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1 **Targeting relaxase genes for classification of the predominant plasmids in**

2 **Enterobacteriaceae**

3

4 **Fabrice Compain¹, Agathe Poisson¹, Simon Le Hello², Catherine Branger³, François-**
5 **Xavier Weill², Guillaume Arlet^{1,4}, Dominique Decré^{1,4*}**
6

7 ¹Université Pierre et Marie Curie-Paris 6, Faculté de Médecine, Site Saint-Antoine,

8 Laboratoire de Bactériologie, ER8, Paris, France; ²Institut Pasteur, Unité des Bactéries

9 Pathogènes Entériques, Centre National de Référence des *Escherichia coli*, *Shigella* et

10 *Salmonella*, WHO Collaborating Centre for Reference and Research on Salmonella, Paris,

11 France; ³Université Paris Diderot, UMR-S 722, Faculté de Médecine, Site Xavier Bichat, F-75018

12 Paris, France; ⁴Laboratoire de Bactériologie, Hôpital Saint-Antoine, Assistance Publique des

13 Hôpitaux de Paris, Paris, France

14

15 *Corresponding author

16 Email addresses:

17 FC: compain.fabrice@wanadoo.fr

18 AP: agatepoisson@gmail.com

19 SLH: slehello@pasteur.fr

20 CB: catherine.branger@lmr.aphp.fr

21 FXW: fxweill@pasteur.fr

22 GA: guillaume.arlet@upmc.fr

23 DD: dominique.decre@upmc.fr; Tel.: +33 1 40 01 14 46; Fax +33 1 49 28 24 72

24 **Abstract**

25 Plasmids are the main vectors of antimicrobial drug resistance and virulence genes, especially
26 in Enterobacteriaceae. Identification and classification of plasmids is essential for analysis of
27 their distribution. The most widely used typing method is PCR-based replicon typing (PBRT).
28 A new classification scheme based on relaxase gene typing has been described recently. We
29 propose a practical application of this method, with the development of a multiplex PCR set
30 targeting relaxase genes found on plasmids most frequently encountered in
31 Enterobacteriaceae. This method, here called “plasmid relaxase gene typing” (PRaseT), was
32 validated with 60 transconjugants and transformants harboring various replicon types. The
33 method was tested with 39 multidrug-resistant clinical isolates including *Escherichia coli*,
34 *Klebsiella pneumoniae* and *Salmonella enterica* subsp. *enterica* carrying 1 to 7 replicons as
35 well as with 17 plasmids non-typeable using PBRT; all replicons were tested in parallel with
36 PBRT for comparison. Six multiplex PCRs and one simplex PCR, including 24 pairs of
37 primers, recognized plasmids of groups A/C, B/O, colE, FIA, FIB, FIC, FV, FIIk, HI1, HI2,
38 I1, K, L/M, N, P1 α , Q1, U, W, X1, X2, X3 and X4. There was perfect correlation between
39 PRaseT and PBRT results in 31/39 (79.5%) clinical isolates. Moreover, 11/17 (64.7%)
40 plasmids non-typeable by PBRT could be typed by PRaseT. Our set of multiplex PCRs
41 showed high sensitivity and specificity for the classification of resistance plasmids. It has
42 proved complementary to the widely used PBRT and will improve the monitoring of plasmid
43 distribution in every-day practice.

44

45 **Keywords**

46 Plasmid; Classification; Replicon typing; Relaxase; Multiplex PCR

47 **Introduction**

48 Plasmids are important agents of gene flux and have found to be responsible for the
49 dissemination of multiple antibiotic resistance genes. Identification and classification of
50 plasmids is essential for analysis of their distribution, their genetic relatedness and evolution,
51 as well as for study of horizontal gene transfer. A classification scheme should be based on
52 genetic traits that are universally present and constant. It should be robust and the
53 corresponding experimental procedure should be easy. The basic replicon locus, which is
54 always present on plasmids, has been used historically for classification. Plasmids were
55 initially classified according to their incompatibility, which is directly related to replication.
56 Incompatibility (Inc) was defined as the inability of two plasmids sharing common replication
57 control (same Inc group) to be maintained in the subsequent lineage during conjugation (Datta
58 and Hedges, 1971 and Novick, 1987). This method which requires plasmid transfer to the
59 same host for testing is time-consuming and not practical for large-scale studies. Couturier *et*
60 *al.* tested a method using hybridization with cloned replication regions as probes but with this
61 method plasmid diversity is underestimated due to cross-hybridization (Couturier *et al.*,
62 1988). In 2005, Carattoli *et al.* developed a PCR scheme of targeting replicons called PCR-
63 based replicon typing (PBRT) (Carattoli *et al.*, 2005). Eighteen pairs of primers were designed
64 in order to perform 5 multiplex and 3 simplex PCRs recognizing the most frequently
65 encountered plasmid incompatibility groups among Enterobacteriaceae. With this method, 27
66 Inc groups are currently recognized (Carattoli, 2009). It has been widely used to study
67 plasmid spread and diversity in Enterobacteriaceae. However, PBRT has several drawbacks:
68 (i) plasmids may carry multiple replicons and/or mosaic replicons, and new replicon types
69 may escape classification with this technique; (ii) false-negative results with some Inc groups
70 (e.g. L/M) have been reported (Carattoli *et al.*, 2005) and (iii) PBRT targets multiple sites

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

77 Bacterial conjugation represents a unique process allowing transfer of plasmid DNA from a
78 donor to a recipient bacterium through cell-to-cell contact. In this process relaxase is a key
79 protein encoded by all transmissible plasmids, i.e. mobilizable and conjugative plasmids
80 involved in horizontal gene transfer (reviewed by Smillie et al., 2010 and Wong et al., 2012).

81 A classification scheme based on the mobilization region of transmissible plasmids has
82 recently been developed (Francia et al., 2004 and Garcillán-Barcia et al., 2009). The scheme
83 classified relaxases in six protein families and 31 subfamilies, depending on their phylogeny;
84 subsequently, 19 degenerate primer pairs targeting the relaxase genes of γ -proteobacterial
85 plasmids were designed (Alvarado et al., 2012). This degenerate primer MOB typing (DPMT)
86 method has been used with success in previous studies (Curiao et al., 2011, Mata et al., 2012,
87 Mata et al., 2010 and Valverde et al., 2009). However, this set of primers was not designed for
88 screening purposes in clinical practice, but rather for experimental purposes in order to
89 discover new relaxases (Alvarado et al., 2012).

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

95

96 **Materials and methods**

97 **Database search and primer design**

98 An *in silico* analysis was carried out using GenBank BLAST (<http://blast.ncbi.nlm.nih.gov/>).

99 For each Inc group, the relaxase/helicase gene was used as template; the presence of relaxase-
100 specific multidomains was checked using CD-Search

101 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/>). Multiple alignments were performed

102 with ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Primer pairs covering

103 most sequences in each family were designed using FastPCR software

104 (<http://primerdigital.com/fastpcr.html>), while minimizing codon degeneracy (Table 1A).

105

106 ***In silico* primer assay**

107 Oligonucleotide primers were tested *in silico* for hybridization with plasmids of the

108 Enterobacteriaceae referenced in GenBank. Some primers were refined to cover a maximum

109 of reported sequences.

110

111 **Bacterial strains**

112 For validation of the PCR assays, experiments were conducted with 60 *Escherichia coli*

113 transconjugants or transformants of Enterobacteriaceae (Table 2). All strains carried replicons

114 of various types that encoded diverse β -lactamases conferring resistance to third-generation

115 cephalosporins or carbapenems. They were part of three collections of, respectively, (i) *E. coli*

116 strains isolated between 1997 and 2002 in various French university hospitals (for further

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

123 All transconjugants, transformants and clinical strains used in this study were analyzed in
124 parallel with PRaseT and the PBRT method applied previously (Carattoli et al., 2005, García-
125 Fernández et al., 2009, Götz et al., 1996, Osborn et al., 2000 and Villa et al., 2010). The
126 transconjugants used as positive controls in PRaseT reactions are given in Table 2. Other
127 controls used in this study included three strains of the ECOR collection (Ochman and
128 Selander, 1984) (ECOR 6, ECOR 10 and ECOR 19 harboring, respectively, pTPqnrS-1a-like-
129 , pcolE1-like- and IncX2-plasmids), as well as reference plasmids pFBAOT6 (IncU) (Rhodes
130 et al., 2004) and RP4 (IncP1 α) (Datta et al., 1971).

131 After optimization on transconjugants or transformants carrying replicon of various types
132 according to PBRT, we applied the PRaseT method to a panel of 39 clinical strains (21 *E.*
133 *coli*, 16 *K. pneumoniae* and 2 *S. enterica*) carrying replicons of one to seven different types
134 (Table 3), and 17 transconjugants or transformants that were non-typeable with PBRT (Table
135 4).

136

137 **DNA extraction and PCR conditions**

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

141 contained pre-optimized concentrations of HotStarTaq DNA polymerase and MgCl₂,
142 deoxynucleotide triphosphate and PCR buffer. To all multiplex PCRs, solution Q (Qiagen)
143 that facilitates the reaction with difficult-to-amplify templates by modifying DNA melting
144 behavior was added. Total DNA in 5µl of bacterial lysate was subjected to multiplex PCR in a
145 50 µl volume. The conditions for multiplex PCR were optimized to ensure that all targets
146 were sufficiently amplified for amplicons to be easily visible on 1.5% agarose gels. The
147 optimal primer concentrations are reported in Table 1A. PCR conditions consisted in initial
148 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
149 for 90 s with a final extension at 72°C for 10 min; for Multiplex IV and V the annealing
150 temperature was elevated to 65°C for 90 sec. Simplex PCR were performed in a 50 µL mix
151 with 2 U of Taq DNA polymerase (Roche Diagnostics), 10× PCR buffer/MgCl₂ (Roche
152 Diagnostics), 200 µM of each deoxynucleotide triphosphate (dNTP Mix Eurobio), 0.2
153 pmol/µL of each primer, 40 µL of sterile water and 2 µL of total DNA extract. PCR
154 conditions consisted in 30 cycles [94°C for 1 min, 55°C for 40 sec, 72°C for 1 min], preceded
155 by one cycle at 94°C for 5 min followed by one cycle at 72°C for 5 min. PCR products were
156 separated at 100 V for 90 minutes in 1.5% agarose gel containing ethidium bromide and
157 visualized using GelDoc (Biorad). PCR products were purified using the Exosap purification
158 kit (illustra ExoStar 1-Step, Dutscher, Brumath, France) and subjected to bidirectional DNA
159 sequencing using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems,
160 Foster City, CA, USA) and an Applied Biosystems 3730 XL capillary sequencer. Sequence
161 analysis was carried out using BLAST and GenBank sequences.

162

163 **Results and discussion**

164 ***In silico* analysis**

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

174 The majority of plasmids had a single putative relaxase locus, which is consistent with the
175 review by Smillie *et al.* (Smillie et al., 2010). In contrast, complete GenBank sequences of
176 IncHI1 and IncHI2 plasmids had one to three putative relaxase/helicase loci on each reported
177 plasmid (Supplementary Table S1). CD-Search results for each protein confirmed the
178 presence of relaxase- or helicase-specific multidomains. We finally used five relaxase clades
179 (arbitrarily designed HI α , HI β , HI γ , HI δ and HI ϵ) present among the various IncHI1 and/or
180 IncHI2 plasmids (Supplementary Table S1). HI β and HI δ relaxases were found to be encoded
181 only on IncHI1 plasmids and HI γ and HI ϵ relaxases only on IncHI2 plasmids while HI α
182 relaxases were encoded on both IncHI1 and IncHI2 plasmids. We therefore designed three
183 oligonucleotide primers covering the HI α , HI β and HI γ relaxase, respectively.

184 *In silico* analysis led to the design of 24 pairs of primers for six multiplex PCRs (targeting the
185 relaxase genes of plasmids belonging to Inc groups A/C, colE, FIA, FIB, FIC, FII, FIik, FV,
186 HI1, HI2, I1, L/M, N, P1 α , Q1, U, W, X1, X2, X3 and X4) and one simplex PCR (targeting
187 the relaxase genes of plasmids belonging to Inc groups B/O and K) (Table 1A).

188

189 **Primer evaluation using transconjugants and transformants**

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

197 All PRaseT results were consistent with the PBRT results, except for two strains which
198 carried an IncR (*K. pneumoniae* strain S51) or an IncFIB/FII replicon (*E. coli* strain 81), that
199 were undetected by the PRaseT method. For the first strain the result was not unexpected as
200 IncR plasmids do not encode relaxases. For the second strain, the result was more surprising
201 as IncF plasmids are known to be conjugative in most cases (Smillie et al., 2010). As it has
202 been previously reported that Mob regions and mating pair formation (MPF) systems were in
203 general of the same type (Smillie et al., 2010), we designed new primers targeting other
204 conserved genes of the type IV secretion system (T4SS), i.e. *traB* (encoding a secretin-like
205 protein) and *traC* (encoding an ATPase) (Supplementary Table S2). The eighteen PRaseT-
206 positive IncF plasmids reported in Table 2 tested positive for *traB* and *traC* while the PRaseT-
207 negative IncFIB/FII plasmid also tested negative with T4SS typing (data not shown).

208 Interestingly, no transconjugant but only transformant was obtained from the parental strain of
209 the later plasmid. We considered three possibilities: (i) a very divergent IncF relaxase gene
210 that could not hybridize with our primers was present, (ii) the relaxase gene was truncated or
211 (iii) the gene was absent. Complete sequencing of the plasmid will be performed to confirm
212 one of these possibilities.

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

220

221

222 **Evaluation of relaxase gene typing using clinical strains**

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

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

236 divergent subgroups (IncP1 α , IncP1 β , IncP1 γ , IncP1 δ , IncP1 ϵ and IncP1 ζ), 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 IncP1 α 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).

258 Six plasmids could be typed neither with PBRT nor with PRaseT (Table 4). Complete
259 sequence is available for 3 of them, i.e. plasmids from *K. pneumoniae* transformant S77
260 (pKpS77, SHV-12), *E. coli* transconjugant 76 (RCS47v1_pI, SHV-2) and *E. coli* transformant
261 93 (RCS63v1_p, CTX-M-3) (unpublished data). Plasmids pKpS77, RCS47v1_pI and
262 RCS63v1_p were, respectively, < 50,000 bp, 117,001 bp and 22,308 bp long. In the
263 sequences of two of them (pKpS77 and RCS63v1_p), no transfer region could be identified.
264 The third plasmid (RCS47v1_pI, *E. coli* transconjugant 76) carried a new relaxase gene which
265 presented 100% query cover and 99% maximum identity with those on plasmids pO111_2
266 (GenBank accession no. AP010962.1) and p12579_1 (GenBank accession no. CP003110.1);
267 its replicase gene also presented 100% query cover and 99% maximum identity with its
268 counterparts on plasmids pO111_2 and p12579_1. The three other non-typeable plasmids
269 (contained in transformants from *K. pneumoniae* S33, *E. coli* 55 and *E. coli* 86) could not be
270 transferred by conjugation, which is in favor of non-transmissible plasmids; this should,
271 however, be confirmed by complete plasmid sequencing.

272

273 **Conclusions**

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

281

282 **Competing interest**

283 The authors declare that they have no competing interests.

284

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381 **Tables**382 **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/ μ L)
Multiplex I	IncFIIK	MRxeFII-K_for	CGATATTCTKGAACCCCGTA	pKPN3	CP000648.1	traI	297	0.2
	IncFIA, IncFIB, IncFII	MRxeFII-K_rev	TCATTGCCATAATYCGTCC					0.2
		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	TTACCCCTCWGCAATATGRCGRA				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	ACAACYCTGTACCATAATC				0.2	
	IncL/M	MRxeLM_for	TCGCTATATCGTTGGTGATG	pCTX-M3	AF550415.2	nikB	491	0.2
	MRxeLM_rev	GGGTATGTTGGGCAATTTTC				0.2		
	IncA/C	MRxeAC_for	TGATTGAGAAAGTGCCGAACTC	pRA1	FJ705807.1	traI	659	0.2
		MRxeAC_rev	CACACCATAGGAGAACTCGT				0.2	
Multiplex III	IncHI1, IncHI2	MRxeHI α _for	CCAAAAGACTGACTTGGGAGCA	pMAK1	AB366440.1	helicase	873	0.2
		MRxeHI α _rev	CCGAGTTAGCCATCAAGGAA				0.2	
	IncHI1	MRxeHI β _for	GATGGTATGTGGATAGTCCG	pNDM-CIT	JX182975.1	traI	368	0.2
		MRxeHI β _rev	GAATATGCCTCACYATCGCT				0.2	
	IncHI2	MRxeHI γ _for	AATGACGTGGTTGAATACGA	R478	BX664015.1	traI	225	0.2
		MRxeHI γ _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	ACGGATTTCGCTTRCCTGGMAGTG	pColEST258	JN247853.1	mobB	997	0.2
		MRxeMobC11_rev	TTCAGSGCGTACTGGTCGAGATCG				0.2	
	Non typeable	MRxeMobQu_for	TCTCCAGAARCAAGCGCTACAG	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	TATCAGRAGWGTGAGYRTCKCKG				0.04		
colE, Non typeable	MRxeMobP5-2_for	TCCGGTGGAATATCTKCTGGGKA	pTPqnrS-1a	AM746977.1	mobA	656	0.2	

	colE, Non typeable	MRxeMobP5-2_rev MRxeMobP5-3_for MRxeMobP5-3_rev	AAGACTGYTCATARATRGCTCC GCTATCAGTCGCCCTGTTTCGTC AGTACCCGATCATCAGTCAGTG	pHUSEC41-4	HE603113.1	mob	147	0.2 0.04 0.04	
Multiplex VI	IncQ1	MRxeQ1_for MRxeQ1_rev	GATTTATCACCTTACGGCGAA TACCTCCCGCAACTCTTTGG	RSF1010	M28829.1	mobA	962	0.2 0.2	
	IncU	MRxeU_for MRxeU_rev	TATGACACTCTAGCCGGGTT TGGATCTTGCTTCCACGGTT	pRA3	DQ401103.1	nic	743	0.2 0.2	
	IncP1 α	MRxeP1 α _for MRxeP1 α _rev	TGAAGTACATCACCGACGAG GTTTCGTGATTGTCACGCTC	RP4	X54459.1	tral	424	0.2 0.2	
	IncW	MRxeW_for MRxeW_rev	CATCGAGTTCGATACCCTTG GCCACATAGTAAACGTCCTT	R388	BR000038.1	trwC	531	0.2 0.2	
	Simplex I	IncB/O, IncK	MRxeBO/K_for MRxeBO/K_rev	GAATGCCATYATCCGMAMAA GTGATATACAGACCATCACTGG	pO113	AY258503.2	nikB	800	0.2 0.2

383

Table 2 - Recipient cells used in validation experiments*

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

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*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.

386

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

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

388 *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	<i>E. coli</i>	Tc	TEM-21	C11
31	<i>E. coli</i>	Tc	TEM-24	P5-3
37	<i>E. coli</i>	Tf	TEM-24	P5-3
55	<i>E. coli</i>	Tf	TEM-52	NT
65	<i>E. coli</i>	Tc	SHV-4	P5-3
66	<i>E. coli</i>	Tc	SHV-12	P5-3
67	<i>E. coli</i>	Tc	SHV-12	P5-3
70	<i>E. coli</i>	Tc	SHV-12	P5-3
71	<i>E. coli</i>	Tc	SHV-2	P5-3
72	<i>E. coli</i>	Tc	SHV-2	P5-3
76	<i>E. coli</i>	Tc	SHV-2	NT, P5-3†
86	<i>E. coli</i>	Tf	CTX-M-3	NT
93	<i>E. coli</i>	Tf	CTX-M-3	NT
99	<i>E. coli</i>	Tc	CTX-M-1	Qu, P5-3†
S33	<i>K. pneumoniae</i>	Tf	DHA-1	NT
S55	<i>K. pneumoniae</i>	Tc	CTX-M-15, OXA-1	FIK
S77	<i>K. pneumoniae</i>	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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396 **Appendix A. Supplementary data**

397 **Supplementary Table S1 – Relaxase and T4SS-ATPase gene sequences** 398 **of completely sequenced IncHI plasmids stored in GenBank**

399

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

401

402 **Supplementary Figure – Relaxase gene typing assays**

403 All PCR products were separated in 1.5% agarose gels. M, molecular size marker (in bp). (a)
404 Multiplex PCR assay of the IncF, IncFIIK and IncFV relaxase genes. Lanes : 1, *K.*
405 *pneumoniae* KpS19 ; 2, *E. coli* 84 ; 3, *E. coli* 53 ; 4, *K. pneumoniae* KpS83 ; 5, *K.*
406 *pneumoniae* KpS19+ *E. coli* 53 ; 6, *E. coli* J53 (negative control). (b) Multiplex PCR assay of
407 the IncA/C, IncI1, IncL/M and IncN relaxase genes. Lanes : 1, *E. coli* 15 ; 2, *E. coli* 34 ; 3, *K.*
408 *pneumoniae* KpS19 ; 4, *K. pneumoniae* FM10 ; 5, *E. coli* 15+ *E. coli* 34+ *K. pneumoniae*
409 KpS19 ; 6, *E. coli* J53 (negative control). (c) Multiplex PCR assay of the Hia α , Hii β and Hii γ
410 relaxase genes. Lanes : 1, *K. pneumoniae* FM10 ; 2, *E. coli* 26 ; 3, *E. coli* 88 ; 4, *E. coli* 106 ;
411 5, *E. coli* 17+*E. coli* 106 ; 6, *E. coli* J53 (negative control). (d) Multiplex PCR assay of the
412 IncX1, IncX2, IncX3 and IncX4 relaxase genes. Lanes: 1, *E. coli* 17; 2, *K. pneumoniae* S90
413 recipient cell; 3, *E. coli* ECOR19; 4, *E. coli* 19; 5, *E. coli* J53 (negative control). (e) Multiplex
414 PCR assay of the MobP5-1, MobP5-2, MobP5-3, MobC11 and MobQu relaxase genes. Lanes
415 : 1, *E. coli* ECOR6 ; 2, *E. coli* ECOR10 ; 3, *E. coli* 16 recipient cell ; 4, *E. coli* 99 recipient
416 cell ; 5, *E. coli* ECOR6+ *E. coli* ECOR10+*E. coli* 16 recipient cell+*E. coli* 70 recipient cell+
417 *E. coli* 99 recipient cell ; 6, *E. coli* J53 (negative control). (f) Multiplex PCR assay of the
418 IncP1 α , IncQ1, IncU and IncW relaxase genes. Lanes : 1, *E. coli* Ec43681 ; 2, *S. enterica*
419 S1922 recipient cell ; 3, pFBAOT6 reference plasmid ; 4, RP4 reference plasmid ; 5, *E. coli*
420 Ec43681+ pFBAOT6 reference plasmid+ RP4 reference plasmid ; 6, *E. coli* J53 (negative
421 control). (g) Simplex PCR assay of the IncB/O and IncK relaxase genes. Lanes : 1, *E. coli* 34 ;
422 2, *E. coli* 66 ; 3, *E. coli* 40 ; 4, *E. coli* 112 ; 5, *E. coli* 53 ; 6, *E. coli* J53 (negative control).

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