Targeting relaxase genes for classification of the predominant plasmids in Enterobacteriaceae

Fabrice Compain, Agathe Poisson, Simon Le Hello, Catherine Branger, François-Xavier Weill, Guillaume Arlet, Dominique Decré

► To cite this version:

Fabrice Compain, Agathe Poisson, Simon Le Hello, Catherine Branger, François-Xavier Weill, et al.. Targeting relaxase genes for classification of the predominant plasmids in Enterobacteriaceae. International Journal of Medical Microbiology Supplements, 2014, 304 (3-4), pp.236 - 242. 10.1016/j.ijmm.2013.09.009 . pasteur-01108896

HAL Id: pasteur-01108896

<https://hal-pasteur.archives-ouvertes.fr/pasteur-01108896>

Submitted on 12 Mar 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution| 4.0 International License

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

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, P1 α , 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.

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

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 (IncP1 α) (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 MgCl₂, 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 5 µl 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/MgCl₂ (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 HI α , HI β , HI γ , HI δ and HI ϵ) present among the various IncHI1 and/or IncHI2 plasmids (Supplementary Table S1). HI β and HI δ relaxases were found to be encoded only on IncHI1 plasmids and HI γ and HI ϵ relaxases only on IncHI2 plasmids while HI α relaxases were encoded on both IncHI1 and IncHI2 plasmids. We therefore designed three oligonucleotide primers covering the HI α , HI β and HI γ 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, P1 α , 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 HI β - and HI γ -primers respectively (e.g. transconjugants S01477 and 102, Table 2). However, when HI α 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 IncP1 α subgroup, while the IncP1 group consists of at least six

divergent subgroups (IncP1 α , IncP1 β , IncP1 γ , IncP1 δ , IncP1 ϵ and IncP1 ζ), many of which are antibiotic resistance vectors in the environment (Alvarado et al., 2012, Bahl et al., 2009 and Heuer et al., 2012). We think that our IncP1 α relaxase primers were unable to classify all IncP1 group replicons because of too great a divergence in gene sequences. PRaseT of IncP group plasmids should be improved in further studies.

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

Relaxase gene typing in recipient cells non-typeable with PBRT

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

281

282 **Competing interest**

283 The authors declare that they have no competing interests.

284

285 **Acknowledgements**

286 We are grateful to Etienne Carbonnelle (Hôpital Européen Georges Pompidou, Paris, France)
287 for providing the three control strains from the ECOR collection, and to Glenn Rhodes
288 (Lancaster Environment Centre, Lancaster, United Kingdom) for providing the IncU-positive
289 control strain.

290 References

- 291 Alvarado A, Garcillán-Barcia MP, de la Cruz F, 2012. A degenerate primer MOB typing (DPMT)
292 method to classify gamma-proteobacterial plasmids in clinical and environmental settings.
293 PLoS One. 7 (7), e40438.
- 294 Bahl MI, Burmølle M, Meisner A, Hansen LH, Sørensen SJ, 2009. All IncP-1 plasmid subgroups,
295 including the novel epsilon subgroup, are prevalent in the influent of a Danish wastewater
296 treatment plant. Plasmid. 62 (2), 134–139.
- 297 Branger C, Zamfir O, Geoffroy S, Laurans G, Arlet G, Thien HV, Gouriou S, Picard B, Denamur E, 2005.
298 Genetic background of *Escherichia coli* and extended-spectrum beta-lactamase type. Emerg
299 Infect Dis. 11 (1), 54–61.
- 300 Burrus V, Marrero J, Waldor MK, 2006. The current ICE age: biology and evolution of SXT-related
301 integrating conjugative elements. Plasmid. 55 (3), 173–183.
- 302 Carattoli A, 2009. Resistance plasmid families in Enterobacteriaceae. Antimicrob Agents Chemother.
303 53 (6), 2227–2238.
- 304 Carattoli A, 2011. Plasmids in Gram negatives: molecular typing of resistance plasmids. Int J Med
305 Microbiol. 301 (8), 654–658.
- 306 Carattoli A, 2013. Plasmids and the spread of resistance. Int J Med Microbiol. S1438-4221(13).
- 307 Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ, 2005. Identification of plasmids by PCR-
308 based replicon typing. J Microbiol Methods. 63 (3), 219–228.
- 309 Couturier M, Bex F, Bergquist PL, Maas WK, 1988. Identification and classification of bacterial
310 plasmids. Microbiol Rev. 52 (3), 375–395.
- 311 Curiao T, Cantón R, Garcillán-Barcia MP, de la Cruz F, Baquero F, Coque TM, 2011. Association of
312 composite IS26-sul3 elements with highly transmissible IncI1 plasmids in extended-spectrum-
313 beta-lactamase-producing *Escherichia coli* clones from humans. Antimicrob Agents
314 Chemother. 55 (5), 2451–2457.
- 315 Dallenne C, Da Costa A, Decré D, Favier C, Arlet G., 2010. Development of a set of multiplex PCR
316 assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae.
317 J Antimicrob Chemother. 65 (3), 490–495.
- 318 Datta N, Hedges RW, 1971. Compatibility groups among fi - R factors. Nature. 234 (5326), 222–223.
- 319 Datta N, Hedges RW, Shaw EJ, Sykes RB, Richmond MH, 1971. Properties of an R factor from
320 *Pseudomonas aeruginosa*. J Bacteriol. 108 (3), 1244–1249.
- 321 Francia MV, Varsaki A, Garcillán-Barcia MP, Latorre A, Drainas C, de la Cruz F, 2004. A classification
322 scheme for mobilization regions of bacterial plasmids. FEMS Microbiol Rev. 28 (1), 79–100.
- 323 García-Fernández A, Carattoli A, 2010. Plasmid double locus sequence typing for IncHI2 plasmids, a
324 subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum
325 beta-lactamase and quinolone resistance genes. J Antimicrob Chemother. 65 (6), 1155–1161.
- 326 García-Fernández A, Chiaretto G, Bertini A, Villa L, Fortini D, Ricci A, Carattoli A, 2008. Multilocus
327 sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in
328 *Escherichia coli* and *Salmonella* of human and animal origin. J Antimicrob Chemother. 61 (6),
329 1229–1233.
- 330 García-Fernández A, Fortini D, Veldman K, Mevius D, Carattoli A, 2009. Characterization of plasmids
331 harbouring qnrS1, qnrB2 and qnrB19 genes in *Salmonella*. J Antimicrob Chemother. 63 (2),
332 274–281.
- 333 García-Fernández A, Villa L, Moodley A, Hasman H, Miriagou V, Guardabassi L, Carattoli A, 2011.
334 Multilocus sequence typing of IncN plasmids. J Antimicrob Chemother. 66 (9), 1987–1991.
- 335 Garcillán-Barcia MP, Francia MV, de la Cruz F, 2009. The diversity of conjugative relaxases and its
336 application in plasmid classification. FEMS Microbiol Rev. 33 (3), 657–687.

- Götz A, Pukall R, Smit E, Tietze E, Prager R, Tschäpe H, van Elsas JD, Smalla K, 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl Environ Microbiol.* 62 (7), 2621–2628.
- Heuer H, Binh CT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T, González B, Top E, Smalla K, 2012. IncP-1 ϵ Plasmids are Important Vectors of Antibiotic Resistance Genes in Agricultural Systems: Diversification Driven by Class 1 Integron Gene Cassettes. *Front Microbiol.* 3 (2).
- Johnson TJ, Bielak EM, Fortini D, Hansen LH, Hasman H, Debroy C, Nolan LK, Carattoli A, 2012. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant Enterobacteriaceae. *Plasmid.* 68 (1), 43–50.
- Marcadé G, Deschamps C, Boyd A, Gautier V, Picard B, Branger C, Denamur E, Arlet G, 2009. Replicon typing of plasmids in *Escherichia coli* producing extended-spectrum beta-lactamases. *J Antimicrob Chemother.* 63 (1), 67–71.
- Mata C, Miró E, Alvarado A, Garcillán-Barcia MP, Toleman M, Walsh TR, de la Cruz F, Navarro F, 2012. Plasmid typing and genetic context of AmpC β -lactamases in Enterobacteriaceae lacking inducible chromosomal ampC genes: findings from a Spanish hospital 1999-2007. *J Antimicrob Chemother.* 67 (1), 115–122.
- Mata C, Miró E, Mirelis B, Garcillán-Barcia MP, de la Cruz F, Coll P, Navarro F, 2010. In vivo transmission of a plasmid coharbouring bla and qnrB genes between *Escherichia coli* and *Serratia marcescens*. *FEMS Microbiol Lett.* 308 (1), 24–28.
- Novick RP, 1987. Plasmid incompatibility. *Microbiol Rev.* 51 (4), 381–395.
- Ochman H, Selander RK, 1984. Standard reference strains of *Escherichia coli* from natural populations. *J Bacteriol.* 157 (2), 690–693.
- Osborn AM, da Silva Tatley FM, Steyn LM, Pickup RW, Saunders JR, 2000. Mosaic plasmids and mosaic replicons: evolutionary lessons from the analysis of genetic diversity in IncFII-related replicons. *Microbiology.* 146 (9), 2267–2275.
- Phan MD, Kidgell C, Nair S, Holt KE, Turner AK, Hinds J, Butcher P, Cooke FJ, Thomson NR, Titball R, Bhutta ZA, Hasan R, Dougan G, Wain J, 2009. Variation in *Salmonella enterica* serovar typhi IncHI1 plasmids during the global spread of resistant typhoid fever. *Antimicrob Agents Chemother.* 53 (2), 716–727.
- Rhodes G, Parkhill J, Bird C, Ambrose K, Jones MC, Huys G, Swings J, Pickup RW, 2004. Complete nucleotide sequence of the conjugative tetracycline resistance plasmid pFBAOT6, a member of a group of IncU plasmids with global ubiquity. *Appl Environ Microbiol.* 70 (12), 7497–7510.
- Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EP, de la Cruz F, 2010. Mobility of plasmids. *Microbiol Mol Biol Rev.* 74 (3), 434–452.
- Valverde A, Cantón R, Garcillán-Barcia MP, Novais A, Galán JC, Alvarado A, de la Cruz F, Baquero F, Coque TM, 2009. Spread of bla(CTX-M-14) is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrob Agents Chemother.* 53 (12), 5204–5212.
- Villa L, García-Fernández A, Fortini D, Carattoli A, 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. *J Antimicrob Chemother.* 65 (12), 2518–2529.
- Wong JJ, Lu J, Glover JN, 2012. Relaxosome function and conjugation regulation in F-like plasmids - a structural biology perspective. *Mol Microbiol.* 85 (4), 602–617.

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	IncFIK	MRxeFII-K_for	CGATATTCTKGAACCCCGTA	pKPN3	CP000648.1	tral	297	0.2
		MRxeFII-K_rev	TCATTGCCATAATYCGTCC					0.2
	IncFIA, IncFIB, IncFII	MRxeF-tot_for	ATCAGGAMCCACAGTTACAC	R100	AP000342.1	tral	753	0.2
		MRxeF-tot_rev	GTTTCATGATRTCRCGACTGAG					0.2
	IncFV	MRxeFV_for	CATCAAAGCGAARGAGYAGTTCAC	pED208	AF411480.1	tral	642	0.2
		MRxeFV_rev	TTACCCCTCWGCAATATGRCGRA					0.2
Multiplex II	IncN	MRxeN_for	CCAGTTTAGAAACCCGATCA	R46	AY046276.1	tral	332	0.2
		MRxeN_rev	CGTAATCGTCGTAAATGCTG					0.2
	IncI1	MRxeI1_for	TTTAATCATGAGCAAACGCC	R64	AB027308.1	nikB	764	0.2
		MRxeI1_rev	ACAACYCTGTCAACATAATC					0.2
	IncL/M	MRxeLM_for	TCGCTATATCGTTGGTGATG	pCTX-M3	AF550415.2	nikB	491	0.2
		MRxeLM_rev	GGGTATGTTGGGCAATTTTC					0.2
	IncA/C	MRxeAC_for	TGATTGAGAAAGTGC GGAACTC	pRA1	FJ705807.1	tral	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	tral	368	0.2
		MRxeHIβ_rev	GAATATGCCTCACATCGCT					0.2
	IncHI2	MRxeHIγ_for	AATGACGTGGTTGAATACGA	R478	BX664015.1	tral	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	CATTACAAAAGCCCGTCTCG					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	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	TATCAGRAGWGTGAGYRTCKGCG					0.04
	colE, Non typeable	MRxeMobP5-2_for	TCCGGTGGAATATCTKCTGGGKA	pTPqnrS-1a	AM746977.1	mobA	656	0.2

colE, Non typeable		MRxeMobP5-2_rev	AAGACTGYTCATARATRGCTCC	pHUSEC41-4	HE603113.1	mob	147	0.2
		MRxeMobP5-3_for	GCTATCAGTCGCCCTGTTCGTC					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	TGGATCTTGTCTTCCACGGTT					0.2
	IncP1 α	MRxeP1 α _for	TGAAGTACATCACCGACGAG	RP4	X54459.1	tral	424	0.2
		MRxeP1 α _rev	GTTTCGTGATTGTCACGCTC					0.2
	IncW	MRxeW_for	CATCGAGTTCGATACCCCTTG	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

383

384 **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	HI α , HI γ
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	HI γ
S00319	<i>S. enterica</i> Havana	Tc	CTX-M-15	HI2	HI γ
S01106	<i>S. enterica</i> Virchow	Tc	SHV-12	I1	I1
S01331	<i>S. enterica</i> Tel el kebiri	Tc	CTX-M-15	HI2	HI γ
S01477†	<i>S. enterica</i> Typhimurium	Tc	CTX-M-1/CMY-2	HI1, I1	I1, HI α , HI β
S01650	<i>S. enterica</i> Brandenburg	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	HI γ
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	HI γ
S07364	<i>S. enterica</i> Miami	Tc	SHV-2	N	N
S09118	<i>S. enterica</i> Keurmassar	Tc	SHV-12	HI2, FI	F, HI α
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

*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.

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	FIHK
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).

392
393

395

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).

423

424