34, Table 3) carried IncFIA/FIB/FII replicons. PCR targeting the T4SS genes *traB* and *traC* was negative, and the plasmid from neither strain could be transferred to a recipient cell by conjugation. Similarly, for the IncU plasmid of strain KpS15 tested negative with PRaseT, primers targeting *virB4*, the gene coding for the ATPase of the T4SS of IncU plasmids (Supplementary Table 2), were used. The result was negative. The two remaining strains (i.e *E. coli* 33 and 101) carried an IncP plasmid which could not be typed by PRaseT. Our primer pair was designed using the reference plasmid RP4 as template. However, this plasmid belongs to the IncP1α subgroup, while the IncP1 group consists of at least six

236 divergent subgroups (IncP1α, IncP1β, IncP1γ, IncP1δ, IncP1ε and IncP1ζ), many of which are
237 antibiotic resistance vectors in the environment (Alvarado et al., 2012, Bahl et al., 2009 and
238 Heuer et al., 2012). We think that our IncP1α relaxase primers were unable to classify all
239 IncP1 group replicons because of too great a divergence in gene sequences. PRaseT of IncP
240 group plasmids should be improved in further studies.

241 Finally, three strains (*K. pneumoniae* KpS63 and FM10, and *E. coli* 105) which were negative
242 with PBRT (after multiple PCR assays) were found to contain IncFIIk, IncHI and IncI1
243 plasmids, respectively, when PRaseT and T4SS typing was used (Table 3). The presence of
244 sequence divergence or mosaic replicons may explain these results that will be clarified by
245 sequencing. These observations underscore the complementarity between the PBRT and
246 PRaseT methods.

247

248 **Relaxase gene typing in recipient cells non-typeable with PBRT**

249 Seventeen recipient cells whose plasmids were found to be non-typeable with PBRT were
250 subjected to typing with PRaseT; parental strains included 14 strains of *E. coli* and 3 of *K.*
251 *pneumoniae.* PCR results are given in Table 4. In the 17 recipient cells, 11 (64.7%) plasmids,
252 mainly pHUSEC41-4-like mobilizable replicons, could be typed with PRaseT. The complete
253 sequence determination of plasmids from 6 recipient cells (i.e. *E. coli* strains 65, 66, 70, 71,
254 72 and 99) confirmed these results (unpublished data). The majority of bla_{SHV} ESBL genes
255 (six out of eight) were localized on pHUSEC41-4-like plasmids. Such colE-like plasmids
256 have been already reported as resistance vectors in Enterobacteriaceae (García-Fernández et
257 al., 2009).

Six plasmids could be typed neither with PBRT nor with PRaseT (Table 4). Complete sequence is available for 3 of them, i.e. plasmids from *K. pneumoniae* transformant S77 (pKpS77, SHV-12), *E. coli* transconjugant 76 (RCS47v1_pI, SHV-2) and *E. coli* transformant 93 (RCS63v1_p, CTX-M-3) (unpublished data). Plasmids pKpS77, RCS47v1_pI and RCS63v1_p were, respectively, < 50,000 bp, 117,001 bp and 22,308 bp long. In the sequences of two of them (pKpS77 and RCS63v1_p), no transfer region could be identified. The third plasmid (RCS47v1_pI, *E. coli* transconjugant 76) carried a new relaxase gene which presented 100% query cover and 99% maximum identity with those on plasmids pO111_2 (GenBank accession no. AP010962.1) and p12579_1 (GenBank accession no. CP003110.1); its replicase gene also presented 100% query cover and 99% maximum identity with its counterparts on plasmids pO111_2 and p12579_1. The three other non-typeable plasmids (contained in transformants from *K. pneumoniae* S33, *E. coli* 55 and *E. coli* 86) could not be transferred by conjugation, which is in favor of non-transmissible plasmids; this should, however, be confirmed by complete plasmid sequencing.

Conclusions

Our set of six multiplex PCRs and one simplex PCR allowed classification of the most frequently encountered transmissible plasmids in Enterobacteriaceae by targeting their relaxase gene. It stands as a promising complement to the widely used PBRT method in understanding plasmid spread and evolution, and can be applied to epidemiological surveys as well. Considering the complexity of constant plasmid evolution, the combined use of two complementary and practical classification tools should be advantageous and reduce the need for systematic full-length plasmid sequencing.

Competing interest

The authors declare that they have no competing interests.

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Tables

Table 1 - Primers used in this study

PCR name	Plasmid type detected	Primer name	Sequence (5'-3')	Plasmid prototype	EMBL accession No.	Target site	Amplicon size (bp)	Primer concentration (pmol/µL)
Multiplex I	IncFIIK	MRxeFII-K_for	CGATATTCTKGAACCCCGTA	pKPN3	CP000648.1	traI	297	0.2
		MRxeFII-K_rev	TCATTGCCCATAATYCGTCC					0.2
	IncFIA, IncFIB, IncFII	MRxeF-tot_for	ATCAGGAMCCACAGTTACAC	R100	AP000342.1	traI	753	0.2
		MRxeF-tot_rev	GTTTCATGATRTCRCGACTGAG					0.2
	IncFV	MRxeFV_for	CATCAAAGCGAARGAGYAGTTCAC	pED208	AF411480.1	traI	642	0.2
		MRxeFV_rev	TTACCCTCWGCAATATGRCGRA					0.2
Multiplex II	IncN	MRxeN_for	CCAGTTTAGAAACCCGATCA	R46	AY046276.1	traI	332	0.2
		MRxeN_rev	CGTAATCGTCGTAAATGCTG					0.2
	IncI1	MRxeI1_for	TTTAATCATGAGCAAACGCC	R64	AB027308.1	nikB	764	0.2
		MRxeI1_rev	ACAACYCTGTCACCATAATC					0.2
	IncL/M	MRxeLM_for	TCGCTATATCGTTGGTGATG	pCTX-M3	AF550415.2	nikB	491	0.2
		MRxeLM_rev	GGGTATGTTGGGCAATTTTC					0.2
	IncA/C	MRxeAC_for	TGATTGAGAAAGTGCGGAACTC	pRA1	FJ705807.1	traI	659	0.2
		MRxeAC_rev	CACACCATAGGAGAACTCGT					0.2
Multiplex III	IncHI1, IncHI2	MRxeHIα_for	CCAAAAGACTGACTTGGGAGCA	pMAK1	AB366440.1	helicase	873	0.2
		MRxeHIα_rev	CCGAGTTAGCCATCAAGGAA					0.2
	IncHI1	MRxeHIβ_for	GATGGTATGTGGATAGTCCG	pNDM-CIT	JX182975.1	traI	368	0.2
		MRxeHIβ_rev	GAATATGCCTCACYATCGCT					0.2
	IncHI2	MRxeHIγ_for	AATGACGTGGTTGAATACGA	R478	BX664015.1	traI	225	0.2
		MRxeHIγ_rev	AGAATAGAAATCAGCGGTCC					0.2
Multiplex IV	IncX1	MRxeX1_for	GAAAATGGTCGTAAGTCAGCT	pOLA52	EU370913.1	taxC	845	0.2
		MRxeX1_rev	TTCAGYCTGACAAGATCACCTGC					0.2
	IncX2	MRxeX2_for	CGAAAATGGTCGTAAGTCTGCA	R6K	X95535.1	taxC	560	0.2
		MRxeX2_rev	CATTACAAAAGCCCGTCCTG					0.2
	IncX3	MRxeX3_for	TGTTGACAAGGAATTCAGGGT	pIncX-SHV	JN247852.1	taxC	736	0.2
		MRxeX3_rev	ATGGTCGTAGCCAACATCAA					0.2
	IncX4	MRxeX4_for	TTAAACGCGCTAAAGGGACTGG	pSH146_32	JX258655.1	taxC	996	0.2
		MRxeX4_rev	GGGCAATATTTTCAATCTGCCAG					0.2
Multiplex V	Non typeable	MRxeMobC11_for	ACGGATTCGCTTRCCTGGMAGTG	pColEST258	JN247853.1	mobB	997	0.2
		MRxeMobC11_rev	TTCAGSGCGTACTGGTCGAGATCG					0.2
	Non typeable	MRxeMobQu_for	TCTCCAGAARCAGCGGCTACAG	pIGWZ12	DQ311641.1	mob	481	0.04
		MRxeMobQu_rev	ATGGTCAGCCCATGATGCACGCCA					0.04
	colE, Non typeable	MRxeMobP5-1_for	AGTCCGGGARCTYATYGATGCCAC	pCOLE1	J01566.1	mob3	367	0.04
		MRxeMobP5-1_rev	TATCAGRGAGWGTCAGYRTCCKGC					0.04
	colE, Non typeable	MRxeMobP5-2 _for	TCCGGTGGAYTATCTKCTGGGKA	pTPqnrS-1a	AM746977.1	mobA	656	0.2

		MRxeMobP5-2 _rev	AAGACTGYTCATARATRGCTCC					0.2
	colE, Non typeable	MRxeMobP5-3_for	GCTATCAGTCGCCCTGTTCGTC	pHUSEC41-4	HE603113.1	mob	147	0.04
		MRxeMobP5-3_rev	AGTACCCGATCATCAGTCAGTG					0.04
Multiplex VI	IncQ1	MRxeQ1_for	GATTTATCACCTTACGGCGAA	RSF1010	M28829.1	mobA	962	0.2
		MRxeQ1_rev	TACCTCCCGCAACTCTTTGG					0.2
	IncU	MRxeU_for	TATGACACTCTAGCCGGGTT	pRA3	DQ401103.1	nic	743	0.2
		MRxeU_rev	TGGATCTTGTCTTCCCACGGTT					0.2
	IncP1α	MRxeP1α_for	TGAAGTACATCACCGACGAG	RP4	X54459.1	traI	424	0.2
		MRxeP1α_rev	GTTTCGTGATTGTCACGCTC					0.2
	IncW	MRxeW_for	CATCGAGTTCGATACCCTTG	R388	BR000038.1	trwC	531	0.2
		MRxeW_rev	GCCACATAGTAAACGTCCTT					0.2
Simplex I	IncB/O, IncK	MRxeBO/K_for	GAATGCCATYATTCCGMAMAA	pO113	AY258503.2	nikB	800	0.2
		MRxeBO/K_rev	GTGATATACAGACCATCACTGG					0.2

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Table 2 - Recipient cells used in validation experiments*

No.	Parental strain	Tc/Tf	β-Lactamase	Plasmid classification	
				PBRT	PRaseT
6†	*E. coli*	Tc	TEM-10	L/M	L/M
40†	*E. coli*	Tc	TEM-24	A/C	A/C
44†	*E. coli*	Tc	TEM-52	I1	I1
48†	*E. coli*	Tc	TEM-52	X1	X1
50	*E. coli*	Tc	TEM-52	I1	I1
52	*E. coli*	Tf	TEM-52	X1	X1
51	*E. coli*	Tc	TEM-3	L/M	L/M
57†	*E. coli*	Tc	SHV-12	F	F
62	*E. coli*	Tc	SHV-2	FIB	F
64†	*E. coli*	Tc	SHV-5	X4	X4
73	*E. coli*	Tc	SHV-4	FIIK	FIIK
81	*E. coli*	Tf	CTX-M-3	FIB, FII	-
85	*E. coli*	Tf	CTX-M-1	FIA	F
91†	*E. coli*	Tc	CTX-M-3	N	N
98	*E. coli*	Tc	CTX-M-1	X1	X1
100	*E. coli*	Tc	CTX-M-1	FIA, FII	F
102†	*E. coli*	Tc	CTX-M-1	HI2	HIα, HIγ
104	*E. coli*	Tf	CTX-M-1	FIA, FIB, FII	F
105	*E. coli*	Tc	CTX-M-1	L/M	L/M
108	*E. coli*	Tf	CTX-M-1	FIA	F
110	*E. coli*	Tc	CTX-M-1	X4	X4
111	*E. coli*	Tc	CTX-M-1	FIA	F
114	*E. coli*	Tc	CTX-M-1	L/M	L/M
118	*E. coli*	Tc	CTX-M-1	FIA, FIB	F
120	*E. coli*	Tc	CTX-M-3	FIB	F
125	*E. coli*	Tc	CTX-M-1	FII	F
126	*E. coli*	Tc	CTX-M-1	N	N
127	*E. coli*	Tc	CTX-M-3	FIB	F
S6	*K. pneumoniae*	Tc	SHV-5	A/C	A/C
S9†	*K. pneumoniae*	Tc	SHV-4	FIIK	FIIK
S16	*K. pneumoniae*	Tc	CTX-M-3	A/C	A/C
S19	*K. pneumoniae*	Tc	CTX-M-15/OXA-1	N	N
S23	*K. pneumoniae*	Tc	CTX-M-3	N, A/C	N, A/C
S24	*K. pneumoniae*	Tc	TEM-3	A/C	A/C
S36	*K. pneumoniae*	Tc	CTX-M-3/OXA-1	FII	F
S43	*K. pneumoniae*	Tc	CTX-M-3	N	N
S46	*K. pneumoniae*	Tc	CTX-M-15	L/M	L/M
S51	*K. pneumoniae*	Tc	SHV-12	FIIK, R	FIIK
S59	*K. pneumoniae*	Tc	CTX-M-15	FIIK	FIIK
S75	*K. pneumoniae*	Tc	CTX-M-15/OXA-1	FIIK	FIIK
S82	*K. pneumoniae*	Tc	CTX-M-15/OXA-1	FII	F
S88	*K. pneumoniae*	Tc	SHV-2a	FIIK	FIIK
S90†	*K. pneumoniae*	Tf	SHV-12, KPC-2	X3	X3
S00056	*S. enterica* Typhimurium	Tc	CTX-M-2	HI2	HIγ
S00319	*S. enterica* Havana	Tc	CTX-M-15	HI2	HIγ
S01106	*S. enterica* Virchow	Tc	SHV-12	I1	I1
S01331	*S. enterica* Tel el kebir	Tc	CTX-M-15	HI2	HIγ
S01477†	*S. enterica* Typhimurium	Tc	CTX-M-1/CMY-2	HI1, I1	I1, HIα, HIβ
S01650	*S. enterica* Brandeburg	Tc	CTX-M-14	FrepB	F
S03207	*S. enterica* Typhimurium	Tf	CTX-M-15	FIA, FIB	F
S03663	*S. enterica* Grumpensis	Tc	CTX-M-15	HI2	HIγ
S03664	*S. enterica* Typhimurium	Tc	CTX-M-15	N	N
S04662	*S. enterica* Virchow	Tc	CTX-M-32	N	N
S05343	*S. enterica* Concord	Tc	CTX-M-15	HI2	HIγ
S07364	*S. enterica* Miami	Tc	SHV-2	N	N
S09118	*S. enterica* Keurmassar	Tc	SHV-12	HI2, FI	F, HIα
S1922†	*S. enterica* Kentucky	Tc	VIM-2	W	W
S1923	*S. enterica* Kentucky	Tc	VIM-2	W	W
S27078	*S. enterica* Carmel	Tc	CTX-M-15	FrepB	F
S7981	*S. enterica* Saintpaul	Tc	OXA-48	L/M	L/M

*Tc, transconjugant, Tf, transformant, PBRT, PCR-based replicon typing, PRaseT, plasmid relaxase gene typing, T4SS, type IV secretion system, ND, no data; †, Tc used as a positive control in relaxase gene typing.

Table 3 - Application of relaxase gene typing in 39 clinical strains of *Enterobacteriaceae**

				Plasmid classification					
				Plasmid relaxase gene typing					
No.	Species	B-Lactamase	PBRT	Multiplex I	Multiplex II	Multiplex III	Multiplex IV	Multiplex VI	Simplex I
3	*E. coli*	TEM-24	A/C, B/O		A/C				K-B/O
15	*E. coli*	TEM-24	A/C, I1		A/C, I1				
17	*E. coli*	TEM-24	A/C, FIB, FII, HI2, X4	F	A/C	HIα, HIγ	X4		
19	*E. coli*	TEM-21	A/C, FIA, FIB, FII, K, N, X1		A/C, N		X1		K-B/O
23	*E. coli*	TEM-24	A/C, FIB, FII, I1	F	A/C, I1				
26	*E. coli*	TEM-24	A/C, FIB, HI2	F	A/C	HIα, HIγ			
28	*E. coli*	TEM-21	A/C, K, X1		A/C		X1		K-B/O
33	*E. coli*	TEM-24	A/C, P		A/C				
34	*E. coli*	TEM-24	A/C, FIA, FIB, FII, K, N, X1		A/C, N		X1		K-B/O
40	*E. coli*	TEM-24	A/C, B/O, FIA	F	A/C				K-B/O
50	*E. coli*	TEM-52	I1		I1				
53	*E. coli*	TEM-3	A/C, B/O, FrepB	FV	A/C				K-B/O
66	*E. coli*	SHV-12	FIB, K	F					K-B/O
84	*E. coli*	CTX-M-1	FIB, HI1, N	F	N	HIβ			
88	*E. coli*	CTX-M1	FIB, FII, HI1, N	F	N	HIβ			
101	*E. coli*	CTX-M-2	HI2, P			HIα, HIγ			
105	*E. coli*	CTX-M-1	K, L/M		L/M, I1				K-B/O
106	*E. coli*	CTX-M-1	FIB, FII, HI1, N	F	N	HIα, HIβ			
112	*E. coli*	CTX-M-3	FIB, FII, K	F					K-B/O
Ec37040	*E. coli*	ND	A/C, FIB, FII	F	A/C				
Ec43681	*E. coli*	ND	FIA, FIB, FII, Q	F				Q1	
KpS3	*K. pneumoniae*	FOX-3	A/C, FIIK, X4	FIIK	A/C		X4		
KpS5	*K. pneumoniae*	DHA-1	FIIK, L/M, R	FIIK	L/M				
KpS15	*K. pneumoniae*	GES-9	FIIK, R, U	FIIK					
KpS19	*K. pneumoniae*	CTX-M-15, DHA-1	FII, FIIK, L/M, N	FIIK, F	L/M, N				
KpS20	*K. pneumoniae*	CTX-M-15,SHV-2a	FII, X4	F			X4		
KpS26	*K. pneumoniae*	SHV-12	FIIK, N, R	FIIK	N				
KpS47	*K. pneumoniae*	CTX-M-3	HI2, L/M		L/M	HIγ			
KpS63	*K. pneumoniae*	DHA-1	L/M, R	FIIK	L/M				
KpS83	*K. pneumoniae*	TEM-3	A/C, FIB, N	F	A/C, N				
KpS88	*K. pneumoniae*	SHV-2a	FIIK	FIIK					
KpS91	*K. pneumoniae*	KPC-2	A/C, FIIK, X3	FIIK	A/C		X3		
KpS92	*K. pneumoniae*	CTX-M-14, VIM-1	FII, FIIK, I1	FIIK, F	I1				
KpS93	*K. pneumoniae*	CTX-M-15, OXA-48	I1, L/M		I1, L/M				
FM-2	*K. pneumoniae*	OXA-48	FIIK, L/M, N	FIIK	L/M, N				
FM-10	*K. pneumoniae*	OXA-48	A/C, L/M		A/C, L/M	HIα			
Kp29537	*K. pneumoniae*	ND	A/C, FIIK	FIIK	A/C				
S10-1477	*S. enterica* Typhimurium	CTX-M-1, CMY-2	HI1, I1		I1	HIα, HIβ			
S10-1526	*S. enterica* Typhimurium	CTX-M-1, CMY-2	HI1, I1		I1	HIα, HIβ			

*PBRT, PCR-based replicon typing, ND, not determined.

389 **Table 4 - Results of relaxase gene typing in 17 recipient cells non-typeable with PBRT***

No.	Parental species	Tc/Tf	β-lactamase	Plasmid relaxase gene typing
16	*E. coli*	Tc	TEM-21	C11
31	*E. coli*	Tc	TEM-24	P5-3
37	*E. coli*	Tf	TEM-24	P5-3
55	*E. coli*	Tf	TEM-52	NT
65	*E. coli*	Tc	SHV-4	P5-3
66	*E. coli*	Tc	SHV-12	P5-3
67	*E. coli*	Tc	SHV-12	P5-3
70	*E. coli*	Tc	SHV-12	P5-3
71	*E. coli*	Tc	SHV-2	P5-3
72	*E. coli*	Tc	SHV-2	P5-3
76	*E. coli*	Tc	SHV-2	NT, P5-3†
86	*E. coli*	Tf	CTX-M-3	NT
93	*E. coli*	Tf	CTX-M-3	NT
99	*E. coli*	Tc	CTX-M-1	Qu, P5-3†
S33	*K. pneumoniae*	Tf	DHA-1	NT
S55	*K. pneumoniae*	Tc	CTX-M-15, OXA-1	FIIK
S77	*K. pneumoniae*	Tf	SHV-12	NT

390 *PBRT, PCR based replicon typing, Tc, transconjugant, Tf, transformant, NT, not typeable; †: recipient cells containing two different
391 plasmids (ESBL was carried by plasmids NT and Qu in strains 76 and 99 respectively).

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Appendix A. Supplementary data

Supplementary Table S1 – Relaxase and T4SS-ATPase gene sequences of completely sequenced IncHI plasmids stored in GenBank

Supplementary Table S2 – Primers used in this study for T4SS-typing

Supplementary Figure – Relaxase gene typing assays

All PCR products were separated in 1.5% agarose gels. M, molecular size marker (in bp). (a) Multiplex PCR assay of the IncF, IncFIIK and IncFV relaxase genes. Lanes : 1, *K. pneumoniae* KpS19 ; 2, *E. coli* 84 ; 3, E. coli 53 ; 4, *K. pneumoniae* KpS83 ; 5, *K. pneumoniae* KpS19+ *E. coli* 53 ; 6, *E. coli* J53 (negative control). (b) Multiplex PCR assay of the IncA/C, IncI1, IncL/M and IncN relaxase genes. Lanes : 1, *E. coli* 15 ; 2, *E. coli* 34 ; 3, *K. pneumoniae* KpS19 ; 4, *K. pneumoniae* FM10 ; 5, *E. coli* 15+ *E. coli* 34+ *K. pneumoniae* KpS19 ; 6, *E. coli* J53 (negative control). (c) Multiplex PCR assay of the Hiα, Hiβ and HIγ relaxase genes. Lanes : 1, *K. pneumoniae* FM10 ; 2, *E. coli* 26 ; 3, *E. coli* 88 ; 4, *E. coli* 106 ; 5, *E. Coli* 17+*E. Coli* 106 ; 6, *E. coli* J53 (negative control). (d) Multiplex PCR assay of the IncX1, IncX2, IncX3 and IncX4 relaxase genes. Lanes: 1, *E. coli* 17; 2, *K. pneumoniae* S90 recipient cell; 3, *E. coli* ECOR19; 4, *E. coli* 19; 5, *E. coli* J53 (negative control). (e) Multiplex PCR assay of the MobP5-1, MobP5-2, MobP5-3, MobC11 and MobQu relaxase genes. Lanes : 1, *E. coli* ECOR6 ; 2, *E. coli* ECOR10 ; 3, *E. Coli* 16 recipient cell ; 4, *E. Coli* 99 recipient cell ; 5, *E. coli* ECOR6+ *E. coli* ECOR10+*E. Coli* 16 recipient cell+*E. Coli* 70 recipient cell+ *E. Coli* 99 recipient cell ; 6, *E. coli* J53 (negative control). (f) Multiplex PCR assay of the IncP1α, IncQ1, IncU and IncW relaxase genes. Lanes : 1, *E. coli* Ec43681 ; 2, *S. enterica* S1922 recipient cell ; 3, pFBAOT6 reference plasmid ; 4, RP4 reference plasmid ; 5, *E. coli* Ec43681+ pFBAOT6 reference plasmid+ RP4 reference plasmid ; 6, *E. coli* J53 (negative control). (g) Simplex PCR assay of the IncB/O and IncK relaxase genes. Lanes : 1, *E. coli* 34 ; 2, *E. coli* 66 ; 3, *E. coli* 40 ; 4, *E. Coli* 112 ; 5, *E. coli* 53 ; 6, *E. coli* J53 (negative control).