Diversity of plasmids in *Escherichia coli and Klebsiella pneumoniae*: a comparison of commensal and clinical isolates

Judith Rodríguez-Navarro [a,b], Elisenda Miró [a], Maryury Brown-Jaque [c], Juan Carlos Hurtado [d], Albert Moreno [b,e], Maite Muniesa [c], Juan José González-López [b,e], Jordi Vila [d], Paula Espinal [a*†] and Ferran Navarro [a,b*†]

[a] Department of Microbiology, Hospital de la Santa Creu i Sant Pau, Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), Barcelona, Spain.

[b] Genetics and Microbiology Department. Universitat Autònoma de Barcelona, Barcelona, Spain.

[c] Department of Genetics, Microbiology and Statistics, University of Barcelona, Barcelona, Spain.

[d] ISGlobal, Hospital Clínic - Universitat de Barcelona, Barcelona, Spain.

[e] Department of Clinical Microbiology, Hospital Vall d'Hebron, Vall d'Hebron Institut de Recerca, Barcelona, Spain.

[†] Contributed equally as senior authors

***Corresponding address:** Dr. Ferran Navarro and Dr. Paula Espinal, Servei de Microbiologia, Hospital de la Santa Creu i Sant Pau, Institut d'Investigació Biomèdica Sant Pau, Barcelona, Spain., C/Sant Quintí, 89, 08041 Barcelona, Spain; Phone: +34935537297; E-mails: fnavarror@santpau.cat and pespinal@santpau.cat

ABSTRACT

In this study, the plasmid content of clinical and commensal strains was analysed and compared. The replicon profile was similar in both, except for L, M, A/C and N (detected only in clinical strains) and HI1 (only in commensal strains). Although I1 and F were the most frequent replicons, only IncI1 ST12 was associated with *bla*$_{CMY-2}$ in both populations. In contrast, the widespread resistant IncF plasmids were not linked to a single epidemic plasmid.

KEYWORDS: pMLST, replicon, antimicrobial resistance, Enterobacteriaceae, plasmid epidemiology.

RUNNING TITLE: Plasmid content in commensal and clinical Enterobacteriaceae

TEXT

The most prevalent mechanism in antimicrobial resistance gene (ARG) acquisition by bacterial pathogens is horizontal gene transfer by plasmids (1, 2). PCR-based replicon typing (PBRT) based on plasmid incompatibility (Inc) is currently the standard method for plasmid identification (3, 4). Plasmid multilocus sequence typing (pMLST) schemes allow to differentiate between plasmids within incompatibility groups and to define epidemiological and evolutionary relatedness (5–10) (http://pubmlst.org/plasmid/).

Several plasmids carrying ARGs have been characterized, most of them recovered from clinically relevant bacteria (11–14). In contrast, there is limited information on plasmids in the commensal microbiota of healthy humans without a selection bias for antimicrobial-resistant bacteria. In this scenario, the aim of this study was to provide a

52 better understanding of resistant plasmid diffusion in a clinical context by comparing
53 plasmids within *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from
54 healthy human faeces and patients with bloodstream infection.

55 One hundred and fifty faecal samples were collected during 2014-2015 from healthy
56 humans who did not consume antibiotics and were not hospitalized. A total of 145 *E.*
57 *coli* and 12 *K. pneumoniae* strains were isolated. In addition, 202 strains from blood
58 cultures, 99 of *E. coli* and 103 of *K. pneumoniae*, from three hospitals in Barcelona
59 were analysed (one per patient). All strains underwent antimicrobial susceptibility
60 testing using disk diffusion (Table S1) and the results were interpreted according to
61 CLSI guidelines (15). The characterization of ESBLs, AmpCs and carbapenemases (16–
62 20) detected in both populations is shown in Table 1. The prevalence of ESBL-
63 producing *E. coli* in healthy carriers (4.7%) was higher than in a previous study in
64 Barcelona in 2005 (3.3%) (21), but still within the 3-6% average of Europe (22).

65 Plasmid identification was performed using the PBRT-kit (Diatheva, 2014) and simplex
66 PCR for ColE, X3, X4, L and M replicons (23–25). Twenty-nine replicons were
67 analysed, and only FIIS, W, T, U and HI2 were not detected in any strain. A total of
68 978 replicons were identified in the 359 studied strains: 84.1% (302/359) harboured
69 from one to four replicons and 10.9% (39/359) from five to seven. In 5% (18/359) of
70 the strains no replicon was detected. Overall, the results suggest that the replicon
71 content of *E. coli* strains followed a similar trend in patients and healthy individuals,
72 and the most prevalent in both sample groups were ColE, FII and FIB (Fig. 1A).
73 Nevertheless, replicons M, A/C and N were only detected in clinical strains, in
74 accordance with the literature (26, 27), while FIIK and HI1 were observed only in faecal
75 strains (Fig. 1A). Hence, it might be hypothesized that the hospital environment, where
76 there is a high antimicrobial use and an intense interhuman transmission, selects for

77 plasmids more adapted to these settings. The plasmid content in *K. pneumoniae* isolates
78 seems to follow similar trends (Fig. 1B) to that of *E. coli*, but this could not be
79 confirmed due to the low number of strains obtained from faecal samples. Notably, both
80 the diversity and frequency of replicons were higher in *E. coli* than in *K. pneumoniae*,
81 except for R and FIIK (Fig. 1C).
82 IncF and IncI1 plasmids have been reported in Enterobacteriaceae as promoters of beta-
83 lactamase gene dissemination in multiple environments, specifically $bla_{\text{CTXM-15}}$ and
84 $bla_{\text{CMY-2}}$ (28–33). In this study, 56 beta-lactamase genes were detected (Table 1). After
85 S1-PFGE and Southern hybridization (19, 34), 75% of ESBL, AmpC and
86 carbapenemase genes identified in *E. coli* (27/36) and *K. pneumoniae* (15/20) were
87 located on a plasmid, the most prevalent being IncF and IncI1 (37% both) in *E. coli*, and
88 IncF (47%) and IncR (20%) in *K. pneumoniae*. The predominant genes were $bla_{\text{CTX-M-}}$
89 $_{15/14/27}$ and $bla_{\text{CMY-2}}$ in IncF and InI1 plasmids of *E. coli* (Fig. S1A) and $bla_{\text{CTX-M-15}}$ in
90 IncF plasmids of *K. pneumoniae* (Fig. S1B). Figure 2 summarises the 49 ESBL-,
91 AmpC- and/or carbapenemase-producing strains detected in the study, the plasmids they
92 harboured, and the location of the beta-lactamase genes.
93 As IncF and IncI1 were two of the most frequently detected plasmids in both faecal and
94 clinical samples (27, 35, 36), they were further characterised using the pMLST method
95 (5, 10). In *E. coli* strains, 29 different IncI1 sequence types (STs) were detected, 59% of
96 which were assigned as new STs. This result reflects the great diversity within this
97 plasmid family, with only ST12 and ST36 being present in both clinical and faecal
98 populations (Fig. S2). Moreover, some of the most frequently reported STs worldwide
99 (ST2, ST12, ST26 and ST36) (37) were only found in *E. coli* from healthy humans. The
100 detection of many newly assigned STs in the clinical isolates and a scarce number of the

most reported STs suggests the latter may have been over-reported due to their involvement in ARG dissemination, resulting in an epidemiological bias.

In addition, IncI1 plasmids have been associated with the carriage of *bla*$_{\text{CMY-2}}$, particularly IncI1 ST2, ST12, and ST23 (5, 38–40). In the current study, all identified ST12 plasmids harboured *bla*$_{\text{CMY-2}}$ and were detected in *E. coli* from both populations (Fig. 2). These results support the suggestion that some IncI1 plasmids have been able to evolve and persist in clinical settings, thanks to particular features that provide resistance, persistence and adaptive success, which would explain why they are more frequently reported and described as epidemic plasmids (38–40).

After defining the final number of IncF plasmids (n=279; 211 in *E. coli* and 68 in *K. pneumoniae*) by Southern hybridization of F-replicons within each strain, subtypification using replicon sequence typing (RST) was performed. In *E. coli* strains, 86 different FAB formulas from 205 typable plasmids (45 from faecal samples, 24 from blood and 17 from both) were defined, where F29:A-:B10, F2:A-:B1, F2:A-:B- and F24:A-:B1 were the most frequent (Fig. S3). Some of these formulas have been previously identified in different environments, such as avian-pathogenic *E. coli* strains and uropathogenic and extraintestinal pathogenic *E. coli* (27, 41), indicating a broad distribution. In *K. pneumoniae*, 16 different FAB formulas from 68 typable plasmids (12 from blood and 4 from both) were detected, K1:A-:B- being the most frequent (Fig. S3).

IncF plasmids, including F2:A-:B-, F2:A1:B-, F31:A4:B1 and F1:A2:B20, have been associated with the worldwide emergence of CTX-M-15 (10, 12, 42–44). Although these four plasmids were detected in our study, only F2:A-:B- harboured the *bla*$_{\text{CTX-M-15}}$ gene in a clinical strain and *bla*$_{\text{CTX-M-27}}$ in a faecal strain. All the other CTX-M-encoding genes were located in different IncF plasmids (Fig. 2). Thus, according to our results

and in agreement with those of previous reports (33, 35), there is no evidence for the persistence of a unique IncF. These highly versatile plasmids are able to adapt to intracellular environments by the rapid evolution of replicon regulatory sequences (10) and they were widely distributed in the Enterobacteriaceae before antimicrobial use, facilitating the persistence and spread of beta-lactamases (10, 33). Their co-existence with other resistance determinants also contributes to the dissemination of IncF-CTX-M plasmids (45).

Additionally, *E. coli* strains were assigned to phylogenetic groups following the procedure of Clermont *et al* (46) (Table 2), and the presence of 15 virulence factors (VFs) was determined (47, 48) (Fig. 3). In commensal *E. coli*, the prevalence of phylogenetic groups varies among studies (36, 49). It has been reported that the highly diverse hosts and environmental factors, the determinants of virulence and the antimicrobial pressure can modify prevalence for a better adaptation to commensal habitats (49). In our study, even though commensal *E. coli* presented a higher diversity of phylogroups compared to the clinical samples (Table 2), a predominance of the phylogroup B2 carrying high rates of VFs was found in both populations. Although no evident association has been reported between plasmids and phylogroups (35), our results indicate a possible association of HI1 plasmids with phylogroup A ($p \leq 0.007$, Bonferroni's correction was applied).

All VFs studied were detected in both populations. As expected, the clinical strains had a higher diversity (9 to15 VFs) and frequency of VFs compared to faecal samples (1 to 8 VFs) (Fig. 3). Finally, as supported by other authors (50), an association between some VFs (*fyuA*, *iutaA*, *hlyF*, *iss* and *traT*) and strains carrying F plasmids was determined ($p \leq 0.003$, Bonferroni's correction was applied).

In conclusion, new information is provided about the plasmid background in strains isolated in a non-hospital setting. Although a similar trend was observed in the Inc groups from both populations, IncL/M, IncA/C and IncN plasmids were only detected in clinical strains, whereas HI1 was only present in faecal strains. Also, two different evolutionary pathways followed by plasmids were observed: specific IncI1 plasmids, such as IncI1 ST12, seem to have evolved by acquiring persistence, adaptive and antibiotic resistance features relevant in clinical settings, whereas the more widespread multireplicon IncF plasmids have randomly acquired resistance genes. Additionally, the findings from this study confirm that strains from healthy individuals have less antimicrobial resistance and fewer VFs and display a higher diversity of phylogenetic lineages (in *E. coli*) than strains causing infection.

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Ethical considerations

The study was approved by the Hospital de la Santa Creu i Sant Pau ethics committee (13/051/1439).

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356 **Figure legends**

357

358 **Figure 1.** Replicon prevalence: (a) comparison between replicons detected in *E. coli*
359 from clinical and faecal samples; (b) comparison between replicons detected in *K.*
360 *pneumoniae* from clinical and faecal samples; (c) comparison between replicons
361 detected in *E. coli* and *K. pneumoniae* from the total strains. Tables show the number of
362 each replicon detected.

363 ND, not detected

364 *Statistical differences ($p<0.05$) between each population, faecal and blood samples or
365 *E. coli* and *K. pneumoniae*.

366

367 **Figure 2.** Heat-map summary of the sources, phylogenetic groups, β-lactamase
368 resistance genes, and the corresponding Inc plasmid types and their sizes for 49 β-
369 lactam-resistant *E. coli* (n=32) and *K. pneumoniae* (n=17) strains from faecal and blood
370 samples. Black and white squares denote the presence and absence of a particular
371 feature, respectively.

372 ND, not determined; CP, carbapenemases

373 [a] Plasmids where the replicon hybridisation occurred in the same plasmid size (hybrid
374 plasmids).

375 [b] *bla*-genes detected in the same plasmid.

376

377 **Figure 3.** Frequency of virulence factors (VFs) analysed in *E. coli* from faecal and
378 blood sample strains and percentages of *E. coli* strains according to the number of VFs
379 they carry. Adhesins *fimH* (mannose-specific adhesin of type 1 fimbriae) and *hra* (heat-
380 resistant agglutinin); siderophores *fyuA* (yersiniabactin), *iutA* (aerobactin), and *iroN*
381 (salmochelin receptor); toxins *hlyD* (α-hemolysin), *hlyF* (hemolysin F), *cnf1* (cytotoxic

necrotizing factor 1), *clbB* and *clbN* (colibactin), and the miscellaneous VF genes *iss* (surface exclusion serum survival protein), *traT* (serum resistance), *ompT* (outer membrane protease), *ibeA* (invasion of brain endothelium) and *usp* (uropathogenic-specific protein).

*Statistical differences ($p<0.05$) between each population, faecal and blood samples

387 **Table 1. ESBL, AmpC and carbapenemase genes detected in *E. coli* and *K.***

388 ***pneumoniae* from faecal and blood samples**

	***E. coli* (n=244)**		***K. pneumoniae* (n=115)**	
	Faecal samples (n=13/145)[a]	**Blood cultures (n=19/99)[b]**	**Faecal samples (n=0/12)**	**Blood cultures (n=17/103)[c]**
ESBLs (n=43)	**n=8, 5.5%** $bla_{CTX\text{-}M\text{-}15}$ (n=4) $bla_{CTX\text{-}M\text{-}14}$ (n=3) $bla_{CTX\text{-}M\text{-}27}$ (n=1) $bla_{SHV\text{-}12}$ (n=1)	**n=17, 17.2%** $bla_{CTX\text{-}M\text{-}15}$ (n=10) $bla_{CTX\text{-}M\text{-}14}$ (n=2) $bla_{CTX\text{-}M\text{-}27}$ (n=2) $bla_{CTX\text{-}M\text{-}32}$ (n=1) $bla_{SHV\text{-}12}$ (n=2)	-	**n=17, 16.5%** $bla_{CTX\text{-}M\text{-}15}$ (n=9) $bla_{CTX\text{-}M\text{-}14}$ (n=2) $bla_{SHV\text{-}28}$ (n=5) $bla_{SHV\text{-}2}$ (n=1)
AmpCs (n=11)	**n=5, 3.5%** $bla_{CMY\text{-}2}$ (n=5)	**n=5, 5.0%** $bla_{CMY\text{-}2}$ (n=4) $bla_{DHA\text{-}1}$ (n=1)	-	**n=1, 1.0%** $bla_{DHA\text{-}1}$ (n=1)
Carbapenemases (n=2)	-	-	-	**n=2, 1.9%** $bla_{KPC\text{-}3}$ (n=2)
Total genes (n=56)	14	22	0	20

389

390 [a] one *E. coli* strain had $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$

391 [b] three *E. coli* strains had $bla_{CTX\text{-}M\text{-}15}$/$bla_{CTX\text{-}M\text{-}32}$, $bla_{CTX\text{-}M\text{-}27}$/$bla_{DHA\text{-}1}$ and $bla_{SHV\text{-}12}$/$bla_{CMY\text{-}2}$.

393 [c] one *K. pneumoniae* strain had $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$, and two strains $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}28}$.

395

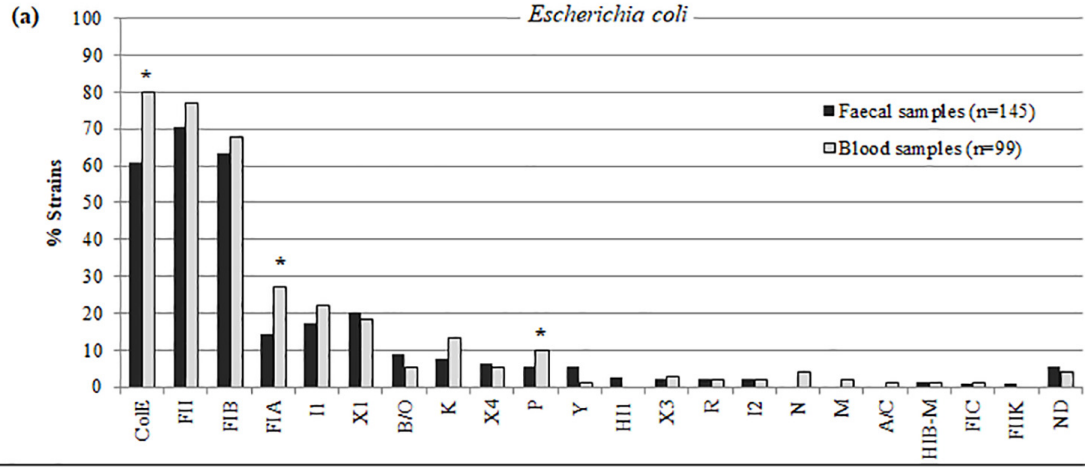
396 **Table 2. Phylogenetic groups detected in *E. coli* strains isolated from faecal and blood samples**

		Phylogenetic groups								
		A	**B1**	**B2**	**C**	**D**	**E**	**F**	**Clade I**	**Unknown**
Total *E. coli*	**n=244 (%)**	26 (10.6)	22 (9)	124 (50.8)	3 (1.2)	32 (13.1)	15 (6.1)	17 (6.9)	4 (1.6)	1 (0.41)
Faecal samples	**n=145**	23 (15.9)[a]	11 (7.6)	59 (40.7)[b]	3 (2.1)	23 (15.9)	8 (5.5)	13 (8.9)	4 (2.7)	1 (0.7)
	S n= 71	16 (22.5)	1 (1.4)	24 (33.8)	3 (4.2)	10 (14.1)	7 (9.9)	9 (12.6)	1 (1.4)	0
	R n= 74	7 (9.5)	10 (13.5)	35 (47.3)[c]	0	13 (17.6)	1 (1.3)	4 (5.4)	3 (4.1)	1 (1.3)
Blood samples	**n= 99**	3 (3)[a]	11 (11.1)	65 (65.7)[b]	0	9 (9.1)	7 (7.1)	4 (4)	0	0
	S n= 9	0	1 (11.1)	6 (66.7)	0	2 (22.2)	0	0	0	0
	R n= 90	3 (3.4)	10 (11.1)	59 (65.5)[c]	0	7 (7.8)	7 (7.8)	4 (4.4)	0	0

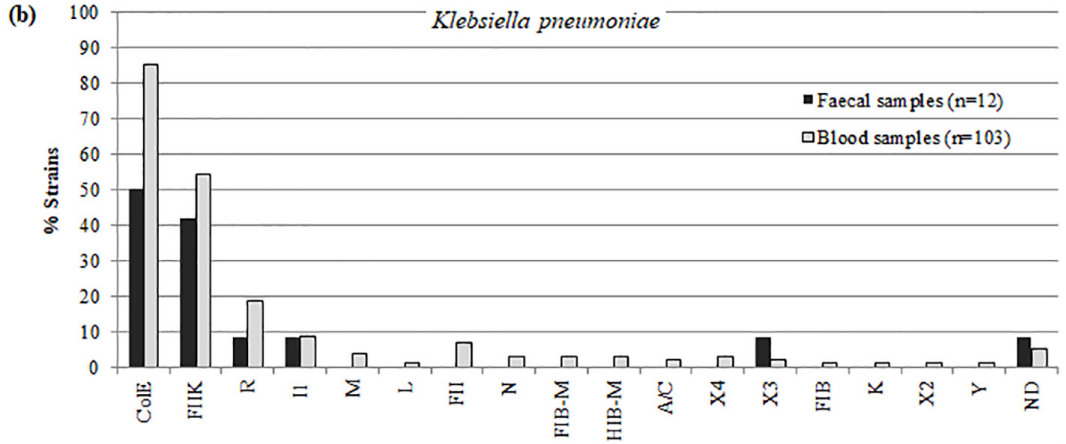
397 S, susceptible to all antimicrobials tested; R, resistant to at least one of the antimicrobials tested.

398 [a,b,c] Statistical differences ($p<0.05$) between each population and the different phylogroups.

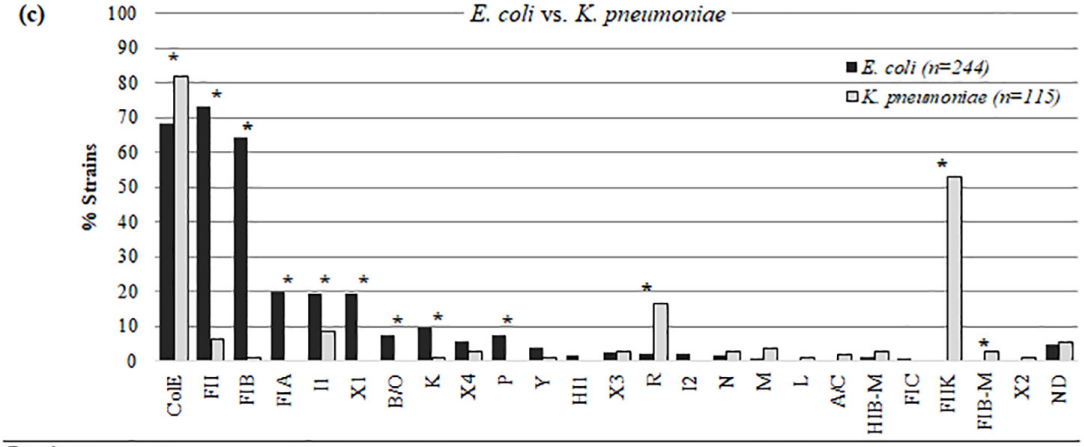
399



	ColE	FII	FIB	FIA	I1	X1	B/O	K	X4	P	Y	HI1	X3	R	I2	N	M	A/C	HIB-M	FIC	FIIK	ND
Faecal samples n=145	88	102	92	21	25	29	13	11	9	8	8	4	3	3	3	0	0	0	2	1	1	8
Blood samples n=99	79	76	67	27	22	18	5	13	5	10	1	0	3	2	2	4	2	1	1	1	0	4



	ColE	FIIK	R	I1	M	L	FII	N	FIB-M	HIB-M	A/C	X4	X3	FIB	K	X2	Y	ND
Faecal samples n=12	6	5	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	1
Blood samples n=103	88	56	18	9	3	4	1	3	3	3	2	3	2	1	1	1	1	5



	ColE	FII	FIB	FIA	I1	X1	B/O	K	X4	P	Y	HI1	X3	R	I2	N	M	L	A/C	HIB-M	FIC	FIIK	FIB-M	X2	ND
E. coli n=244	167	178	157	48	47	47	18	24	14	18	9	4	6	5	5	4	2	0	1	3	2	1	0	0	12
K. pneumoniae n=115	94	7	1	0	10	0	0	1	3	0	1	0	3	19	0	3	4	1	2	3	0	61	3	1	6

Specie	Source: Faecal	Source: Blood	Strain	Phylogroup	ESBL: *bla* CTX-M-15	ESBL: *bla* CTX-M-14	ESBL: *bla* CTX-M-27	ESBL: *bla* CTX-M-32	ESBL: *bla* SHV-12	ESBL: *bla* SHV-28	ESBL: *bla* SHV-2	AmpC: *bla* CMY-2	AmpC: *bla* DHA-1	CP: *bla* KPC-3	PBRT: ColE	PBRT: I1	PBRT: FIA	PBRT: FIB	PBRT: FII	PBRT: B/O	PBRT: I2	PBRT: P	PBRT: FIC	PBRT: X1	PBRT: X4	PBRT: R	PBRT: K	PBRT: Y	PBRT: FIIK	PBRT: N	PBRT: L/M	PBRT: FIB-M	*bla*-gene location: *bla*-gene	*bla*-gene location: Plasmid type	*bla*-gene location: Plasmid size (Kb)
Escherichia coli	■		UB37	B1								■				■																	*bla* CMY-2	IncI1 (ST12)	72
	■		VH10	B1		■									■	■		■	■							■							*bla* CTX-M-14	IncI1 (ST238)	48.5
	■		VH17	B1				■									■	■	■				■										*bla* SHV-12	F2:A1:B56	72
	■		UB2	B2								■			■	■		■	■													*bla* CMY-2	ND	-
	■		HC20	B2	■										■		■	■	■						■								*bla* CTX-M-15	F1:A1:B16	97
	■		VH2	B2								■			■	■		■	■	■		■				■						*bla* CMY-2	IncI1 (ST237)	97
	■		VH3	B2								■			■	■		■	■			■				■						*bla* CMY-2	IncI1 (ST12)	97
	■		SP44	D			■												■														*bla* CTX-M-27	F2:A-:B-	72
	■		HC12	D	■	■									■	■	■	■	■							■							*bla* CTX-M-14/*bla* CTX-M-15 [b]	IncI1 (ST170)	72
	■		VH7	D		■													■		■			■	■			■					*bla* CTX-M-14	F35:A-:B-	48.5
	■		SP12	E	■										■	■			■					■									*bla* CTX-M-15	ND	-
	■		SP13	F	■										■		■	■	■														*bla* CTX-M-15	ND	-
	■		HC27	F								■				■	■	■	■														*bla* CMY-2	IncI1 (ST239)	48.5
		■	HSP65.H	A	■			■								■	■							■							■		*bla* CTX-M-15/*bla* CTX-M-32 [b]	IncI1 (ST80)	145.5
		■	HSP03.H	B2	■										■		■	■	■							■							*bla* CTX-M-15	ND	
		■	HSP06.H	B2			■								■		■		■						■		■						*bla* CTX-M-27	F1:A6:B-	48.5
		■	HSP13.H	B2	■										■		■	■	■								■						*bla* CTX-M-15	ND	-
		■	HSP26.H	B2		■									■		■	■															*bla* CTX-M-14	F-:A6:B1	97
		■	HSP34.H	B2				■							■			■	■					■							■		*bla* SHV-12	IncN (ST1)	97
		■	HSP56.H	B2								■			■	■		■															*bla* CMY-2	F-:A-:B29 / IncI1 (ST292) / IncK [a]	145.5
		■	HSP61.H	B2		■											■		■														*bla* CTX-M-14	F1:A6:B-	145.5
		■	HSP74.H	B2	■										■		■	■	■														*bla* CTX-M-15	ND	-
		■	HC01.H	B2								■						■									■						*bla* CMY-2	IncK	48.5
		■	HVH03.H	B2	■										■		■	■	■														*bla* CTX-M-15	ND	-
		■	HVH07.H	B2	■										■	■	■	■			■												*bla* CTX-M-15	ND	-
		■	HVH08.H	B2	■										■			■	■	■				■									*bla* CTX-M-15	F2:A-:B-	97
		■	HVH11.H	B2	■										■		■	■	■					■									*bla* CTX-M-15	ND	-
		■	HVH13.H	B2	■										■			■	■					■									*bla* CTX-M-15	ND	-
		■	HSP48.H	E				■				■			■	■		■	■														*bla* SHV-12/*bla* CMY-2 [b]	F18:A-:B1 / IncI1 (ST12) [a]	170
		■	HSP52.H	E								■				■																	*bla* CMY-2	IncI1 (ST258)	48.5
		■	HSP67.H	E			■						■	■			■	■	■												■		*bla* CTX-M-27	F1:A6:B20	121
																																	bla DHA-1	IncM	48.5
		■	HSP51.H	F	■										■	■		■	■			■											*bla* CTX-M-15	F2:A-:B10	194
Klebsiella pneumoniae		■	HSP31.H		■										■	■										■		■					*bla* CTX-M-15	IncR	48.5
		■	HSP57.H											■	■	■	■									■		■					*bla* KPC-3	ND	48.5
		■	HSP66.H										■		■											■		■		■			*bla* DHA-1	IncM	48.5
		■	HSP70.H							■					■											■		■					*bla* SHV-28	K8:A-:B- / IncR [a]	48.5
		■	HSP86.H		■	■									■											■		■					*bla* CTX-M-14/*bla* CTX-M-15 [b]	K7:A-:B-	245
		■	HSP89.H		■										■											■		■					*bla* CTX-M-15	IncR	72
		■	HSP93.H								■				■										■								*bla* SHV-2	IncR	97
		■	HSP96.H			■									■												■							*bla* CTX-M-14	IncK	194
		■	HSP98.H											■	■													■					*bla* KPC-3	K1:A-:B-	267
		■	HSP104.H		■										■															■	■		*bla* CTX-M-15	ND	-
		■	HC08.H		■				■						■											■		■					*bla* CTX-M-15	K7:A-:B-	
																																	bla SHV-28	ND	170
		■	HC11.H		■				■						■											■		■		■			*bla* CTX-M-15	K9:A-:B-	170
		■	HC19.H		■				■						■													■					*bla* CTX-M-15	K7:A-:B- / IncR [a]	
																																	bla SHV-28	ND	218
		■	HVH40.H						■																			■					*bla* SHV-28	ND	-
		■	HVH48.H		■										■													■			■		*bla* CTX-M-15	K7:A-:B-	170
		■	HVH50.H		■										■			■								■		■					*bla* CTX-M-15	F2:A-:B-	145.5
		■	HVH60.H							■					■			■	■														*bla* SHV-28	ND	-