

1 **Mobile genetic elements related to the diffusion of plasmid-mediated AmpC β -**
2 **lactamases or carbapenemases from *Enterobacteriaceae*: findings from a multicenter**
3 **study in Spain**

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18 **Running title:** Genetic context of plasmid-mediated AmpC β -lactamases or carbapenemases
19 in *Enterobacteriaceae*

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24

25 **ABSTRACT**

26 We examined the genetic context of 74 acquired *ampC* genes and 17 carbapenemase genes
27 from 85 out of 640 *Enterobacteriaceae* isolates collected in 2009. Using S1-PFGE and
28 Southern hybridization, 37 out of 74 *bla*_{AmpC} genes were located on large plasmids of different
29 sizes belonging to six Inc groups. We used sequencing and PCR mapping to investigate the
30 regions flanking the acquired *ampC* genes. The *bla*_{CMY-2like} genes were associated with
31 *ISEcp1*, the surrounding *bla*_{DHA} genes were similar to *Klebsiella pneumoniae* plasmid
32 pTN60013 associated with IS26 and the *psp* and *sap* operons, and *bla*_{ACC-1} genes were
33 associated with IS26 elements inserted into *ISEcp1*. All the carbapenemase genes (*bla*_{VIM-1},
34 two *bla*_{IMP-22} and *bla*_{IMP-28}) were located in class 1 integrons. Therefore, although plasmids are
35 the main cause of the rapid dissemination of *ampC* genes among *Enterobacteriaceae*, we need
36 to be aware that other mobile genetic elements, such as insertion sequences, transposons or
37 integrons, can be involved in the mobilization of these genes of chromosomal origin.
38 Additionally, three new integrons are described in this study (In846 to In848).

39

40 **INTRODUCTION**

41 β -lactam resistance in *Enterobacteriaceae* due to acquired Amp-C β -lactamases (pAmpC) or
42 carbapenemases represents an emerging and increasing problem that limits therapeutic
43 options. pAmpC confer resistance to most β -lactams, except cefepime and carbapenems,
44 whereas carbapenemases, including class A, B and D, can confer resistance to most β -
45 lactams, including carbapenems. The number of *Enterobacteriaceae* carrying these enzymes
46 is lower than ESBLs-producing isolates, but it has increased over the last few years,
47 particularly the pAmpC CMY-2 and DHA, and carbapenemases NDM, VIM, IMP and OXA-
48 48 types (1, 2).

49 Both families of enzymes (pAmpC and carbapenemases) are normally codified in plasmids,
50 and their genes are associated with mobile genetic elements (MGE) such as insertion
51 sequences, transposon-like elements and class 1 integrons. All these MGE can transfer these
52 genes into mobilizable and conjugative plasmids and subsequently disseminate them into
53 many bacterial species that naturally lack these genes (3-5).

54 As previously described (3), 100,132 *Enterobacteriaceae* isolates were collected from
55 February to July 2009 from 35 Spanish hospitals. Among them, we found a total of 674
56 *Enterobacteriaceae* with acquired *ampC* and/or carbapenemase genes. The enzyme types
57 found were: CMY-2-like (74.3%), followed by DHA (17.8%), ACC (1.5%), FOX (0.6%),
58 VIM (4.3%) and IMP (1.5%) (3). Although a great genetic diversity among pAmpC-
59 producing strains was observed, some clonal relationships were established between these
60 isolates, mainly in carbapenemase-producing strains (3).

61 This study aimed to describe the plasmid families and the surrounding regions involved in the
62 dissemination of a great diversity of acquired *ampC* and metallo- β -lactamases genes in
63 *Enterobacteriaceae* isolates lacking inducible chromosomal AmpC enzymes.

64

65 MATERIAL AND METHODS

66 **Clinical isolates.** To characterize the plasmids and flanking regions implicated in the
67 expansion of these genes, we selected 85 strains from the collection cited above (3). The
68 selection was made on the basis of prevalence, and strains that produced new enzymes were
69 also included.

70 **PCR-based replicon typing.** PCR-based Inc/rep typing (PBRT) was used to identify the
71 major Inc groups of the plasmids present (4,6).

72 **Plasmid profiles and Southern blot analysis.** Plasmid analysis was carried out by DNA
73 linearization with the S1 enzyme followed by pulsed-field gel electrophoresis (PFGE), as

74 previously described (7). Plasmid sizes were estimated using Fingerprinting II Informatix™
75 software (Bio-rad)(7). A PCR DIG Probe synthesis kit (Roche Diagnostics GmbH,
76 Mannheim, Germany) was used to obtain *bla*_{AmpC} or Inc probes for hybridization of the S1-
77 PFGE blots. These probes were labelled with the commercial kit (DIG high prime DNA
78 labelling and detection starter kit II (Roche Diagnostics GmbH, Mannheim, Germany)).
79 The chromosomal location of *bla* genes was analysed by digesting the genomic DNA with the
80 ICEuI enzyme, followed by PFGE and hybridization blots, as described above.

81 **Genetic environment characterization of acquired *ampC* and carbapenemase genes.** The
82 genetic context was investigated by exploring the regions surrounding acquired AmpC and
83 carbapenemase genes frequently reported in the literature (8-14), employing PCR and
84 sequencing with previously described primers. Additionally, primers designed in accordance
85 with accessible DNA sequences in the GenBank (AY581207, AJ870924, Y11068, AJ971345
86 and EF577408) were used to ascertain the presence of genes linked to the acquired *bla*_{ampC}
87 and carbapenemase genes (Table S1). Sequencing reactions were performed with the BigDye
88 Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an
89 ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were
90 then compared with those available at the GenBank (www.ncbi.nih.gov/BLAST).

91

92 **RESULTS**

93 Among the 85 selected strains (66 pAmpC-producing, 13 IMP/VIM-producing, 4 producing
94 both enzymes and 2 strains that produced two pAmpC), we characterized the plasmids and
95 flanking regions of 91 genes, 74 *bla*_{ampC} and 17 *bla*_{IMP/VIM} genes (Table 1).

96 The studied *bla*_{ampC} genes included 40 *bla*_{CMY-2-like}, 22 *bla*_{DHA}, 8 *bla*_{ACC} and 4 *bla*_{FOX}, while
97 the metallo-β-lactamase genes included 14 *bla*_{VIM-1}, 2 *bla*_{IMP-22} and 1 *bla*_{IMP-28}.

98 **Plasmid characterization.** Analysis by S1-PFGE and Southern hybridization allowed us to
99 determine the plasmid size for 74.7% (68/91) of the studied genes, leaving 23 genes (25.3%)
100 with a possible chromosomal location (positive hybridization in ICEuI-PFGE). Nevertheless,
101 we were able to describe the incompatibility group by PCR-based replicon (PBRT-PCR)
102 among these 68 plasmidic genes in only 41 cases (60.3%).

103 We found that 39 out of 74 *bla*_{AmpC} genes (50%) were located in large plasmids of different
104 sizes belonging to eight Inc groups, including: A/C, FIB, FIIA, I1, K, HI2, N and U. The most
105 representative, present alone or together with other replicons, were: I1 (n=18, one also with
106 FIB), K (n=7; two also with FIB), A/C (n=6), FIIA (2), HI2 (2) and N (2) (Table 1). In 13 out
107 of 74 *bla*_{AmpC} genes (17.5%), the plasmid replicon was not identified (in 10 cases the PBRT-
108 PCR was positive for different replicons, but their hybridization bands did not match the
109 *bla*_{AmpC} band, and in 3 cases the PBRT-PCR was negative). Finally, in 22 cases (29.7%) a
110 possible chromosomal location of these genes was confirmed by ICEuI-PFGE.

111 The plasmids carrying *bla*_{CMY-2-like} genes belonged to the following Inc groups: I1 [16/40
112 (37.5%): sizes ranged from 43.7 to 145.5 kb), K (7/40 (17.5%): sizes ranged from 48.5 to
113 105.1 kb), and A/C [5/40 (12.5%): sizes ranged from 177.5 to 300.7 kb). In three cases the
114 FIB plasmid was also found to be associated with one IncI1 or two IncK plasmids (Table 1).

115 Seven out of 22 plasmids carrying *bla*_{DHA} genes were characterized, with the following Inc
116 groups being found: I1 (9%: sizes 77.6 and 87.3 kb), FIIA (9%: size 76.6 and 218 kb), HI2
117 (9%: size 291 kb) and A/C (4.5%: 203.7 kb). The remaining 15 cases were not resolved, in 11
118 cases because no incompatibility group probes hybridized and in four cases because the
119 PBRT-PCR was negative.

120 Only two of the eight plasmids carrying *bla*_{ACC-1} were identified and they belonged to the
121 IncN group, with sizes varying from 32.5 to 80 kb. *bla*_{FOX-3} genes were found in plasmids of

122 72.5 and 80.5 kb, both of the IncU group, and the *bla*_{FOX-8} gene (15) was probably located on
123 the chromosome.

124 Finally, the sizes of the fourteen plasmids carrying the *bla*_{VIM-1} gene ranged from 48 to 72.5
125 kb and one of them belonged to the IncU group; *bla*_{IMP-22} genes were in plasmids of 485 kb
126 with an unidentified Inc group. The *bla*_{IMP-28} gene was probably located on the chromosome,
127 as previously described (16).

128 **Detection of the flanking regions of acquired *ampC* and metallo- β -lactamase genes.**

129 The variable genetic environments detected for the most prevalent enzymes (CMY-2-like,
130 DHA, ACC and metallo- β -lactamase genes) are shown in Figures 1-3.

131 The analysis of the genetic environment revealed that *bla*_{CMY-2-like} genes (*bla*_{CMY-2}, *bla*_{CMY-4},
132 *bla*_{CMY-7}, *bla*_{CMY-27}, *bla*_{CMY-48}, *bla*_{CMY-54/57}, *bla*_{CMY-59} and *bla*_{CMY-60}) were associated with
133 *ISEcp1*, responsible for the transfer of the *bla*_{CMY-2-like}-*blc-sugE* region from the chromosome
134 of *Citrobacter freundii* to plasmids (8). In our study, 16 strains contained the *ISEcp1* and *blc-*
135 *sugE-ecnR* upstream and downstream of *bla*_{CMY-2-like} genes, respectively. However, truncation
136 of *ISEcp1*(Δ *ISEcp1*) was observed in 8 strains: four at the 3' end and four at the 5' end. In the
137 former, primers described to explore this region (*ISEcp1*/CMY2Ri) (Table S1) amplified a
138 product of 1,560 bp instead of the expected 2,160 bp. In the latter, the amplicons were not
139 obtained using *ISEcp1*/CMY2Ri primers and we required a new pair of primers
140 (TnpA1L/CMY2Ri). Finally, twelve strains did not contain *ISEcp1* upstream, and in six
141 strains the region downstream of the *bla*_{CMY-2-like} gene could not be amplified by PCR with the
142 primers used. Only in two strains, with complete *bla*_{CMY-2-like} gene, the genetic environment
143 was unknown (Figure 1).

144 The surrounding regions of *bla*_{DHA} genes (*bla*_{DHA-1}, *bla*_{DHA-6} and *bla*_{DHA-7}) were similar to
145 those previously described in *Klebsiella pneumoniae* plasmid pTN60013 (AJ971345) (5),
146 although a certain variability was detected, in accordance with the literature data (11,17). This

147 variability mainly concerned the absence or presence of *sapB*, *sapA* and *sdr* genes (Figure 2).
148 The quinolone resistance determinant *qnrB4* and additional *aadA1* (streptomycin and
149 spectinomycin resistance) genes were detected in most of the strains. This linkage between
150 *bla_{DHA-1}* and *qnrB4* genes has been previously described in isolates of *K. pneumoniae* (11).
151 In the environment of the *bla_{ACC}* gene, *ISEcp1* and *gdhA* gene were detected upstream and
152 downstream, respectively. In all cases, *ISEcp1* was truncated in the 5'(13). Six out of eight
153 *bla_{ACC}* genes showed two IS26 copies in the same orientation; one of these strains contained a
154 truncated 5' *gdhA* and one contained a *mpR* gene of Tn5393 upstream of the *gdhA* gene.
155 Four *bla_{FOX}* genes, *bla_{FOX-3}* (n=2) and *bla_{FOX-8}* (n=2), were located in a class I integron, at the
156 5' of the integrase *intI1*, and several attempts to identify the 3' end by PCR were unsuccessful.
157 All the metallo- β -lactamase genes (14 *bla_{VIM-1}*, 2 *bla_{IMP-22}* and 1 *bla_{IMP-28}*) were located in
158 class I integrons. In this study, we detected five different structures harbouring *bla_{VIM}* genes
159 (Figure 3), with *In846* (GenBank accession number KC417378), *In847* (KC417379) and
160 *In848* (KC417377) being described for the first time.

161

162 **DISCUSSION**

163 We have characterized the genetic context of the largest available collection of acquired
164 AmpC β -lactamases and metallo- β -lactamases in *Enterobacteriaceae* lacking inducible
165 chromosomal AmpC enzymes, recovered during 2009 from 35 Spanish hospitals (3).
166 Several authors have related the spread of different acquired AmpC genes with the expansion
167 of plasmids of certain incompatibility groups (1, 2, 4,5,7,18-21). In this context, the *bla_{CMY-2}*
168 gene is associated with plasmids of I1, A/C, and K incompatibility groups (7, 18-21). Our
169 results match these data, but differences were found in the percentage of each incompatibility
170 group. In a previous study (7), carried out during 1999-2007, A/C was the most predominant
171 incompatibility group found (33%) among plasmids carrying *bla_{CMY-2}*, followed by I1 (23%)

172 and K (10%). In this study (with strains isolated in 2009), the most prevalent incompatibility
173 group was II (40%), followed by K (17.5%) and A/C (12.5%). The fact that different Inc
174 plasmids have been found to carry the same resistance gene is an indication of a successful
175 and widespread distribution; moreover, this variability contributes to the genetic environment
176 of these genes (20). The IncA/C and IncI1 are considered to be epidemic plasmids, because
177 they are found in different countries, in bacteria of diverse origin and carrying a range of
178 resistance mechanisms (4). IncA/C plasmids have been described carrying ESBLs TEM-type
179 or VEB, as well as NDM-1 carbapenemase. On the other hand, IncI1 has an efficient
180 conjugative system that could also contribute to the dissemination of different resistance
181 mechanisms, such as ESBLs CTX-M-type and TEM-type (4).

182 The genetic environment of *bla*_{CMY-2} and its variants was highly conserved. 60% of isolates
183 carried the transposon-like elements *ISEcp1*(*ISEcp1*/ Δ *ISEcp1*-*bla*_{CMY}-*blc*-*sugE*), as
184 documented in previous reports (8,10,18). As these *bla*_{CMY-2}-derived *bla*_{CMY} genes differ from
185 one other by only a few nucleotide substitutions, it is possible that these differences could
186 have evolved within the same Inc plasmid (11) (20). The genes, *bla*_{CMY-55} and *bla*_{CMY-56}, were
187 found in the A/C plasmid, and the *bla*_{CMY-54} gene in a K plasmid, in this case cointegrated with
188 FIB.

189 *bla*_{DHA-1} genes were initially associated with IncFII plasmids (20), but recent studies link them
190 with IncL/M plasmids and *qnrB* determinants (5,7). Among our DHA-producing strains, 19
191 showed the *qnrB4* determinant (data not shown) and none were associated with the IncL/M
192 plasmids. In fact, we were only able to characterize the plasmid in seven cases (38.8%), with
193 I2, FIIA and HI2 being the incompatibility groups found. The genetic organization of *bla*_{DHA}
194 genes was more variable. Mobilization of this enzyme has been associated with IS26 or class
195 1 integron-bearing ISCR1 elements (11,18). Among *bla*_{DHA}-carrying isolates, 86% were
196 associated with IS26.

197 In the literature, characterization of plasmids carrying *bla*_{ACC-1} genes is scarce. In a previous
198 study, a *bla*_{ACC-1} gene in an *Escherichia coli* strain was found in an IncI1 plasmid (7), but
199 other authors could not type it (21). Regarding the genetic context of *bla*_{ACC-1} genes, an
200 *ISEcpI* element truncated at the 5' end with an IS26 insertion sequence was found in all of our
201 *bla*_{ACC-1}-carrying isolates, as described in previous reports (12,13).

202 *bla*_{FOX} and all carbapenemases detected in this study, including previously undescribed
203 structures (*bla*_{FOX}), were located in a class 1 integron, and present in the most recent IncU
204 plasmids.

205 There is little data on the types of plasmids involved in the spread of metallo- β -lactamases. In
206 the literature, *bla*_{IMP} and *bla*_{VIM} genes are described in plasmids of incompatibility groups II,
207 N, W and HI2 (4). In this study, the *bla*_{IMP-22} gene was located in a 485kb plasmid of an
208 uncharacterised incompatibility group, and two *bla*_{VIM-1} genes were found in IncU plasmids,
209 curiously both isolated from different species but in the same hospital. Finally, the location of
210 the *bla*_{IMP-28} gene seems to be chromosomal, as *bla*_{IMP-28} positive hybridization was found in
211 the PFGE-ICeuI membrane.

212 Accordingly, the high number of unidentified replicons could be associated with plasmids
213 other than those tested or, alternatively, they could be associated with one of the tested
214 plasmids, albeit with some genetic variability, as has been described for the carbapenemase
215 NDM in plasmids with a variant of the IncN or IncHI1 groups (4).

216 In conclusion, although plasmids have proven to be one of the main causes of the rapid
217 dissemination of *bla*_{AmpC} and carbapenemase genes among bacteria, other MGE must play an
218 important role in the increasing prevalence of these enzymes. Further studies, focused not
219 only on plasmids but also on other MGE, such as insertion sequences, transposons or ICES,
220 are needed to gain a better understanding of the complex process involved in the
221 dissemination of antibiotic resistance genes worldwide.

222

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299

300 **Figure legend**

301 **Figure 1.** The genetic environment of *bla*_{CMY-2like} and *bla*_{ACC} genes, which are represented by
302 solid black arrows, while the surrounding genes are represented by white arrows. The
303 discontinued lines represent the areas that could not be amplified by PCR. Eight isolates
304 indicate truncated versions of *ISEcp1* at 3' (pattern A.1) and 5' end (pattern A.2). Truncated
305 genes are represented by disrupted arrows. One isolate (pattern C) carrying the ACC gene
306 showed an identical structure to AJ870924. In one isolate (pattern D), IS26 was truncated by a
307 *tmpR* gene of insertion Tn5393. The primers used for PCR amplification are also shown.

308 .

309 **Figure 2.** The genetic environment of the *bla*_{DHA} gene, which is represented by solid black
310 arrows, while the surrounding genes are represented by white arrows. Continuous lines
311 indicate an absence of the corresponding DNA fragments.

312

313 **Figure 3.** Structure of *bla*_{VIM-1}, *bla*_{IMP-22} and *bla*_{IMP-28} genes carrying the integrons described
314 in this work. Carbapenemase genes are represented by solid black arrows and the surrounding
315 genes are represented by white arrows. The locations of the primers used for PCR
316 amplification are also shown.

317 **Table 1.** Flanking regions and plasmid families associated with acquired AmpC and
 318 carbapenemases in *Enterobacteriaceae*

<i>bla</i> genes	Flanking region ^a	Plasmid size (kb) ^b	Replicon	Microorganism (n)	
<i>bla</i> _{ACC-1}	ACC-1(B)	64.5	N	<i>K. pneumoniae</i> (1); <i>E. coli</i> (1)	
		Chromosomal		<i>E. coli</i> (1); <i>P. mirabilis</i> (1)	
		80	Unidentified	<i>K. pneumoniae</i> (1)	
	ACC-1(C)	32.5	Unidentified	<i>P. mirabilis</i> (1)	
	ACC-1(D)	48.5	Unidentified	<i>K. pneumoniae</i> (1)	
<i>bla</i> _{ACC-1} + <i>bla</i> _{FOX-3}	ACC-1(A) + FOX (A)	64.5 + 80.5	Unidentified + U	<i>E. coli</i> (1)	
<i>bla</i> _{CMY-2}	CMY-2(A)	177.5 to 300.7	A/C	<i>K. pneumoniae</i> (2); <i>P. mirabilis</i> (1)	
		43.7 to 83.1	II	<i>C. koseri</i> (1); <i>E. coli</i> (4)	
		69.3	II + FIB	<i>E. coli</i> (1)	
		48.5 to 83.1	K	<i>E. coli</i> (3); <i>K.pneumoniae</i> (1)	
		Chromosomal		<i>E. coli</i> (1); <i>K. oxytoca</i> (1)	
		CMY-2(B)	55.4	K	<i>E. coli</i> (1)
			76.2	II	<i>E. coli</i> (1)
		CMY-2(C)	145.5	II	<i>K. pneumoniae</i> (1)
			Chromosomal		<i>P. mirabilis</i> (3); <i>K. pneumoniae</i> (1); <i>P. penneri</i> (1)
		CMY-2(D)	83.1 to 97.6	II	<i>E. coli</i> (1); <i>K. pneumoniae</i> (1)
<i>bla</i> _{CMY-4}	CMY-2(A)	105.1	K + FIB	<i>E. coli</i> (1)	
		Chromosomal		<i>E. coli</i> (1)	
	CMY-2(B)	80.8	II	<i>E. coli</i> (1)	
	CMY-2(C)	88.9	II	<i>K. pneumoniae</i> (1)	
<i>bla</i> _{CMY-7}	CMY-2(A)	80.8	II	<i>E. coli</i> (1)	
<i>bla</i> _{CMY-27}	CMY-2(A)	113.2	II	<i>E. coli</i> (2)	
<i>bla</i> _{CMY-43}	CMY-2(A)	Chromosomal		<i>E. coli</i> (1)	
<i>bla</i> _{CMY-48}	CMY-2(B)	Chromosomal		<i>E. coli</i> (1)	
<i>bla</i> _{CMY-54}	CMY-2(C)	105.1	K + FIB	<i>E. coli</i> (1)	
<i>bla</i> _{CMY-55}	CMY-2(A)	282.9	A/C	<i>E. coli</i> (1)	
<i>bla</i> _{CMY-56}	CMY-2(A)	299.1	A/C	<i>K. pneumoniae</i> (1)	
<i>bla</i> _{CMY-57}	CMY-2(C)	97.0	II	<i>E. coli</i> (1)	
<i>bla</i> _{CMY-2} + <i>bla</i> _{DHA-1}	CMY-2(A) + DHA-1(G)	Chromosomal + 218	FIIA	<i>K. pneumoniae</i> (1)	
<i>bla</i> _{CMY-2} + <i>bla</i> _{VIM-1}	CMY-2(C) + VIM-1(B)	Chromosomal + 48	Unidentified	<i>P. mirabilis</i> (1)	
<i>bla</i> _{DHA-1}	DHA-1(A)	72.8 to 87.3	Unidentified	<i>C. koseri</i> (1); <i>E. coli</i> (1); <i>K. pneumoniae</i> (2)	
		203.7	A/C	<i>K. pneumoniae</i> (1)	
		Chromosomal		<i>C. koseri</i> (1); <i>E. cloacae</i> (1)	
	DHA-1(B)	72.8	Unidentified	<i>K. oxytoca</i> (2)	

		77.6	II	<i>E. coli</i> (1)
	DHA-1(C)	Chromosomal		<i>P. mirabilis</i> (3)
	DHA-1(D)	72.8	Unidentified	<i>K. oxytoca</i> (1)
	DHA-1(E)	72.8	Unidentified	<i>Salmonella</i> spp. (1)
	DHA-1(F)	87.3	Unidentified	<i>E. coli</i> (1)
	DHA-1(H)	Chromosomal		<i>E. coli</i> (1)
<i>bla</i> _{DHA-6}	DHA-1(G)	87.3	II	<i>E. coli</i> (1)
<i>bla</i> _{DHA-1} + <i>bla</i> _{VIM-1}	DHA-1(B) + VIM-1 (D) (<i>In846</i>)	76.6 kb + Chromosomal	FIIA	<i>K. pneumoniae</i> (1)
<i>bla</i> _{DHA-7} + <i>bla</i> _{VIM-1}	DHA-1(D) + VIM-1(E) (<i>In847</i>)	310.4 kb + 48.5 kb	HI2 + Unidentified	<i>E. cloacae</i> (2)
<i>bla</i> _{FOX-3}	FOX(A)	72.5	U	<i>E. coli</i> (1)
<i>bla</i> _{FOX-8}	FOX(A)	Chromosomal		<i>E. coli</i> (2)
<i>bla</i> _{IMP-22}	IMP-22(A)	485.0	Unidentified	<i>K. pneumoniae</i> (2)
<i>bla</i> _{IMP-28}	IMP-28(A) (<i>In767</i>)	Chromosomal		<i>K. oxytoca</i> (1)
<i>bla</i> _{VIM-1}	VIM-1(A) (<i>In488</i>)	48.0	Unidentified	<i>K. oxytoca</i> (1)
	VIM-1(B) (<i>In624</i>)	48.0 and 66.2	Unidentified	<i>E. cloacae</i> (2); <i>K. oxytoca</i> (2); <i>K. pneumoniae</i> (2)
	VIM-1(C) (<i>In846</i>)	48.0 and 72.5	U	<i>K. pneumoniae</i> (1); <i>E. cloacae</i> (1)
	VIM-1(D) (<i>In848</i>)	48.0	Unidentified	<i>E. coli</i> (1)

319 a, between brackets the type of surrounding regions found for each *bla* gene. More detailed

320 data in Figures 1-3.

321 b, the plasmid size was determined after the hybridisation procedure.

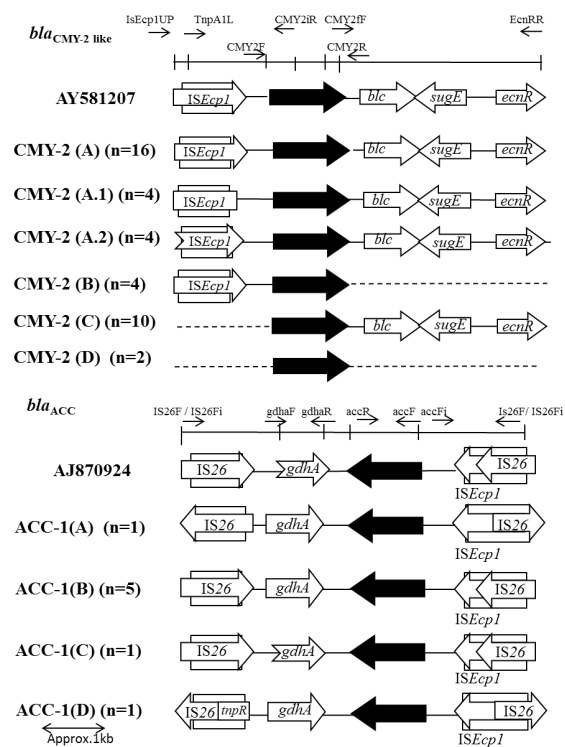


Figure 1. The genetic environment of *bla*_{CMY-2like} and *bla*_{ACC} genes, which are represented by solid black arrows, while the surrounding genes are represented by white arrows. The discontinued lines represent the areas that could not be amplified by PCR. Boxes corresponds to *TnpA* genes of each IS. Eight isolates indicate truncated versions of *ISEcp1* at 3' (pattern A.1) and 5' end (pattern A.2). Truncated genes are represented by disrupted arrows. One isolate (pattern C) carrying the ACC gene showed an identical structure to AJ870924. In one isolate (pattern D), *IS26* was truncated by a *tnpR* gene of insertion Tn5393. The primers used for PCR amplification are also shown.

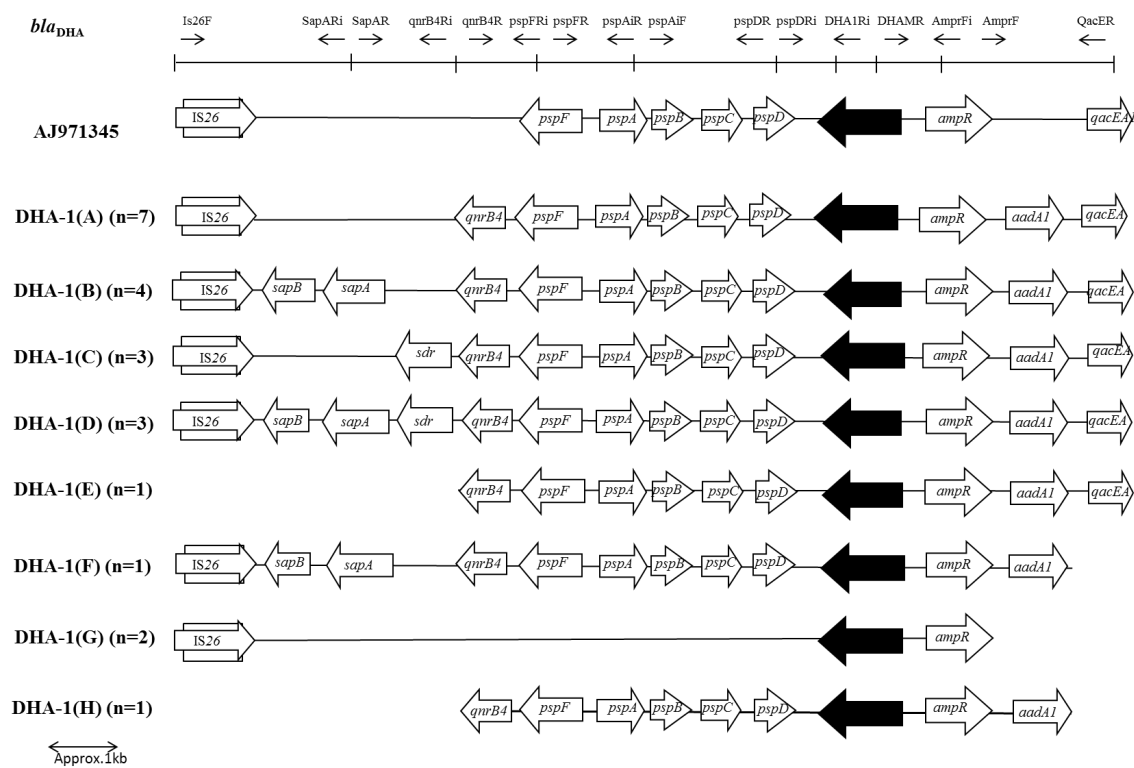


Figure 2. The genetic environment of the *bla*_{DHA} gene, which is represented by solid black arrows, while the surrounding genes are represented by white arrows. Continuous lines indicate an absence of the corresponding DNA fragments. Boxes corresponds to *TnpA* genes of each IS.

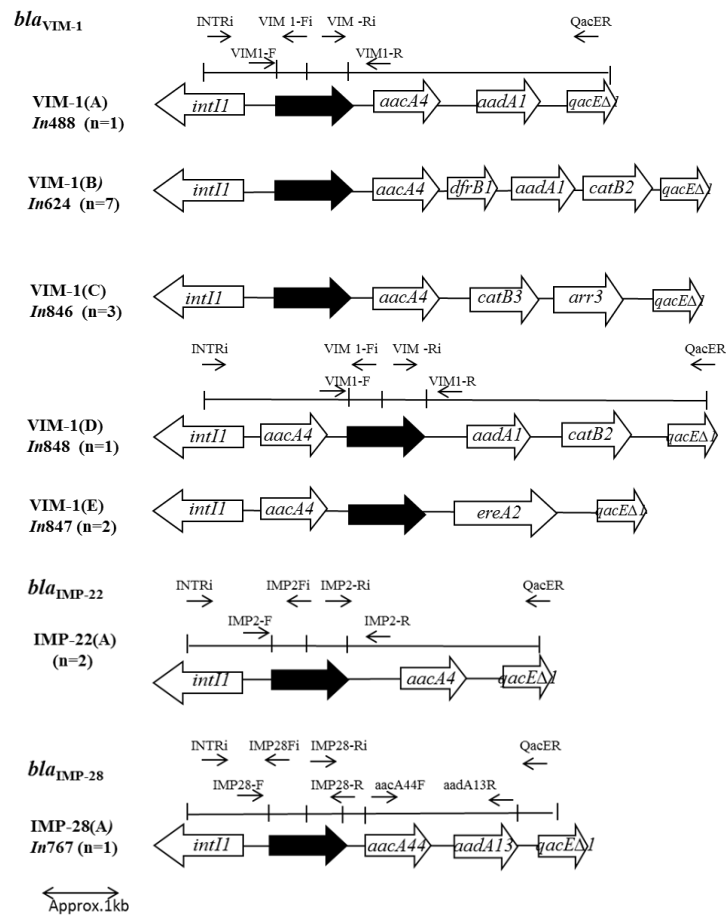


Figure 3. 3. Structure of *bla*_{VIM-1}, *bla*_{IMP-22} and *bla*_{IMP-28} genes carrying the integrons described in this work. Carbapenemase genes are represented by solid black arrows and the surrounding genes are represented by white arrows. The locations of the primers used for PCR amplification are also shown.