Universitat Autònoma de Barcelona

Facultat de Biociències

Departament de Genètica i de Microbiologia

Epidemiologia molecular de les betalactamases AmpC plasmídiques en enterobacteris aïllats a l'Hospital de la Santa Creu i Sant Pau i la seva difusió horitzontal

Memòria realitzada per a optar al grau de
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RESULTATS

4.1. CAPACITAT DELS CENTRES ESPANYOLS PER A DETECTAR I INFORMAR FENOTIPS DE RESISTÈNCIA A BETALACTÀMICS (ANNEX I)

El propòsit d'aquest estudi fou avaluar la capacitat dels laboratoris espanyols per a detectar i informar correctament la producció de BLEA i betalactamases AmpC, tant cromosòmiques com plasmídiques, en aïllats clínics d' *E. coli* i *K. pneumoniae*.

Les 12 soques seleccionades, detallades en l'apartat de material i mètodes, foren distribuïdes als 57 centres participants, repartits per tot l'àmbit nacional. Els resultats obtinguts pels diferents centres es compararen amb els valors obtinguts pels centres de referència (Taula 12 de l'apartat de material i mètodes).

4.1.1. TÈCNIQUES D'IDENTIFICACIÓ I ESTUDI DE SENSIBILITAT MICROBIANA UTILITZADES PELS CENTRES PARTICIPANTS

El 61,4% dels centres participants varen utilitzar sistemes automatitzats per a la identificació, incloent el sistema MicroScan WalkAway (Dade Behring MicroScan, West Sacramento, CA, EEUU; n=25), el sistema Vitek 2 (bioMérieux-Vitek, Hazelwood, Montana, EEUU; n=8) y el sistema Phoenix (Becton Dickinson Diagnostic Systems, Sparks, MD, EEUU; n=2). El 38,6% restants varen utilitzar sistemes semi-automatitzats o manuals, incloent el sistema Wider (Francisco Soria Melguizo, Madrid, Espanya; n=16), el sistema MicroScan AutoScan 4 (Dade Behring MicroScan, West Sacramento, CA, EEUU; n=2), el sistema API20E (bioMérieux, França; n= 2), el

sistema BBL Enterotube II (Becton Dickinson Diagnostic Systems, EEUU; n=1), o no especificat (n=1). Tots els centres que utilitzaren un sistema automatitzat o semi-automatitzat per a la identificació l'utilitzaren també per a l'estudi de sensibilitat microbiana, exceptuant dos usuaris de Vitek 2, que utilitzaren la tècnica manual de difusió amb discs. En el cas dels que utilitzaren la tècnica manual per a la identificació, la tècnica utilitzada per a l'antibiograma va ser la tècnica de disc-difusió, exceptuant els dos centres que utilitzaren el sistema API20E per a la identificació, que utilitzaren sistemes automatitzats, un el sistema Vitek 2 i l'altre el Sensititre (TREK Diagnostic Systems, West Sussex, Inglaterra) (Taula 28).

Taula 28. Metodologia utilitzada pels 57 laboratoris participants per a la identificació i l'estudi de sensibilitat antimicrobiana.

IDENTIFICACIÓ	
Tècnica	**Nº de centres (%)**
▪ **Manual o semi-automatitzada**	22 (38,6)
Wider (Francisco Soria Melguizo)	16
MicroScan Autoscan 4 (Dade Behring MicroScan)	2
API20E (bioMérieux)	2
BBL Enterotube II (Becton Dickinson Diagnostic Systems)	1
▪ **Sense especificar**	1
▪ **Automatitzada**	35 (61,4)
MicroScan Walk-Away (Dade Behring MicroScan)	25
Vitek 2 (bioMérieux)	8
Phoenix (Becton Dickinson Diagnostic Systems)	2
ESTUDI DE SENSIBILITAT ANTIMICROBIANA	
Tècnica	**Nº de centres (%)**
▪ **Manual o semi-automatitzada**	22 (38,6)
Wider	16
MicroScan Autoscan 4	2
Sinèrgia amb doble disc	4
▪ **Automatitzada**	35 (61,4)
MicroScan Walk-Away	25
Vitek 2	7
Phoenix	2
Sensititre (TREK Diagnostic Systems)	1

4.1.2. RESULTATS DE LA IDENTIFICACIÓ BACTERIANA PER PART DELS LABORATORIS PARTICIPANTS

La identificació bacteriana a nivell de gènere va ser correcta en el 99,3% dels casos i en el 99,1% a nivell d'espècie. Les úniques soques que varen ser identificades erròniament foren la soca CCG04, identificada incorrectament per dos centres com a *K. ozaenae* i com a *Klebsiella* spp. per un altre centre; la soca CCG06, identificada incorrectament per un centre com a *E. aerogenes*; la soca CCG07, identificada incorrectament per un centre com a *K. oxytoca* i la soca CCG12 identificada incorrectament per un centre com a *E. coli*. El 40% (2/5) dels errors en la identificació varen ocórrer en un únic centre usuari del sistema automàtic MicrosScan WalkAway. Les tres identificacions incorrectes restants varen ocórrer en tres centres diferents. Les tècniques utilitzades pels centres foren el sistema automàtic MicrosScan WalkAway (un centre), el sistema semi-automàtic MicrosScan AutoScan 4 (un centre) o la tècnica manual BBL-Enterotube (un centre).

4.1.3. CAPACITAT DELS CENTRES ESPANYOLS PER A REALITZAR I INFORMAR CORRECTAMENT ELS RESULTATS DE SENSIBILITAT ANTIMICROBIANA

Per a l'anàlisi de les dades corresponents a l'estudi de la sensibilitat antimicrobiana es varen utilitzar les categories clíniques interpretades subministrades pels participants. Els criteris aplicats foren els del CLSI amb les matisacions detallades en l'apartat de material i mètodes (Taula 14) .

La distribució de les discrepàncies segons el tipus d'error, per a cada soca i per a cada tipus d'antimicrobià, es mostren en la taula 29 (per a les

cefalosporines i l'aztreonam) i la taula 30 (per a carbapenèmics i combinacions de penicil·lina amb inhibidors de betalactàmics).

En general, es varen observar percentatges d'errors baixos amb les cefalosporines i l'aztreonam en el grup de les soques productores de BLEA, degut fonamentalment a l'elevat nombre de centres que foren capaços de detectar la producció de BLEA en aquestes soques. Tots els VME observats en el cas de les cefalosporines foren deguts a la falta d'identificació del fenotip BLEA, excepte un VME per a la cefepima, degut a que un centre no va corregir la categoria clínica de l'antimicrobià a pesar que el seu sistema expert li suggeria la possible presència de BLEA. En el cas de l'aztreonam, dels tres VME observats, un va ser degut a la falta d'identificació del fenotip BLEA, mentre que en els dos casos restants van ser deguts a que els centres no corregien la categoria clínica d'aquest antimicrobià a pesar d'haver detectat la presència de BLEA. Els majors percentatges d'errors per al grup de les soques productores de BLEA es varen observar per a les combinacions de penicil·lines-inhibidors de betalactamases. No obstant, en més de la meitat dels casos (69,5% per a amoxicil·lina-àcid clavulànic i 64,4% per a piperacil·lina-tazobactam), aquests errors no estaven relacionats amb la metodologia emprada per a l'estudi de sensibilitat antimicrobiana, sinó amb la interpretació que feia el microbiòleg pel fet de detectar una BLEA (canvis de categoria clínica de S a R o de I a R). No es va observar cap discrepància amb els carbapenèmics en el grup de soques productores de BLEA.

En el cas de les soques productores del fenotip AmpC, la majoria d'errors detectats per a cefalosporines, aztreonam o piperacil·lina-tazobactam no estigueren relacionats directament amb la metodologia emprada, sinó que

foren conseqüència de la interpretació del microbiòleg del fenotip de resistència. En 13 casos, els errors foren fruit de confondre la producció d'AmpC amb una BLEA, amb el consegüent canvi de la categoria clínica a resistent en totes les cefalosporines i aztreonam. En altres ocasions, encara que es va identificar correctament el mecanisme de resistència subjacent, la falta de recomanacions clares sobre com informar aquest tipus de soques, va fer que els criteris aplicats foren molt variats, amb gran tendència a deixar els carbapenèmics i els antimicrobians no betalactàmics, en cas de sensibilitat, com a única alternativa terapèutica, de forma que alguns centres informaven totes les cefalosporines, incloent la cefepima i la combinació piperacil·lina-tazobactam com a resistents amb independència del valor de la seva CIM.

Les dues úniques discrepàncies observades per als carbapenèmics afectaren a l'ertapenem i la soca CCG09. En un dels centres el valor de CIM obtingut amb tires d'Etest va ser de 3 mg/l, corresponent-li per tant la categoria intermèdia. En l'altre, es va obtindre un valor de CIM de 4 mg/l (MicroScan WalkAway), corresponent a la categoria clínica intermèdia, encara que el microbiòleg ho va corregir a la categoria de resistent.

No s'observaren discrepàncies significatives respecte a les soques control recomanades pel CLSI. Les úniques discrepàncies es detectaren en les combinacions de penicil·lina-inhibidor de betalactamases i la soca control CCG11 (productora de TEM-1). L'anàlisi detallat d'aquestes discrepàncies va revelar que sols en un cas l'error fou degut a la tècnica, essent els dos altres errors deguts a l'errònia interpretació que feia el microbiòleg al considerar que la producció de TEM-1 implica un canvi de categoria clínica de sensible a intermèdia.

Taula 29. Distribució de les discrepàncies en la interpretació dels valors de sensibilitat antimicrobiana i taxa d'errors per a cefalosporines i aztreonam.

SOCA	ESPÈCIE/ BETALACTAMASA	CEFTRIAXONA			CEFOTAXIMA			CEFTAZIDIMA			CEFEPIMA				AZTREONAM		
		n[a]	Errors (%)[b]		n[a]	Errors (%)[b]		n[a]	Errors (%)[b]		n[a]	Errors (%)[b]			n[a]	Errors (%)[b]	
			MiE	VME		MiE	VME		MiE	VME		MiE	MaE	VME		MiE	VME
CCG01	*E. coli* /CTX-M-14	9	0 (0)	0 (0)	53	0 (0)	0 (0)	57	0 (0)	0 (0)	57	0 (0)	NA	0 (0)	38	0 (0)	1 (3)
CCG02	*E. coli* /CTX-M-9	9	0 (0)	0 (0)	52	0 (0)	0 (0)	57	0 (0)	1 (2)	57	0 (0)	NA	1 (2)	38	0 (0)	2 (5)
CCG03	*E. coli* /CTX-M-10	9	0 (0)	0 (0)	53	0 (0)	0 (0)	57	0 (0)	0 (0)	57	0 (0)	NA	0 (0)	38	0 (0)	0 (0)
CCG04	*E. coli* /SHV-12	9	0 (0)	0 (0)	53	0 (0)	0 (0)	57	0 (0)	0 (0)	57	0 (0)	NA	0 (0)	38	0 (0)	0 (0)
CCG05	*K. pneumoniae*/TEM-4	9	0 (0)	1 (11)	53	0 (0)	0 (0)	57	0 (0)	0 (0)	57	0 (0)	NA	2 (4)	38	0 (0)	0 (0)
CCG06	*K. pneumoniae*/CTX-M-10	8	0 (0)	0 (0)	49	0 (0)	0 (0)	52	0 (0)	0 (0)	52	0 (0)	NA	0 (0)	35	0 (0)	0 (0)
CCG12	*K. pneumoniae* ATCC 700603/SHV-18	9	0 (0)	0 (0)	53	0 (0)	1 (2)	57	0 (0)	0 (0)	57	0 (0)	NA	3 (5)	38	0 (0)	0 (0)
Total soques BLEA positives		62	0 (0)	1 (2)	366	0 (0)	1 (0,3)	394	0 (0)	1 (0,3)	394	0 (0)	NA	6 (2)	263	0 (0)	3 (1)
CCG07	*K. pneumoniae*/ FOX-5	9	6 (67)	NA	53	32 (60)	NA	57	4 (7)	0 (0)	56	2 (4)	9 (16)	NA	38	22 (56)	NA
CCG08	*E. coli* /AmpC cromosòmica	9	4 (44)	NA	53	23 (43)	NA	57	20 (35)	0 (0)	57	2 (4)	10 (18)	NA	38	20 (53)	NA
CCG09	*E. coli* /CMY-2	9	0 (0)	NA	53	2 (4)	NA	57	4 (7)	**1 (2)[c]**	57	1 (2)	15 (26)	NA	38	31 (82)	NA
Total soques AmpC positives		27	10 (37)	NA	159	57 (36)	NA	171	28 (16)	1 (0,6)	170	5 (3)	34 (20)	NA	114	73 (64)	NA

MiE: error menor; **MaE:** error major; **VME:** error màxim; **NA:** no aplicable

[a] Nombre de determinacions realitzades per a aquesta combinació microorganisme-antimicrobià.

[b] Els percentatges es calcularen considerant com a denominador el nombre de determinacions de la sensibilitat realitzades a aquest antimicrobià.

[c] Error produït en el valor brut de sensibilitat. CIM obtinguda pel centre: 8 mg/l (S); CIM obtinguda pels centres de referència: 32 mg/l (R). (Tècnica per a l'estudi de sensibilitat del centre: Wider).

Taula 30. Distribució de les discrepàncies en la interpretació dels valors de sensibilitat antimicrobiana i taxa d'errors per als carbapenèmics i les combinacions de betalactàmics/inhibidors de betalactamases.

SOCA	ESPÈCIE/ BETALACTAMASA	ERTAPENEM			AMOXICIL.LINA/ÀCID CLAVULÀNIC			PIPERACIL.LINA/ TAZOBACTAM	
		n[a]	Errors (%)[b]		n[a]	Errors (%)[b]		n[a]	Errors (%)[b]
			MiE	MaE		MiE	VME		MiE
CCG01	*E. coli* /CTX-M-14	28	0 (0,0)	0 (0,0)	56	37 (66,1)	0 (0,0)	56	15 (26,8)
CCG02	*E. coli* /CTX-M-9	28	0 (0,0)	0 (0,0)	56	19 (33,9)	0 (0,0)	56	15 (26,8)
CCG03	*E. coli* /CTX-M-10	28	0 (0,0)	0 (0,0)	56	19 (33,9)	0 (0,0)	56	15 (26,8)
CCG04	*E. coli* /SHV-12	28	0 (0,0)	0 (0,0)	56	18 (32,1)	0 (0,0)	56	14 (25)
CCG05	*K. pneumoniae*/TEM-4	28	0 (0,0)	0 (0,0)	56	22 (39,3)	0 (0,0)	56	**47 (83,9)**
CCG06	*K. pneumoniae*/CTX-M-10	25	0 (0,0)	0 (0,0)	51	16 (31,4)	0 (0,0)	51	11 (21,6)
CCG12	*K. pneumoniae* ATCC 700603/SHV-18	28	0 (0,0)	0 (0,0)	56	20 (35,7)	0 (0,0)	56	15 (26,8)
Total soques BLEA positives		193	0 (0,0)	0 (0,0)	387	**151 (39)**	0 (0,0)	387	**132 (34,1)**
CCG07	*K. pneumoniae*/ FOX-5	28	0 (0,0)	0 (0,0)	56	6 (10,7)	0 (0,0)	55	20 (36,4)
CCG08	*E. coli* /AmpC cromosòmica	28	0 (0,0)	0 (0,0)	56	9 (16,1)	0 (0,0)	56	17 (30,4)
CCG09	*E. coli* /CMY-2	28	1 (3,6)	1 (3,6)	56	8 (14,3)	**2 (3,6)[c]**	56	22 (39,3)
Total soques AmpC positives		84	1 (1,2)	1 (1,2)	168	23 (13,7)	2 (1,2)	167	**59 (35,3)**
CCG04	*E. coli* ATCC 25922	28	0 (0,0)	0 (0,0)	55	0 (0,0)	0 (0,0)	56	0 (0,0)
CCG05	*E. coli* ATCC 35218/TEM-1	29	0 (0,0)	0 (0,0)	56	2 (3,6)	0 (0,0)	56	1 (1,8)
Total soques BLEA I AmpC positives		57	0 (0,0)	0 (0,0)	111	2 (1,8)	0 (0,0)	112	1 (0,9)

MiE: error menor; **MaE:** error major; **VME:** error màxim;

[a] Nombre de determinacions realitzades per a aquesta combinació microorganisme-antimicrobià.
[b] Els percentatges es calcularen considerant com a denominador el nombre de determinacions de la sensibilitat realitzades a aquest antimicrobià.
[c] Error produït en el valor brut de sensibilitat. CIM obtingudes pels dos centres: 8/4 mg/l (S); CIM obtinguda pels centres de referència: 32/16 mg/l (R). (Tècniques per a l'estudi de sensibilitat dels dos centres: Wider).

4.1.4. CAPACITAT DELS CENTRES ESPANYOLS PER A DETECTAR CORRECTAMENT EL MECANISME DE RESISTÈNCIA

4.1.4.1. Detecció del mecanisme BLEA

Dels 53 centres que utilitzaven sistemes automatitzats o semi-automatitzats per a l'estudi de la sensibilitat antimicrobiana, el 37,7% va especificar que davant la sospita de producció de BLEA realitzava sempre proves confirmatòries, utilitzant el test de sinèrgia de doble disc (10 laboratoris) o tires Etest (10 laboratoris).

El 86% (49/57) dels laboratoris va identificar correctament la producció de BLEA en totes les soques productores d'aquests enzims. Altres tres laboratoris no varen especificar la producció de BLEA als seus informes, però varen realitzar la correcció de les categories clíniques de les cefalosporines i aztreonam a resistent, pel que podrien incloure's entre les anteriors. El percentatge d'èxit en la detecció de BLEA s'elevaria doncs al 91,2%. Tant sols cinc centres varen errar en la identificació d'alguna de les soques productores de BLEA. Els errors en la detecció d'aquests enzims es produïren amb les soques CCG02 (un centre), CCG05 (2 centres) i CCG12 (2 centre).

L'error en la detecció de BLEA detectat per a la soca CCG02 (productora de CTX-M-9) es va produir en un centre que utilitzava el sistema Vitek 2. En aquest cas, les targetes utilitzades no contenien ni cefotaxima ni ceftriaxona, i les lectures de CIM de les altres cefalosporines i aztreonam foren inferiors a 1 mg/l. Un dels errors en la detecció de la soca CCG05 (productora de TEM-4) es va produir en un centre que utilitzava la tècnica de sinèrgia amb doble disc, i que va obtenir un test de sinèrgia amb àcid clavulànic negatiu, pel que va identificar el microorganisme com un possible hiperproductor de SHV-1. En

l’altre cas, es tractava d’un usuari del sistema Wider que a pesar d’obtindre valors de CIM de tots els betalactàmics dins de la categoria resistent, a excepció de cefepima i carbapenèmics, no va sospitar la presència de BLEA.

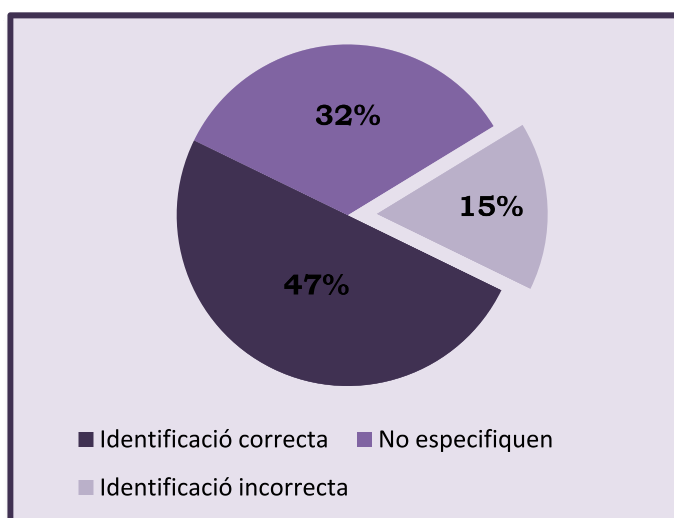
En aquest mateix centre tampoc es va detectar la presència de BLEA en la soca CCG12 (control de qualitat recomanat pel CLSI per a la detecció de BLEA/productora de SHV-18), a pesar d’obtindre un antibiograma amb CIM elevades d’algunes cefalosporines i aztreonam. En aquest cas el seu sistema expert li suggeria la possibilitat de presència de BLEA associada a alteracions de permeabilitat o presència de betalactamasa AmpC, però l’usuari no va realitzar cap tipus de comprovació i no va corregir la categoria clínica de cefepima a resistent.

L’altre error produït en la soca CCG12 es va donar en un usuari del sistema MicroScan AutoScan 4. En aquest cas del l’error també es va produir en la interpretació que feia el microbiòleg, ja que no va sospitar la presència de BLEA a pesar d’obtindre valors de CIM elevats d’alguna de les cefalosporines i l’aztreonam i la suggerència de BLEA per part del seu sistema expert.

4.1.3.2. Detecció del mecanisme AmpC

Degut a la falta de mètodes aplicables en rutina per a la detecció de betalactamases AmpC, la detecció de soques amb aquest fenotip de resistència resulta sovint complicat. Així, la possible presència d'una pACBL va ser suggerida per a les soques CGG02 (productora de FOX-5) i CGG03 (productora de CMY-2) pel 51% i el 60% dels laboratoris respectivament. En el cas de la soca CGG01 (hiperproductora de la seva AmpC cromosòmica), la possibilitat d'hiperproducció d'una AmpC va ser senyalada pel 53% dels centres. Tan sols en el 47% dels centres es varen identificar correctament les tres soques productores d'AmpC. El 32% dels centres no va especificar el mecanisme de resistència i el 15% ho va fer incorrectament. Els principals errors foren deguts a la confusió del fenotip AmpC amb una BLEA, en el 8% dels casos, o a la confusió amb una hiperproducció de la penicil·linasa associada o no a alteracions de permeabilitat en el 7% dels casos (Figura 31).

Figura 31. Capacitat dels centres espanyols per a detectar el fenotip AmpC.



4.2. TRANSMISSIÓ *IN VIVO* D'UN PLASMIDI PORTADOR DE *bla*$_{DHA-1}$ I *qnr*B ENTRE UNA SOCA D'*Escherichia coli* I UNA DE *Serratia marcescens* (Annex II)

La detecció de pACBL és molt problemàtica degut a la falta de mètodes fenotípics estandarditzats, complicant-se encara més quan aquestes es troben presents en microorganismes productors d'una AmpC cromosòmica natural.

En una mostra d'orina d'un home de 65 anys, es va aïllar una *S. marcescens* i una *E. coli* amb un patró fenotípic compatible amb una betalactamasa AmpC induïble. Encara que en el cas de la soca de *S. marcescens* podria tractar-se de la seva AmpC cromosòmica natural, l'observació d'abundants colònies dins dels halos d'inhibició de les cefalosporines de tercera generació i aztreonam va fer sospitar la presència d'una pACBL.

Per a determinar la transmissibilitat d'aquesta resistència es va procedir a la realització de tècniques de conjugació i es va determinar el patró de sensibilitat per disc difusió tant de les soques clíniques com dels transconjugants obtinguts. Ambdues soques clíniques presentaren el mateix patró de sensibilitat a antibiòtics tant betalactàmics com no betalactàmics, a excepció de la cefotaxima i l'àcid nalidíxic. *S. marcescens* mostrava sensibilitat disminuïda a cefotaxima (14 mm) mentre que *E. coli* era sensible a aquest antibiòtic (25 mm). En canvi, *E. coli* presentava sensibilitat disminuïda a l'àcid nalidíxic (15 mm) mentre que *S. marcescens* es mostrava sensible (21 mm). L'estudi de sensibilitat dels transconjugants obtinguts tant de la soca de *S. marcescens* com d'*E. coli* va ser idèntic. Els transconjugants d'ambdós

aïllats presentaren sensibilitat disminuïda a l'àcid nalidíxic (14-15 mm). La transmissibilitat de la resistència a àcid nalidíxic via conjugació va fer sospitar la presència de gens *qnr* en aquestes soques.

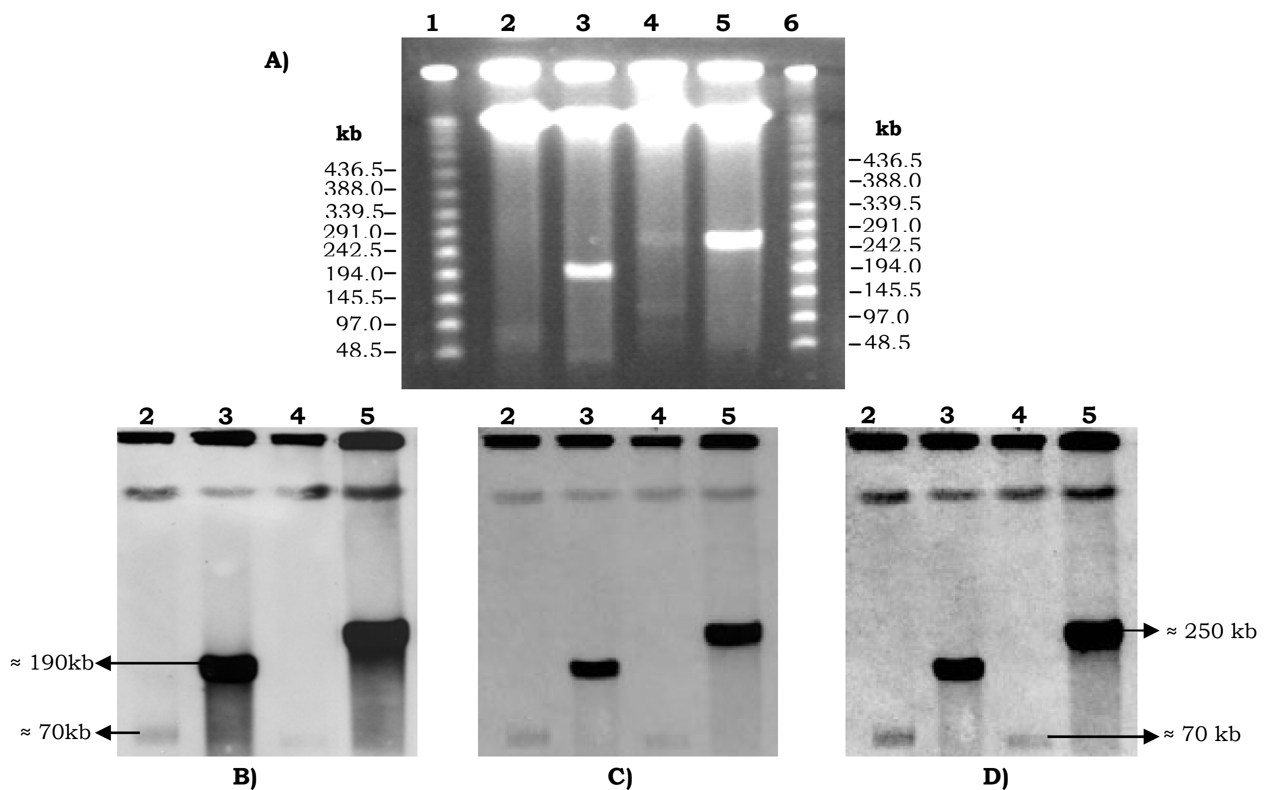
La presència de gens bla_{DHA-1} i *qnr*B4, tant en les soques clíniques com en els transconjugants, es va demostrar mitjançant tècniques de PCR i seqüenciació. Aquest és el primer estudi on es detecta la presència d'una pACBL en una soca de *S. marcescens*.

L'anàlisi plasmídica es va dur a terme mitjançant la tipificació del replicó i de la relaxasa per PCR, S1-PFGE, *Southern blot* i hibridació. Encara que es va observar més d'un plasmidi en les soques donadores en el gel S1-PFGE (Figura 28), els resultats obtinguts de la tipificació del replicó i de la relaxasa únicament varen donar resultats positius per a un únic replicó IncL/M i una única subfamília de relaxases MOB_{P13} respectivament. Les seqüències nucleotídiques dels amplicons obtinguts dels replicons L/M (684 pb) van ser idèntiques tant per a la soca de *S. marcescens* com la d'*E. coli*, així com també els seus respectius transconjugants. Aquestes seqüències presentaren un 96% d'homologia amb els plasmidis IncL/M pEL60 (AY422214), pCTX-M3 (AF550415) i pCTXM360 (EU938349). Les seqüències dels amplicons obtinguts de la relaxasa (177 pb) tant de les soques clíniques com dels seus transconjugants també foren idèntiques, mostrant un 86% d'homologia amb els mateixos plasmidis pEL60, pCTX-M3 i pCTXM360.

Les anàlisis de *Southern blot* i hibridació varen revelar la co-localització dels gens de resistència bla_{DHA-1} i *qnr*B4 en el mateix plasmidi conjugatiu (Figura 32). En les soques clíniques de *S. marcescens* i *E. coli*, els gens bla_{DHA-1}

i *qnr*B4 hibridaren en un plasmidi d'unes 70 kb, mentre que els plasmidis portadors d'aquestes resistències en els transconjugants presentaren mides molt majors, essent de 190 kb per al transconjugant de *S. marcescens* i d'unes 250 kb per al transconjugant d'*E. coli* (Figura 32).

Figura 32. S1-PFGE hibridat amb les sondes bla_{DHA-1}, *qnr*B4 i IncL/M.



Carrils 1 i 6: marcador de pesos moleculars λ; **carril 2:** N4112 (*S. marcescens*); **carril 3:** N4112 Tc; **carril 4:** N4114 (*E. coli*); **carril 5:** N4114 Tc; **A)** S1-PFGE; **B)** S1-PFGE hibridat amb bla_{DHA-1}; **C)** S1-PFGE hibridat amb el replicó L/M; **D)** S1-PFGE hibridat amb *qnr*B4.

El fet que tant la soca de *S. marcescens* com la d'*E. coli* s'aïllaren d'un mateix pacient, que presentaren el mateix patró de sensibilitat, que ambdues presentaren els gens de resistència bla_{DHA-1} i *qnr*B4 en un mateix plasmidi IncL/M-MOB_{P13} d'unes 70 Kb i que ambdós plasmidis foren conjugatius al laboratori, suggereix una possible transmissió d'aquestes resistències *in vivo*.

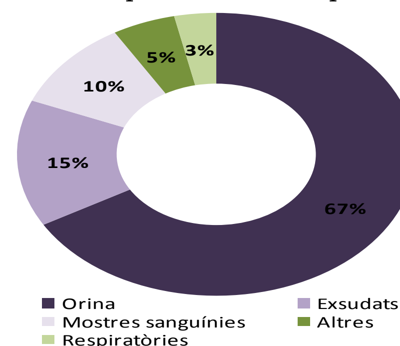
4.3. CARACTERITZACIÓ I EPIDEMIOLOGIA DE LES pACBL (Annex III)

4.3.1. CARACTERITZACIÓ DE LES pACBL

De la totalitat d'enterobacteris sense betalactamasa cromosòmica AmpC induïble aïllats durant 1999-2007, 437 compliren el criteri d'inclusió detallat en el punt 3.2.1 de l'apartat de material i mètodes, com a possibles productors d'una pACBL. La seva caracterització genotípica, mitjançant la tècnica de PCR múltiple descrita per Pérez-Pérez *et al.*, va permetre la detecció de 117 pACBL en soques d'*E. coli* (n=75), *P. mirabilis* (n=20), *K. pneumoniae* (n=16), *K. oxytoca* (n=4) i *S. enterica* (n=2). Els tipus d'enzims detectats foren CMY-2, DHA-1, ACC-1, CMY-4 i tres noves variants de CMY-2 descrites per primer cop en aquest estudi: CMY-25, CMY-27 i CMY-40. Set d'aquests aïllats (6%) eren a més a més productors d'una BLEA (dues soques d'*E. coli* amb DHA-1 i CTX-M-14; una *K. pneumoniae* amb CMY-2 i CTX-M-1; tres *K. pneumoniae* amb DHA-1 i CTX-M-15 i un *P. mirabilis* amb CMY-2 i CTX-M-2).

El 66,7% dels aïllats procediren de mostres d'orina, el 14,5% de diferents exsudats, el 10,3% de sang, el 3,4% de mostres del tracte respiratori i el 5,1% restant de diferents orígens com femtes entre d'altres.

Figura 33. Origen de les mostres productores de pACBL.



El 68,3% (69/101) dels pacients que presentaren una infecció per enterobacteris portadors de pACBL eren persones d'edat avançada, entre els 70 i els 89 anys. L'edat dels pacients portadors de les 16 soques restants de la col·lecció és desconeguda.

4.3.2. RELACIÓ CLONAL DE LES SOQUES

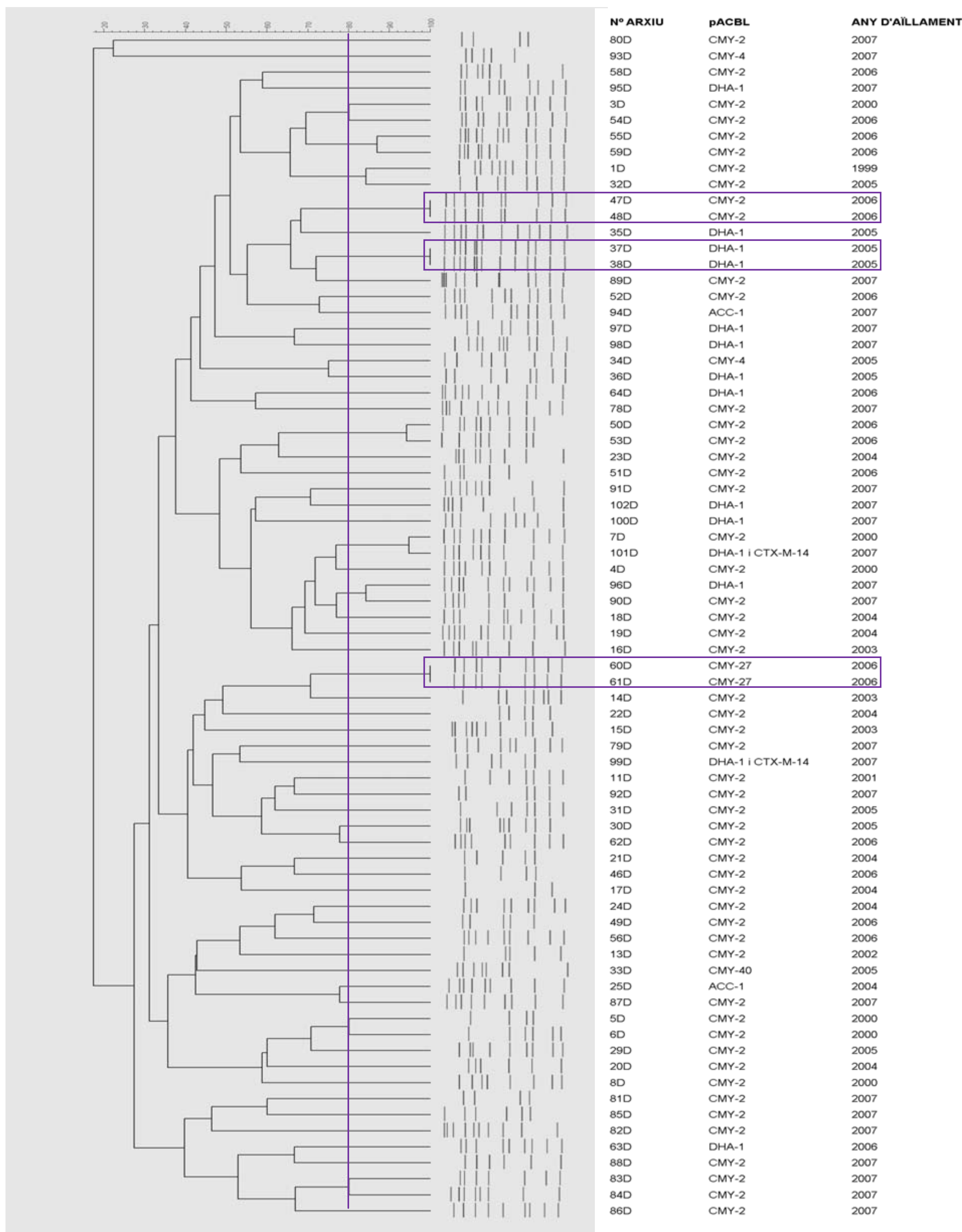
Tal i com es menciona en l'apartat de material i mètodes, les dues tècniques utilitzades per a determinar la clonalitat de les soques foren l'ERIC-PCR per als aïllats d'*E. coli* i el PFGE per a la resta d'enterobacteris. En aquelles soques d'*E. coli* amb patrons d'ERIC-PCR amb homologies superiors o iguals al 80% també es va realitzar el PFGE ja que és considerada com la tècnica de tipificació estàndard per a aquest tipus d'anàlisis.

Del total de soques d'*E. coli* (n=75) estudiades per ERIC-PCR (Figura 30), es varen obtenir 69 patrons diferents i tres *clusters* de dues soques cadascun amb patrons idèntics, tant per ERIC-PCR com per PFGE (Figura 34). El contagi entre pacients es va suggerir en dos dels *clusters*, donat que en ambdós casos es tractava de persones que havien compartit sala i període d'hospitalització. En el *cluster* composat pels dos aïllats d'*E. coli* amb DHA-1 no es va establir cap tipus de relació epidemiològica entre els pacients.

De les 48 soques restants estudiades per PFGE (20 *P. mirabilis*, 16 *K. pneumoniae*, quatre *K. oxytoca* i dos *S. enterica*), s'obtingueren patrons de bandes diferents en tots els casos, a excepció de dues soques de *K. pneumoniae* portadores de DHA-1 i una BLEA CTX-M-15, cinc soques de *P. mirabilis* amb CMY-2 (quatre idèntiques per PFGE i una probablement relacionada) i dues soques de *K. oxytoca* amb DHA-1. El possible contagi entre els dos pacients portadors de les soques de *K. pneumoniae* es va poder determinar, mentre que en el cas de les cinc soques de *P. mirabilis*, no es va poder establir cap tipus de relació entre els pacients per epidemiologia clínica. Les dues soques de *K. oxytoca* pertanyien al mateix pacient, encara que foren

aïllades en un interval de temps de vuit mesos, fet que suggereix que aquesta soca es troba formant part de la microbiota habitual del pacient i que podria estar actuant com a reservori d'aquesta resistència.

Figura 34. Dendrograma de les 75 soques d'*E. coli* analitzades per ERIC-PCR.



4.4. PREVALENÇA I DISTRIBUCIÓ DE LES pACBL

Des de l'aparició a l'any 2000 de la pACBL a l'Estat espanyol (34), molt pocs han sigut els estudis realitzats per a determinar la prevalença d'aquests enzims a Espanya.

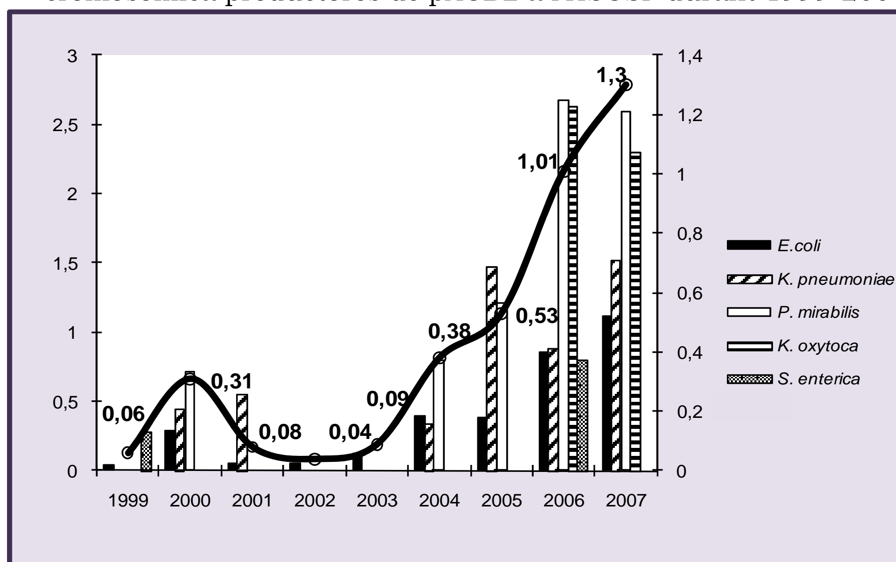
Amb la finalitat de conèixer la situació d'aquestes betalactamases al nostre entorn, es varen avaluar totes les soques d'enterobacteris sense betalactamasa cromosòmica AmpC induïble, aïllades a l'Hospital de la Santa Creu i Sant Pau durant el període 1999-2007.

Del total de les 27.119 soques clíniques aïllades durant aquest període, el 73% (19929/27.119) foren *E. coli*, el 9% (2387/27.119) *K. pneumoniae*, el 8% (2095/27.119) *P. mirabilis*, el 7% (1771/27.119) *S. enterica*, el 2% (509/27.119) *K. oxytoca* i el 2% restant (428/27.119) d'altres espècies d'enterobacteris com *C. koseri* (n=211), *P. vulgaris* (n=108), *Shigella* spp. (n=101) i *P. penneri* (n=8).

De la totalitat de soques analitzades, 437 compliren el criteri d'inclusió, de les quals 117 foren productores de pACBL. Les 320 soques restants foren soques productores de BLEA, hiperproductores d'enzims AmpC (*E. coli*) o hiperproductores d'enzims de classe A (*Klebsiella* spp.). La prevalença global en aquest període va ser del 0,4%. A pesar de que els valors de prevalença són relativament baixos, s'observa un increment significatiu ($p<0,001$; taula de contingència Xi-quadrat; software SPSS V15) d'aquests enzims any darrere any, passant d'una prevalença del 0,06% l'any 1999 fins a valors de l'1,3% el 2007 (Figura 35).

Aquest increment significatiu s'observa en la pràctica totalitat d'espècies estudiades (Taula 31). Per al cas d'*E. coli*, s'observa una prevalença molt baixa durant el primer any d'estudi (0,04%), donant-se un increment a l'any següent (0,3%). No obstant, el nombre de soques portadores de pACBL durant els anys 2000-2003 torna a baixar, i no és fins a l'any 2004 que s'observa un increment significatiu any rere any de la prevalença, fins a assolir valors màxims el 2007 (1,1%). En el cas de *K. pneumoniae*, ocorre un fenomen semblant, encara que els valors de prevalença són més elevats que els observats per a *E. coli.* S'observen dos pics màxims de prevalença l'any 2005 i 2007 amb valors de l'1,5% en ambdós anys. En el cas de *P. mirabilis*, únicament es descriu una soca productora de pACBL durant els primers anys, però és a partir del 2004 quan es dispara de forma exponencial el nombre de soques productores d'aquesta resistència, arribant a ser l'espècie més prevalent del nostre estudi, amb valors del 2,7% l'any 2006 (Taula 31). En *K. oxytoca*, únicament s'aïllen soques productores de pACBL durant 2006-2007 amb valors de prevalença del 2,6% i 2,3% respectivament. En *S. enterica* es descriuen únicament dues soques en tot el període d'estudi, una l'any 1999 i l'altra el 2006 amb valors de prevalença màxims del 0,8% el 2006 (Taula 31).

Figura 35. Dades de prevalença global de soques d'enterobacteris sense AmpC cromosòmica productores de pACBL a l'HSCSP durant 1999-2007.



Encara que *E. coli* fou l'espècie on es caracteritzaren un major nombre de soques portadores de pACBL (n=75), *P. mirabilis* fou l'espècie portadora de pACBL més prevalent (1%), seguida de soques de *K. oxytoca* (0,8%) i *K. pneumoniae* (0,7%) respectivament. *S. enterica* fou l'espècie menys prevalent (0,1%) (Taula 31).

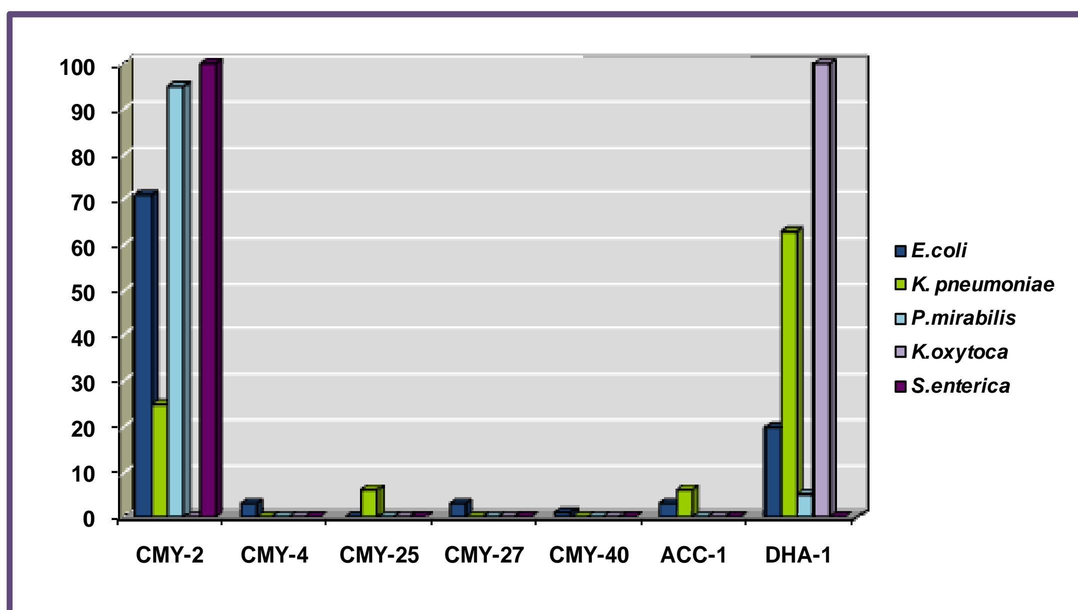
Taula 31. Prevalença i distribució de les pACBL durant 1999-2007.

ESPÈCIE I pACBL	PERÍODE D'ESTUDI									TOTAL (%)
	1999	2000	2001	2002	2003	2004	2005	2006	2007	
E. coli										
Nº soques aïllades	2283	2068	1820	2109	2440	2285	2385	2315	2224	**N= 19929**
CMY-2	1	6	1	1	3	8	4	14	15	53 (71%)
CMY-4							1		1	2 (3%)
CMY-40							1			1 (1%)
CMY-27								2		2 (3%)
ACC-1						1			1	2 (3%)
DHA-1							4	3	6	13 (17%)
DHA-1+ CTX-M-14									2	2 (3%)
%	**0,04%**	**0,3%**	**0,05%**	**0,04%**	**0,1%**	**0,4%**	**0,4%**	**0,8%**	**1,1%**	**75 (0,4%)**
K. pneumoniae										
Nº soques aïllades	214	222	181	181	288	295	273	339	394	**N= 2387**
CMY-2		1				1	1			3 (19%)
CMY-25								1		1 (6%)
DHA-1							2	2	3	7 (44%)
ACC-1							1			1 (6%)
CMY-2 + CTX-M-1			1							1 (6%)
DHA-1 + CTX-M15									3	3 (19%)
%		**0,4**	**0,5**			**0,3**	**1,5**	**0,9**	**1,5**	**16 (0,7%)**
P. mirabilis										
Nº soques aïllades	280	140	201	178	267	249	248	262	270	**N= 2095**
CMY-2		1				2	2	6	7	18 (90%)
DHA-1								1		1 (5%)
CMY-2+ CTX-M-2							1			1 (5%)
%		**0,7**				**0,8**	**1,2**	**2,7**	**2,6**	**20 (1%)**
K. oxytoca										
Nº soques aïllades	0	0	45	68	65	70	98	76	87	**509**
DHA-1								2	2	4 (100%)
%								**2,63**	**2,3**	**4 (0,8%)**
S. enterica										
Nº soques aïllades	352	148	290	208	182	231	141	125	94	**N= 1771**
CMY-2	1							1		2 (100%)
%	**0,3**							**0,8**		**2 (0,1%)**

Del total de pACBL caracteritzades en aquest estudi, CMY-2 fou l'enzim predominant, amb valors del 67% (78/117), seguida de DHA-1, amb una representació del 26% (30/117). Altres pACBL caracteritzades amb menor proporció foren: ACC-1, CMY-4, CMY-25, CMY-27 i CMY-40, essent aquestes tres últimes variants de CMY-2 descrites per primer cop en aquest estudi (Taula 31).

L'espècie que va mostrar una major variabilitat de pACBL fou *E. coli*, essent CMY-2 l'enzim caracteritzat amb una major proporció (71%), seguit de DHA-1 (20%). En el cas de *K. pneumoniae* trobem la situació inversa, essent DHA-1 l'enzim majoritàriament descrit (63%) seguit de CMY-2 (25%). En menor proporció també trobem CMY-25 i ACC-1. Per al cas de *P. mirabilis*, pràcticament la totalitat de soques eren portadores de CMY-2 (95%) descrivint-se únicament una soca portadora de DHA-1. En *K. oxytoca* tots els enzims detectats foren DHA-1, mentre que en les dues úniques soques aïllades de *S. enterica*, ambdues eren portadores de CMY-2 (Taula 31, Figura 36).

Figura 36. Distribució dels tipus de pACBL en funció de l'espècie aïllada.



4.5. CARACTERITZACIÓ DELS VECTORS IMPLICATS EN LA DISSEMINACIÓ DE LES pACBL (Annex III)

L'estudi dels vectors implicats en la mobilització de les pACBL és de vital importància a l'hora d'establir les mesures de control i prevenció necessàries per a intentar reduir la disseminació d'aquests gens de resistència.

4.5.1. DETERMINACIÓ DE LA FREQÜÈNCIA D'AUTOTRANSFERÈNCIA

El primer pas per a determinar si les nostres pACBL d'estudi es trobaven vehiculades per plasmidis i amb quina freqüència es mobilitzaven a una cèl·lula receptora es va realitzar mitjançat la tècnica de conjugació.

Del total de les 117 soques estudiades, la transferència dels gens *amp*C per conjugació va tindre lloc en el 83% dels casos (97/117), amb freqüències d'autotransferència que oscil·laren entre 10^{-2} i 10^{-8}. De les set soques que a més a més de presentar una pACBL eren portadores també d'una BLEA, s'obtingueren transconjugants en sis dels casos, transferint-se ambdós gens de resistència tan sols en quatre aïllats. No s'obtingueren transconjugants de les soques portadores de CMY-4, CMY-25 i CMY-40. El percentatge de transconjugants obtinguts i la seva freqüència de conjugació en funció de la pACBL transferida, es troba reflectida en la taula 32.

Taula 32. Percentatge i freqüència de conjugació de les pACBL.

pACBL/(n)	% CONJUGACIÓ	FREQ. CONJUGACIÓ
CMY-2 (78)	**85%**	**10^{-2} a 10^{-8}**
DHA-1 (30)	**87%**	**10^{-5} a 10^{-8}**
ACC-1 (3)	**67%**	**10^{-5} a 10^{-7}**
CMY-27 (2)	**100%**	**10^{-5} a 10^{-7}**
CMY-4 (2), CMY-25 (1) i CMY-40 (1)	**-**	**-**

4.5.2. CARACTERITZACIÓ DELS PLASMIDIS PORTADORS DELS GENS *amp*C

La primera aproximació per a caracteritzar els plasmidis portadors dels gens *amp*C va ser mitjançant PBRT, S1-PFGE i hibridació amb sondes específiques, tant per al replicó obtingut com per al tipus de pACBL en qüestió.

Del total dels 18 replicons testats, 10 replicons, incloent I1/Iγ, L/M, N, FIA, FIC, A/C, FIIA, K i F, varen estar implicats en la disseminació dels gens *amp*C. Els grups Inc més àmpliament representats, sols o formant part de multireplicons foren: A/C (n=30), L/M (n=27), I1/Iγ (n=26), K (n=10) i el grup F (n=10). Aquesta primera aproximació va permetre la caracterització dels plasmidis portadors dels gens *amp*C en el 78% (91/117) de les soques d'estudi.

Com a segona aproximació per a caracteritzar les 26 soques restants no resoltes seguint aquesta metodologia, es va realitzar la PBRT dels grups ColE, IncU i IncR i la caracterització de la regió *mob* que codifica per a la relaxasa. Onze soques foren positives per al replicó ColE mentre que cap de les soques testades va ser positiva per als replicons de tipus IncU o IncR. Els resultats obtinguts de la tipificació de la regió *mob* foren: 10 mostres positives per a la subfamília de relaxases MOB_{H12}, set MOB_{P11}, quatre MOB_{F12}, tres MOB_{P12} i tres MOB_{P131}.

La hibridació amb sondes específiques per al replicó ColE i per a les relaxases obtingudes va permetre la caracterització de quatre plasmidis portadors de gens *amp*C: dos plasmidis MOB_{F11}, un plasmidi MOB_{P12} i un cointegrat entre un plasmidi tipus ColE i una relaxasa MOB_{P11}.

Els motius pels quals les 22 soques restants no es varen poder resoldre van ser diversos (Taula 33), incloent:

1) En nou aïllats, no es va observar cap plasmidi en el gel de S1-PFGE, o cap dels plasmidis presents va hibridar amb la sonda del gen *amp*C.
2) En tres aïllats, els plasmidis portadors dels gens *amp*C no es varen poder caracteritzar ni per PBRT ni per la tipificació de la relaxasa.
3) En sis aïllats, múltiples plasmidis presents en la soca hibridaren tant per al replicó com per al gen *amp*C en qüestió.
4) Quatre aïllats es varen lisar durant el S1-PFGE i per tant no es varen poder analitzar (dues *E. coli* amb CMY-2 i dues *E. coli* amb DHA-1).

Taula 33. Resultats de l'anàlisi plasmídica de les soques no resoltes.

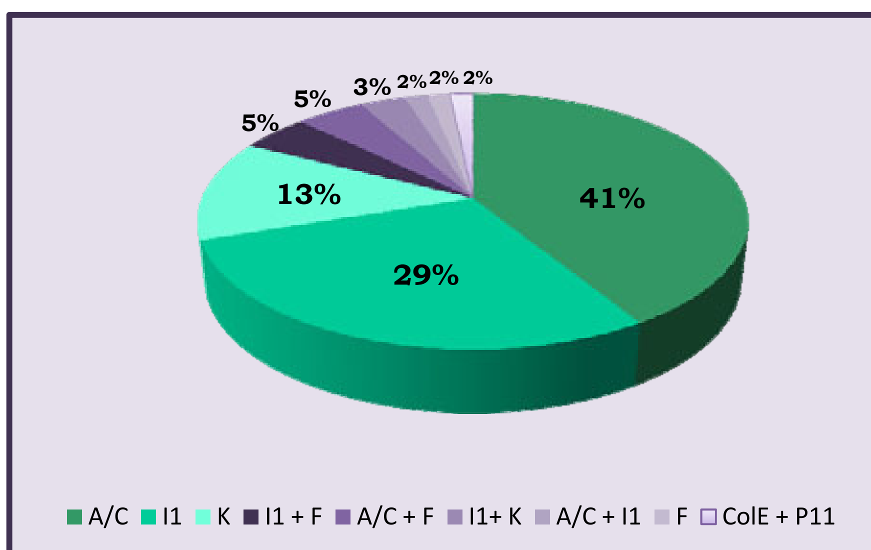
SOQUES (D/Tc)[a]	ESPÈCIE	pACBL/BLEA	PBRT/RELAXASA/GENS DE RESISTÈNCIA I MIDA DEL PLASMIDI (kb)
27D	*P. mirabilis*	CMY-2	-
44D	*P. mirabilis*	CMY-2+CTX-M-2	ColE
72D	*P. mirabilis*	CMY-2	ColE
74D	*P. mirabilis*	CMY-2	MOB_{H12} (SXT/R391)[b]
75Tc	*P. mirabilis*	CMY-2	ColE (40), MOB_{H12} (SXT/R391)[b]
76D	*P. mirabilis*	CMY-2	MOB_{H12} (R391)
112Tc	*P. mirabilis*	CMY-2	IncP+ColE (40), MOB_{H12} (SXT/R391)[b]
117Tc	*P. mirabilis*	CMY-2	ColE+MOB_{P11} (40)
33D	*E. coli*	CMY-40	ColE (230), MOB_{F11}
46Tc	*E. coli*	CMY-2	pNT[c] CMY-2 (373)
93D	*E. coli*	CMY-4	pNT[c] CMY-4 (40)
65D	*K. pneumoniae*	CMY-25	pNT[c] CMY-25 (380)
6Tc	*E. coli*	CMY-2	A/C $^{CMY-2}$ (179 i 359)
30D	*E. coli*	CMY-2	A/C+F $^{CMY-2}$ (97Kb), A/C $^{CMY-2}$(135 i 170), A/C+F+FIB $^{CMY-2}$ (230)
12Tc	*K. pneumoniae*	CMY-2+CTX-M-1	A/C+N $^{CMY-2+CTX-M-1}$ (200 i 425)
39Tc	*K. pneumoniae*	CMY-2	A/C $^{CMY-2}$ (173 i 353)
37Tc	*E. coli*	DHA-1	L/M $^{DHA-1}$ (100 and 312)
62Tc	*E. coli*	DHA-1	L/M $^{DHA-1}$ (74 and 164)

[a] **D:** donadores per la impossibilitat d'obtindre transconjugants; **Tc:** transconjugants.
[b] La seqüenciació dels amplicons MOB_{H12} va ser compatible amb la relaxasa d'un ICE de la família SXT/R391.
[c] **pNT:** plasmidi no tipificable ni per PBRT ni per la relaxasa.

4.5.2.1. Plasmidis portadors dels gens *bla*$_{CMY-2}$

Els plasmidis portadors dels gens *bla*$_{CMY-2}$ presentaren mides molt diverses que oscil·laren entre les 81 i les 414 kb, essent per regla general els més grans aquells que presentaven més d'un replicó. Del 81% (63/78) dels plasmidis portadors de *bla*$_{CMY-2}$ caracteritzats, el 41% (26/63) pertanyeren als grups IncA/C (amb mides que oscil·laven entre les 150 i les 170 kb en el 69% dels casos), el 29% (18/63) a IncI1/Iγ (amb mides que oscil·laven entre les 82 i les 100 kb en el 88% dels casos) i el 13% (8/63) a IncK (amb mides que oscil·laven entre les 81 i les 86 kb en el 63% dels casos). Una de les soques d'*E. coli* portava el gen *bla*$_{CMY-2}$ en un plasmidi tipus F i una soca de *P. mirabilis* en un cointegrat entre un plasmidi tipus ColE i un plasmidi MOB$_{P11}$. La seqüenciació de la regió *mob* va indicar que es tractava d'un plasmidi tipus IncP1α, plasmidi *helper* freqüentment associat a plasmidis mobilitzables. En el 16% (10/63) dels casos, els plasmidis portadors de *bla*$_{CMY-2}$ presentaven més d'un replicó (Figura 30). En el 19% (15/78) dels casos, els vectors implicats en la disseminació dels gens *bla*$_{CMY-2}$ no es varen poder caracteritzar (Taula 34).

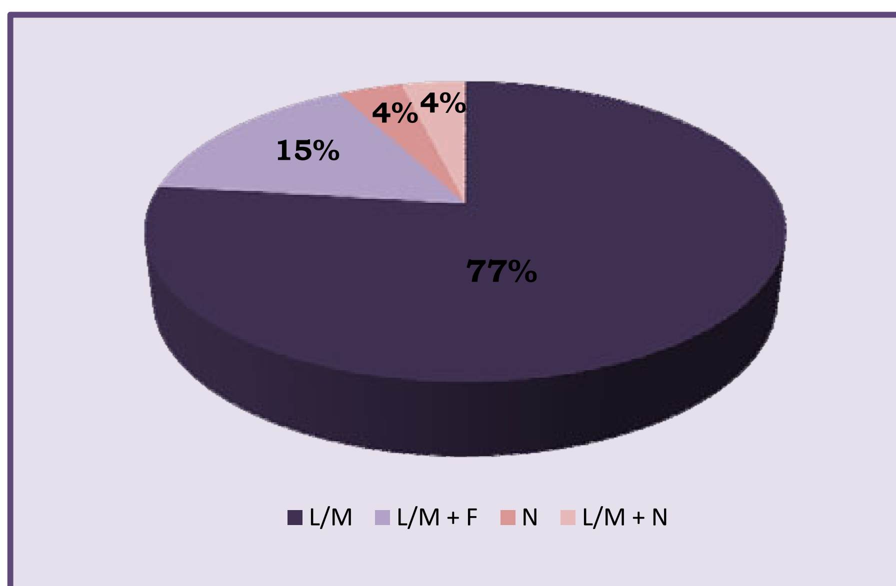
Figura 37. Tipus de plasmidis portadors de *bla*$_{CMY-2}$.



4.5.2.2. Plasmidis portadors dels gens *bla*$_{DHA-1}$

Els plasmidis portadors dels gens *bla*$_{DHA-1}$ presentaren també mides molt diverses que oscil·laren entre les 52 i les 293 kb. Del 87% (26/30) dels plasmidis caracteritzats, tots menys un foren assignats al grup IncL/M. En el 77% (20/26) dels casos, el plasmidi de tipus L/M va ser l'únic replicó trobat (amb mides que oscil·laren entre les 70 i les 80 kb en el 56% dels casos) o formant multireplicons en el 19% (5/26) dels casos, trobant-se quatre soques amb els replicons L/M + F (15%) i una soca amb L/M + N (4%). L'únic plasmidi no pertanyent al grup L/M fou del tipus IncN (Figura 38). En dos dels tres casos on a més a més també és va transferir una BLEA junt amb la DHA-1, ambdues resistències es trobaren localitzades en el mateix plasmidi. En el 13% (4/30) dels casos, els vectors implicats en la disseminació dels gens *bla*$_{DHA-1}$ no es varen poder caracteritzar (Taula 34).

Figura 38. Tipus de plasmidis portadors de *bla*$_{DHA-1}$.



4.5.2.3. Plasmidis portadors dels gens *bla*$_{ACC-1}$

La tècnica de PBRT i posterior hibridació va permetre la caracterització únicament d'un dels plasmidis portadors de *bla*$_{ACC-1}$ en un plasmidi de 95 kb pertanyent al grup IncI1/Iγ. Les dues soques restants es varen resoldre mitjançant la tipificació de la regió *mob*, essent ambdues soques positives per PCR per a la subfamília MOB_{F11}. La seqüenciació dels amplicons de les relaxases tipus MOB_{F11} va ser compatible en un dels casos amb la relaxasa del plasmidi R46 (tipus IncN), mentre que en l'altre cas es va descriure una relaxasa nova (Nº de GeneBank: FJ421285) en un plasmidi de 75 kb, amb una homologia aminoacídica del 79% amb la regió TraI del plasmidi R46 i d'un 51% amb la regió TraI del plasmidi pCT14. Aquesta nova relaxasa va rebre el nom de VirD2, per la seva homologia amb la relaxasa del sistema model d'*Agrobacterium* (Taula 34).

4.5.2.4. Plasmidis portadors dels gens *bla*$_{CMY-4, -25, -27 i -40}$

De les sis soques portadores de les diferents variants de *bla*$_{CMY-2}$, dues soques presentaren *bla*$_{CMY-4}$ i *bla*$_{CMY-27}$ en plasmidis de tipus F, amb mides de 121 i 73 kb respectivament, mentre que en l'altra soca portadora de *bla*$_{CMY-27}$, el gen *amp*C es va localitzar en un plasmidi MOB_{P12} de 249 kb. La seqüenciació de la regió *mob* va indicar que es tractava d'un plasmidi tipus IncI1. Els plasmidis portadors de *bla*$_{CMY-25}$ i el restant *bla*$_{CMY-4}$ no es varen poder caracteritzar ni per la tipificació del replicó ni per la tipificació de la relaxasa (Taula 34). Finalment en la soca portadora de *bla*$_{CMY-40}$ cap dels plasmidis presents en la soca varen hibridar amb la sonda del gen *amp*C, indicant la seva possible localització cromosòmica (Taula 33).

Taula 34. Famílies plasmídiques associades amb diferents tipus de pACBL en *Enterobacteriaceae*.

ENZIMS (n)	REPLICONS/RELAXASES	AÏLLATS (n)	Nº SOQUES	MIDES (kb)	TOTAL (%)
CMY-2/ CMY-2+BLEA (78)	A/C	*E. coli* (15), *K. pneumoniae* (2), *P. mirabilis* (8), *S. enterica* (1)	26	≈ 97 a 365	33%
	I1	*E. coli* (16), *P. mirabilis* (1), *S. enterica* (1)	18	≈ 82 a 350	23%
	K	*E. coli* (7), *P. mirabilis* (1)	8	≈ 81 a 113	10%
	I1 + K	*E. coli* (2)	2	≈ 90 a 93	3%
	I1 + F	*E. coli* (3)	3	≈ 84 a 133	4%
	A/C + F	*E. coli* (3)	3	≈ 162 a 339	4%
	A/C + I1	*E. coli* (1)	1	≈ 414	1%
	F	*E. coli* (1)	1	≈190	1%
	ColE + MOB_{P11}	*P. mirabilis* (1)	1	≈ 97	1%
	No resolt	*P. mirabilis* (8), *E. coli* (5), *K. pneumoniae* (2)	15	-	19%
DHA-1/ DHA-1+BLEA (30)	L/M	*E. coli* (8), *K. pneumoniae* (9), *K. oxytoca* (3)	20	≈ 70 a 293	67%
	L/M + F	*E. coli* (2), *K. pneumoniae* (1), *K. oxytoca* (1)	4	≈ 108 a 171	13%
	N	*E. coli* (1)	1	≈ 52	3%
	L/M + N	*P. mirabilis* (1)	1	≈ 103	3%
	No resolt	*E. coli* (4)	4	-	13%
ACC-1 (3)	I1	*E. coli* (1)	1	≈ 95	33%
	MOB_{F11}	*E. coli* (1), *K. pneumoniae* (1)	2	≈ 64 i 76	67%
CMY-4 (2)	F	*E. coli* (1)	1	≈ 121	50%
	No resolt	*E. coli* (1)	1	≈ 40	50%
CMY-27 (2)	F	*E. coli* (1)	1	≈ 73	50%
	MOB_{P12}	*E. coli* (1)	1	≈ 249	50%
CMY-25 (1)	No resolt	*K. pneumoniae* (1)	1	≈ 380	100%
CMY-40 (1)	No resolt	*E. coli* (1)	1	-	100%

4.5.3. CORRELACIÓ ENTRE LA PBRT I LA TIPIFICACIÓ DE LA RELAXASA

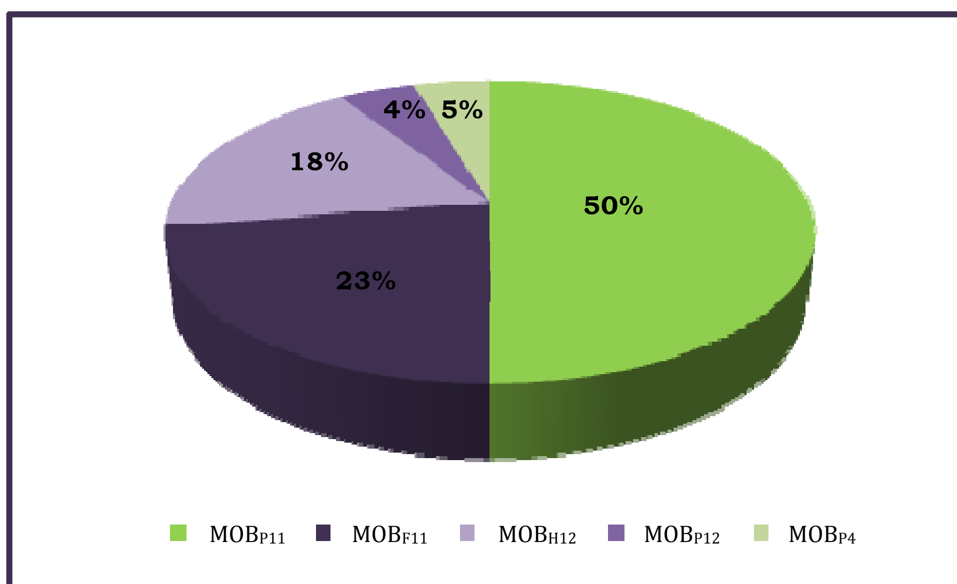
La caracterització plasmídica mitjançant la tipificació de la relaxasa és una nova tècnica en procés de desenvolupament que encara no ha estat àmpliament testada per a una gran col·lecció de soques clíniques. Amb l'objectiu de comprovar si els resultats obtinguts per PBRT eren reproduïbles amb aquesta nova tècnica, tots aquells grups Inc obtinguts per la PBRT varen ser posteriorment analitzats per a la corresponent subfamília de relaxases MOB específiques. A més a més, la caracterització de les 19 subfamílies de relaxases detallades en la taula 23 de l'apartat de material i mètodes 3.2.6.1 també es va realitzar en totes aquelles soques que eren negatives per als 18 replicons testats per PBRT i aquelles soques on existia alguna discrepància de resultats entre ambdues tècniques.

Entre els 138 replicons detectats per PBRT en les 117 soques de la col·lecció, la subfamília específica de relaxases es va caracteritzar en 127 dels casos, mostrant una correlació del 92% (127/138) entre ambdues tècniques. Curiosament, dels 11 casos on la relaxasa específica no es va detectar, 10 dels casos eren de grups IncF, mentre que el grup restant fou IncL/M.

L'estudi de totes de les subfamílies de relaxases es va dur a terme en 25 soques: 14 soques amb resultats negatius per a tots els replicons testats i 11 soques on la PCR de la relaxasa específica era negativa. La tipificació de la regió *mob* ens va permetre detectar 20 plasmidis més que la tècnica de PBRT, incloent 11 plasmidis pertanyents a la subfamília MOB_{P11} (relaxases associades a plasmidis IncP-1), quatre a MOB_{H12} (IncA/C i ICE SXT/R391),

tres MOB_{F11} (IncN i IncW), un MOB_{P12} (IncI, K i B/O) i un MOB_{P4} (IncU). Entre la diversitat plasmídica observada en els 14 aïllats negatius per a tots els replicons testats es varen trobar: sis MOB_{P11}, quatre MOB_{H12} i tres MOB_{F11}, trobant-se els gens *amp*C en tres dels plasmidis detectats per aquesta nova tècnica (dos MOB_{F11} i un MOB_{P11}) (taula 35).

Taula 35. Nova diversitat plasmídica detectada per la tècnica de la relaxasa.



4.5.4. LOCALITZACIÓ CROMOSÒMICA DELS GENS *amp*C

Per tal d'esbrinar si els gens *amp*C de les 18 soques no resoltes, excloent les quatre soques lisades durant la realització del S1-PFGE, presentaven una localització cromosòmica, es va procedir a la digestió del DNA total de les soques amb I-Ceu-I seguida del PFGE, *Southern blot* i hibridació de les membranes amb les sondes específiques per als gens *amp*C implicats.

Entre les 18 soques no resoltes (Taula 33), l'anàlisi amb I-Ceu-I va confirmar la localització cromosòmica dels gens *amp*C en totes les soques testades, a excepció de la soca d'*E. coli* (93D) portadora de *bla*$_{CMY-4}$ en un plasmidi no tipificable (pNT) de 40 kb. Una de les vuit soques de *P. mirabilis* portadora de *bla*$_{CMY-2}$ i la soca de *K. pneumoniae* portadora de *bla*$_{CMY-25}$ varen presentar múltiples còpies del gen *amp*C al cromosoma.

4.5.4.1. Mobilització de *bla*$_{CMY-2}$ per ICE de la família SXT/R391 en soques de *P. mirabilis* (Annex IV)

El fet que tres de les vuit soques de *P. mirabilis* on *bla*$_{CMY-2}$ va ser localitzat al cromosoma foren conjugatives i que a més a més quatre d'elles presentaren una relaxasa compatible amb un ICE de la família SXT/R391 (Taula 33), va fer sospitar que la mobilització dels gens *amp*C en aquestes soques podia estar vehiculada per aquest tipus d'elements.

Per a poder demostrar la implicació d'aquests MGE en la mobilització de *bla*$_{CMY-2}$, es va procedir a la detecció per PCR de gens que codifiquen per a la integrasa (*int)* i per al sistema toxina/antitoxina (*TA*), ambdós altament conservats en els ICE pertanyents als membres d'aquesta família. A continuació es va procedir a hibridar el gel d'I-Ceu-I-PFGE, realitzat tant per a les soques salvatges com per als transconjugants obtinguts, amb els amplicons de *bla*$_{CMY-2}$, *int* i *prf*C (diana on s'integra SXT/R391 al cromosoma bacterià).

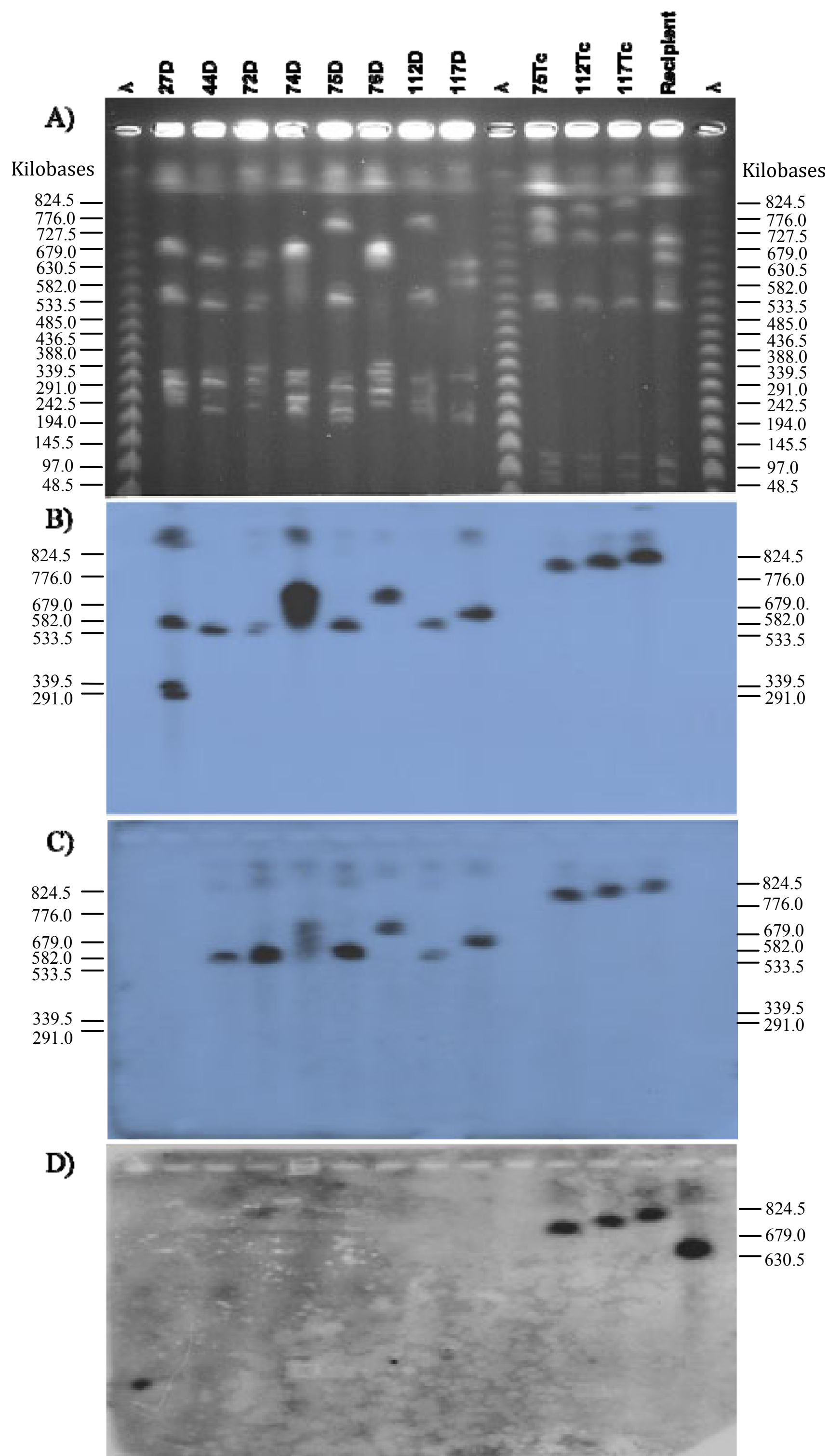
En set dels vuit *P. mirabilis* analitzats per PCR es varen obtenir amplicons tant per a *int* com per a *TA*. La hibridació amb *bla*$_{CMY-2}$ i *int* va confirmar que tant els gens *bla*$_{CMY-2}$ com l'ICE de la família SXT/R391 es

localitzaven en la mateixa porció del genoma en les set soques amb amplicons positius per a *int* i *TA*. L'única soca (27D) no mobilitzada per un ICE d'aquesta família, va presentar almenys quatre còpies de *bla*$_{CMY-2}$ al cromosoma (Figura 32).

Per altra banda s'observaren patrons diferents d'I-Ceu-I-PFGE entre les tres soques transconjugants (75Tc, 112Tc i 117Tc) i la soca receptora HB101 (Figura 39). Es va observar una inserció al cromosoma en els tres transconjugants; la banda de 660 kb present en la soca receptora desapareixia en tots els transconjugants apareixent una banda de major grandària, que era diferent per a cada transconjugant. El gen *prf*C va hibridar en la banda de 660 kb en la soca receptora i en les diferents insercions dels transconjugants. No es va observar hibridació amb *prf*C en les soques donadores de *P. mirabilis* perquè l'amplicó utilitzat per a fer la sonda de *prf*C es va obtindre d'una soca d'*E. coli* (Figura 39).

Una de les set soques portadores de *bla*$_{CMY-2}$ vehiculades per un ICE, era portadora a més a més de *bla*$_{CTX-M-2}$ (soca 44D, taula 33). La hibridació amb la sonda de *bla*$_{CTX-M-2}$ en el gel d'I-Ceu-I-PFGE, va confirmar la seva localització al cromosoma però a un lloc diferent d'on es trobava localitzat l'ICE.

Figura 39. Hibridació del gel I-Ceu-I-PFGE amb *bla*$_{CMY-2}$, *int* i *prf*C.



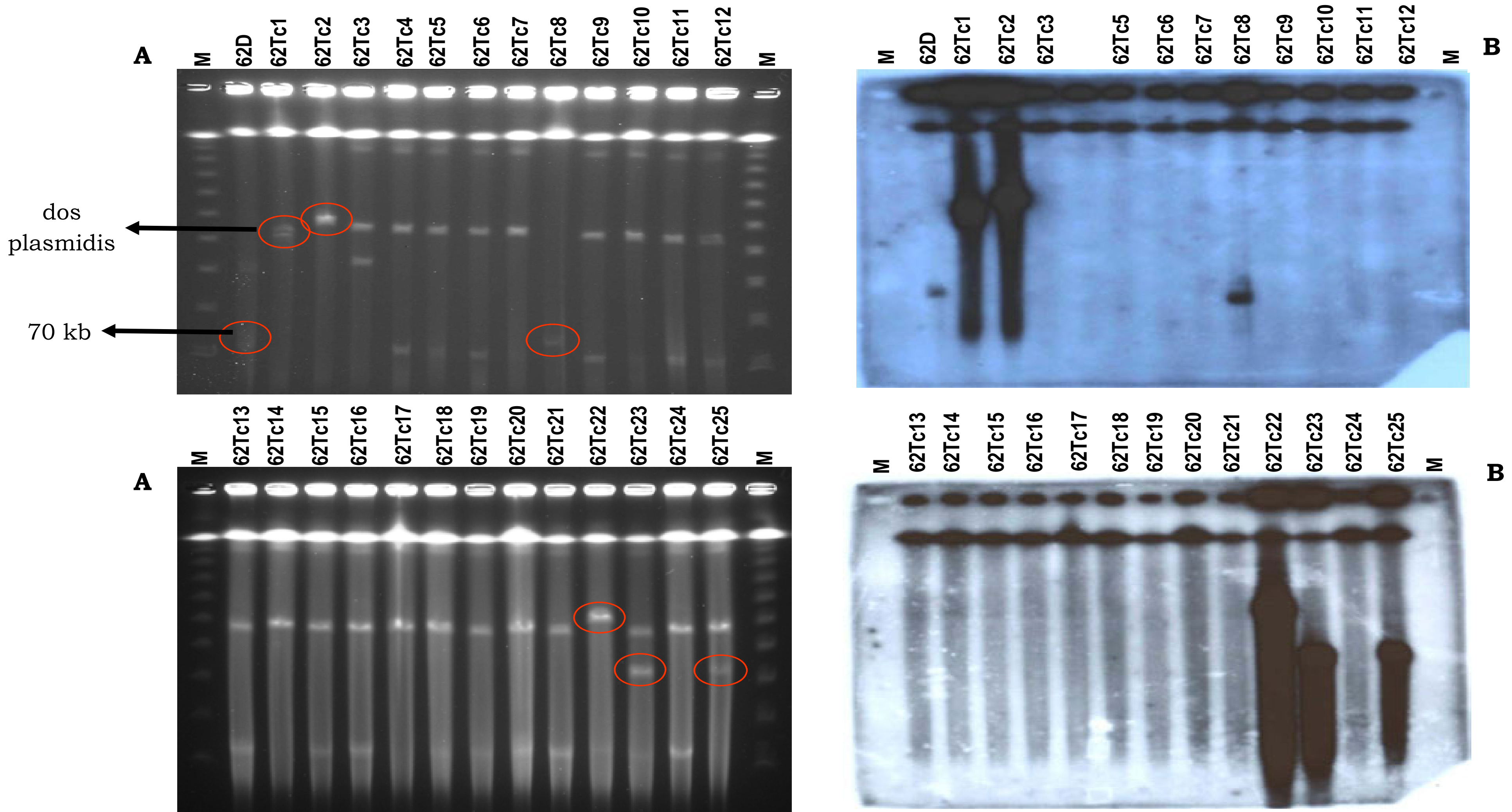
A) I-Ceu-I PFGE; **B)** I-Ceu-I PFGE hibridat amb *bla*$_{CMY-2}$; **C)** I-Ceu-I PFGE hibridat amb la integrasa (*int*) de la família SXT/R391; **D)** I-Ceu-I PFGE hibridat amb *prf*C.

4.5.4.2. Localització cromosòmica dels gens *amp*C en els aïllats amb múltiples hibridacions

Entre els sis aïllats on més d'un plasmidi hibridava tant per al replicó com per al gen *amp*C en qüestió, es va seleccionar una soca (62D) de forma aleatòria. La conjugació d'aquesta soca es va tornar a repetir i en aquest cas es seleccionaren a més a més 25 transconjugants. Per tal de veure si els primers resultats obtinguts eren reproduïbles, es va procedir a l'anàlisi plasmídica tant de la soca salvatge com dels 25 transconjugants seleccionats aleatòriament. L'anàlisi cromosòmica també es va dur a terme tant per a la soca donadora com per als 25 transconjugants.

Els resultats obtinguts varen revelar que el gen *amp*C es trobava originàriament localitzat tant al cromosoma com en un plasmidi d'unes 70 kb en la soca donadora, mentre que per al cas dels transconjugants s'observaren diferents patrons plasmídics on els gens *amp*C hibridaven en un o més plasmidis de diferents tamanys en funció del transconjugant específic (Figura 40). En molts dels transconjugants no es va observar cap hibridació dels gens *amp*C en cap dels plasmidis presents en la soca, però en tots ells el gen *amp*C es va trobar localitzat al cromosoma bacterià.

Figura 40. Hibridació dels gels S1-PFGE amb la sonda de *bla*$_{DHA-1}$.



A: Gels d'S1-PFGE; **B:** Gels d'S1-PFGE hibridats amb la sonda de *bla*$_{DHA-1}$; Els cercles en vermell mostren els plasmidis que han hibridat amb la sonda de *bla*$_{DHA-1}$; **M:** marcador lambda; **D:** donadora; **Tc:** transconjugant.

4.6. CARACTERITZACIÓ DE L'ENTORN GENÈTIC DE LES pACBL (Annex IV)

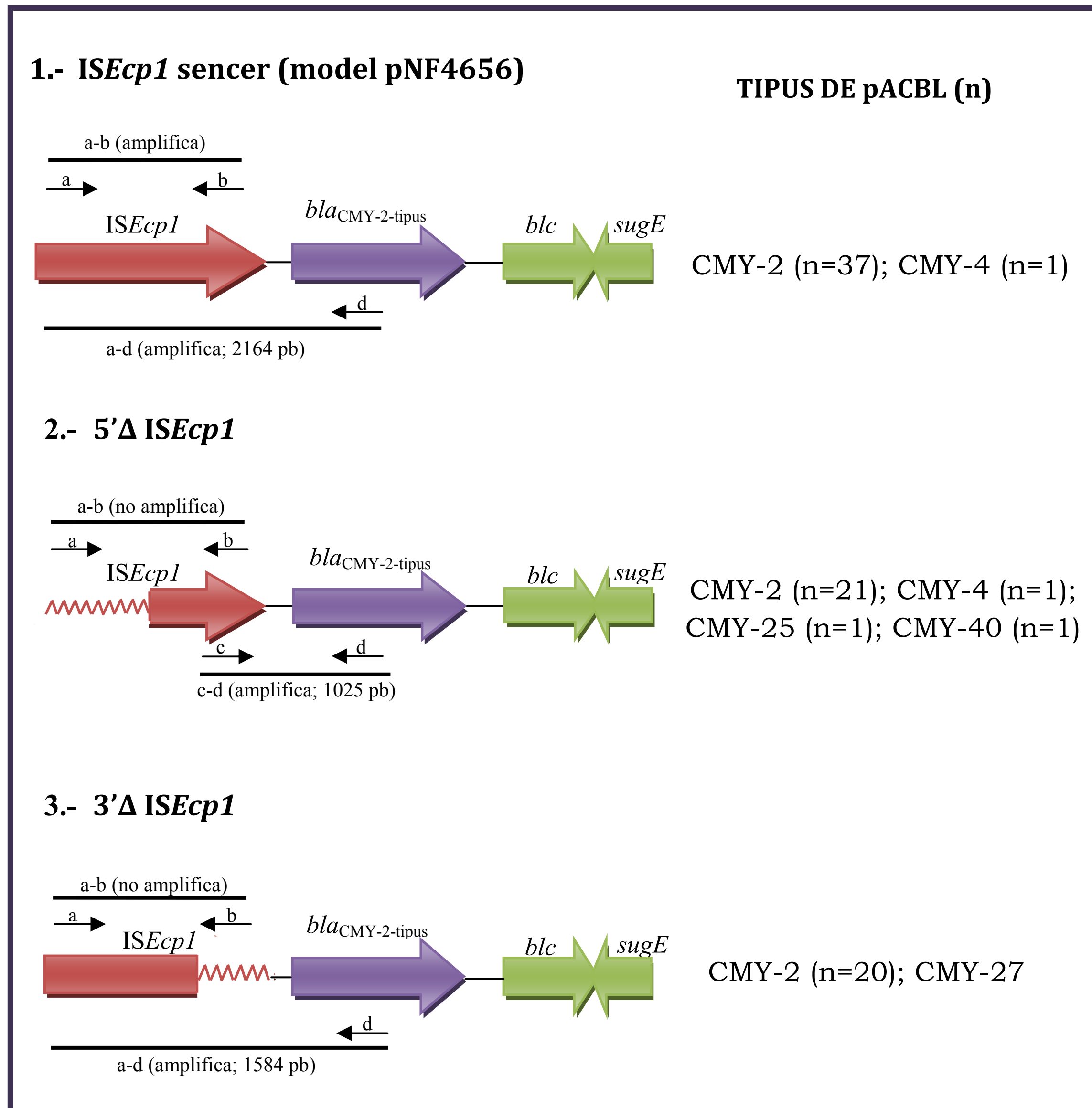
Les regions adjacents als gens *amp*C també varen ser estudiades amb l'objectiu d'analitzar amb més detall els vectors implicats en la seva disseminació. Aquest estudi es va realitzar mitjançant PCR creuades explorant les regions adjacents més freqüentment descrites en la literatura.

4.6.1. ORGANITZACIÓ GENÈTICA DE $bla_{CMY-2, -4, -25, -27 \text{ i } -40}$

Les regions adjacents explorades per als gens bla_{CMY-2} (n=78), bla_{CMY-4} (n=2), bla_{CMY-25} (n=1), bla_{CMY-27} (n=2) i bla_{CMY-40} (n=1) foren IS*Ecp1* i els gens *blc* (lipoproteïna de membrana externa) i *sug*E (canal d'expulsió per a la sortida de drogues), ambdós procedents del cromosoma de *C. freundii*.

L'element transposable IS*Ecp1* es va localitzar *upstream* del gen *amp*C mentre els gens *blc* i *sug*E, foren localitzats *downstream* en totes les soques estudiades (Figura 34). No obstant, l'element IS*Ecp1* va aparèixer truncat en el 55% (46/84) dels aïllats. El truncament es trobava a l'extrem 5' en el 29% (24/84) dels casos mentre que en el 26% (22/84) dels aïllats restants, aquest truncament es va observar a l'extrem 3' (Figura 41).

Figura 41. Organització genètica de *bla*$_{CMY-2, -4, -25, -27 i -40}$.



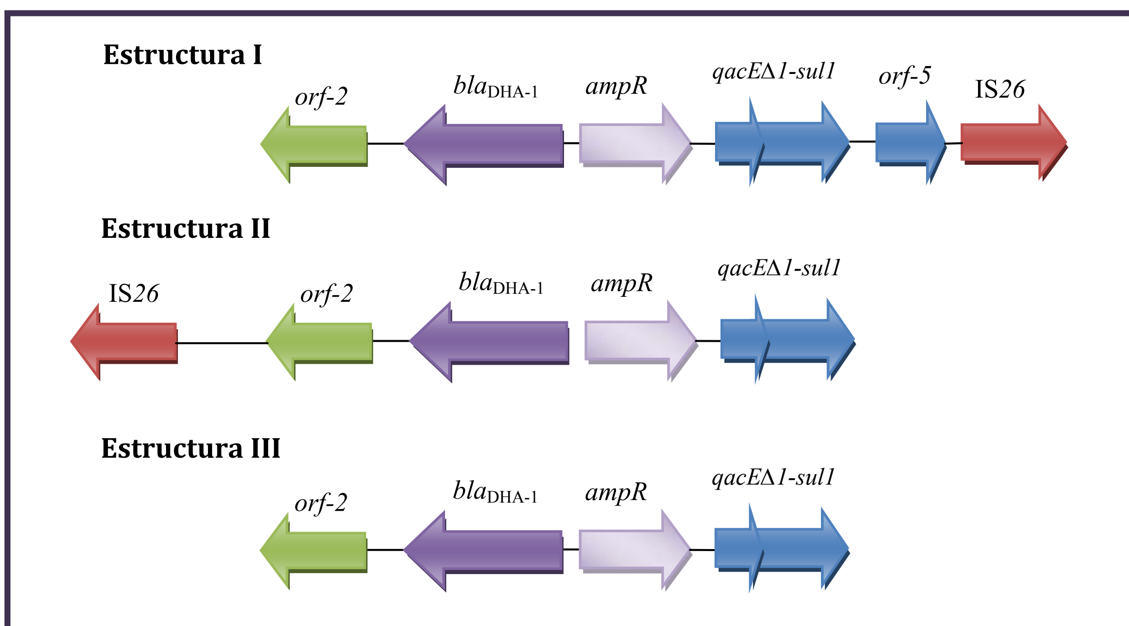
Els gens *amp*C estan representants per fletxes liles, els elements transposables per fletxes de color vermell i les regions adjacents procedents del cromosoma de *C. freundii* per fletxes de color verd. Els gens truncats estan representats per fletxes incompletes. **1)** el 45% dels aïllats són idèntics a l'estructura descrita en el plasmidi pNF4656 (AY581207); **2)** el 29% dels aïllats tenen l'IS*Ecp1* truncat en l'extrem 5'; **3)** el 26% dels aïllats tenen l' IS*Ecp1* truncat en l'extrem 3'. a, b, c i d fan referència a la posició dels iniciadors utilitzats per a amplificar la regió de l'element transposable (iniciadors descrits en la Taula 26 de material i mètodes).

4.6.2. ORGANITZACIÓ GENÈTICA DE *bla*$_{DHA-1}$

Els gens explorats en les 30 soques portadores de *bla*$_{DHA-1}$ foren IS*CR1* (*orf513*), IS*26*, *orf*2, *amp*R, *qac*EΔ1 i *sul*1.

Es va detectar un estructura composada per set ORF consecutius en el 93% (28/30) de les soques portadores de *bla*$_{DHA-1}$ (estructura I) (Figura 42). En aquest cas, *upstream* del gen *amp*C es varen detectar: el seu regulador *amp*R, gens conservats característics d'un integró de classe 1, *qac*EΔ1, *sul*1 i *orf*-5, i l'element transposable IS*26. Downstream* del gen *amp*C es va trobar un ORF de funció desconeguda procedent del cromosoma bacterià de *Morganella* (Figura 42). En una de les soques restants l'element IS*26* es va trobar *downstream* (estructura II), mentre que en l'altra soca, aquest element no es va trobar ni *upstream* ni *downstream* del gen *amp*C (estructura III) (Figura 42). L'element IS*CR1* no es va detectar en cap de les soques a excepció d'un aïllat. En aquest cas, aquest element no es va trobar adjacent del gen *amp*C.

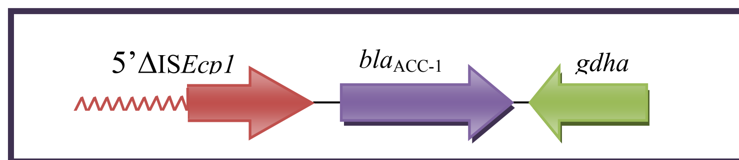
Figura 42. Organització genètica de *bla*$_{DHA-1}$.



4.6.3. ORGANITZACIÓ GENÈTICA DE bla_{ACC-1}

Les tres soques productores dels gens *bla*$_{ACC-1}$ presentaren l'element IS*Ecp1,* truncat al seu extrem 5', *upstream* del gen *amp*C, mentre que el gen *gdha* va ser localitzat *downstream* (Figura 43). Tots tres aïllats foren negatius per a la presència de l'element IS*26*.

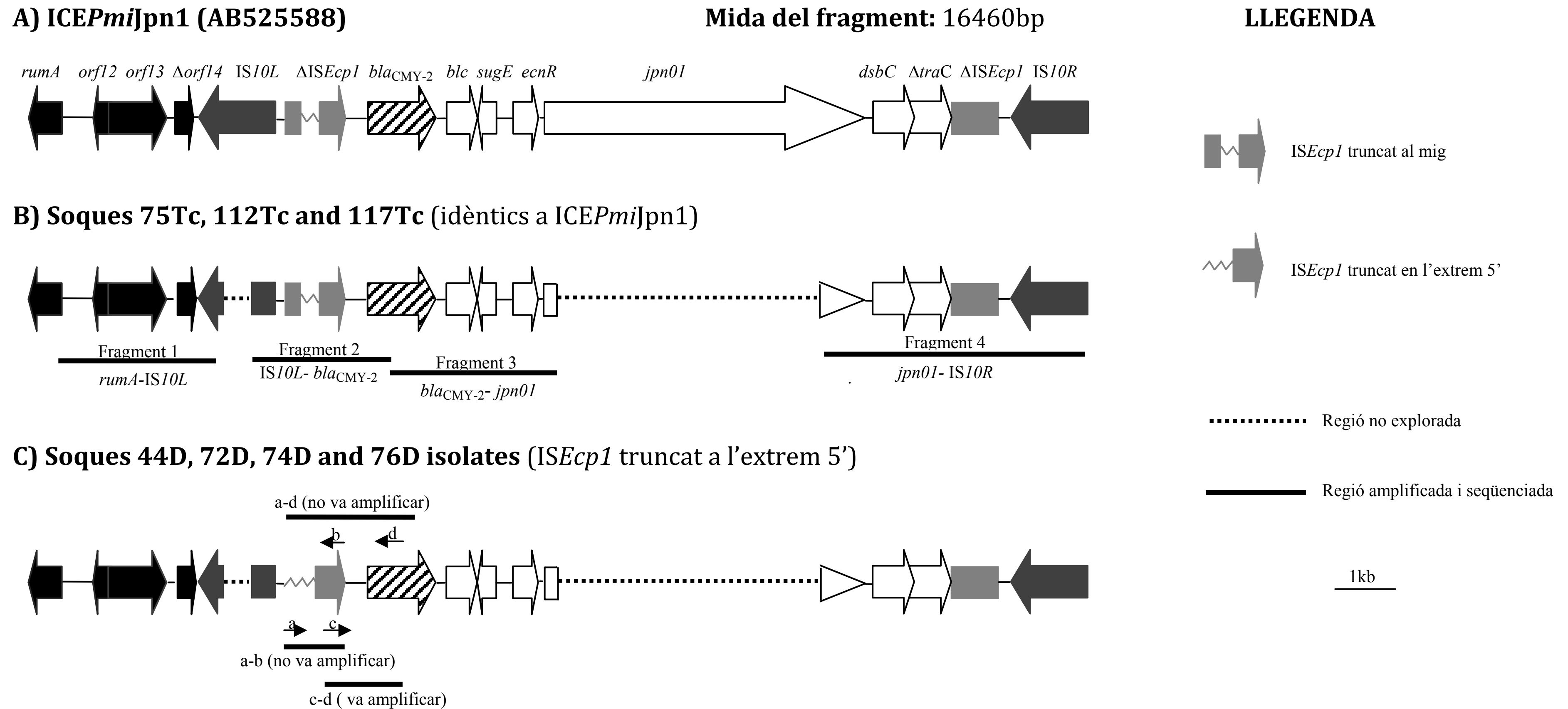
Figura 43. Organització genètica de *bla*$_{ACC-1}$.



4.6.4. ORGANITZACIÓ GENÈTICA DE bla_{CMY-2} MOBILITZATS PER ICE (Annex V)

L'organització genètica de les set soques portadores de *bla*$_{CMY-2}$ en un ICE de la família SXT/R391 es va analitzar utilitzant com a model l'ICE*Pmi*Jpan1 (AB525688), recentment descrit a Japó. Es va procedir a realitzar PCR creuades cobrint la major part de la regió que va des del gen *rum*A, gen conservat de l'ICE, fins a la seqüència IS*10* dreta del transposó Tn*10* compost (16.460 pb) (Figura 44). Els productes purificats de PCR obtinguts dels transconjugants (75Tc, 112Tc i 117Tc) foren seqüenciats. Les set soques de *P. mirabilis* varen mostrar la mateixa organització genètica que la descrita per a l'ICE*Pmi*Jpan1, encara que es varen detectar algunes diferències en referència a la posició del truncament de l'IS*Ecp1* localitzada *downstream* del gen *amp*C. L' IS*Ecp1* present en 44D, 72D, 74D i 76D es va trobar truncat en el seu extrem 5', mentre que en 75Tc, 112Tc i 117Tc el truncament era idèntic al que s'observa per a l'ICE*Pmi*Jpan1 (Figura 44).

Figura 44. Organització genètica dels gens *bla*$_{CMY-2}$ en soques de *P. mirabilis* amb ICE de la família SXT/R391.



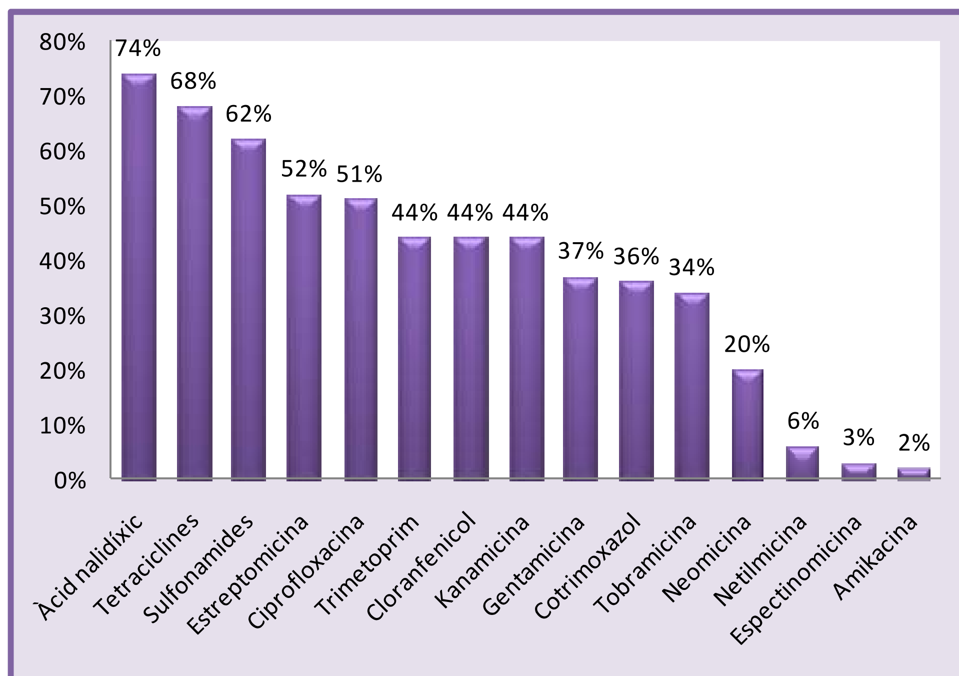
Les fletxes negres representen els gens conservats de l'ICE. Les fletxes gris obscur representen el transposó Tn*10*. Les fletxes gris clar representen l'I*SEcp1*. Les fletxes amb línies diagonals representen al gen *bla*$_{CMY-2}$. Les fletxes blanques representen altres gens presents en aquesta regió **A)** Regió de 16.460 pb de l'ICE*Pmi*Jpn1 utilitzat com a model per a explorar l'entorn genètic del gen *bla*$_{CMY-2}$ en els aïllats d'aquest estudi. **B)** Representació esquemàtica de les regions amplificades i seqüenciades. Les soques 75Tc, 112Tc i 117Tc són idèntiques a ICE*Pmi*Jpn1. **C)** Aquestes soques presenten la mateixa organització genètica que ICE*Pmi*Jpn1, a excepció de l'element IS*Ecp1* que apareix truncat a l'extrem 5'.

4.7. SENSIBILITAT A ANTIMICROBIANS NO BETALACTÀMICS

L'estudi de sensibilitat a antibiòtics no betalactàmics en les soques clíniques portadores de pACBL va mostrar un elevat grau de resistència a la majoria dels antimicrobians testats (Figura 45).

Els percentatges de resistència més elevats en aquestes soques foren detectats per a antimicrobians com l'àcid nalidíxic, amb el 74% de soques resistents, les tetraciclines, amb un 68% de soques resistents, les sulfonamides, amb un 62% de soques resistents, i la ciprofloxacina, amb un 51% de soques resistents. El 44% de les soques foren també resistents a cloranfenicol, trimetoprim i kanamicina, un 37% de les soques foren resistents a gentamicina, un 34% a tobramicina i un 20% a neomicina. Tan sols un 6% de les soques productores de pACBL foren resistents a netilmicina, un 3% a espectinomicina i un 2% a amikacina.

Figura 45. Percentatge de soques resistents a antibiòtics no betalactàmics.



Amb l'objectiu d'avaluar si aquests determinants de resistència eren transferits per conjugació, conjuntament amb els diferents tipus de pACBL, es va estudiar també el patró de sensibilitat de tots els transconjugants obtinguts. En aquest cas, com que la receptora utilitzada per als experiments de conjugació era resistent a una majoria d'aminoglucòsids, la transferència d'aquests antibiòtics no es va poder avaluar.

Tal i com es mostra en la taula 36, les soques clíniques portadores de pACBL, a excepció de la soca portadora de CMY-25, foren resistents a la gran majoria d'antibiòtics no betalactàmics. No obstant, el percentatge de soques transconjugants mostrant co-resistència a aquests antibiòtics fou molt menor.

Taula 36. Percentatge de resistència a antibiòtics no betalactàmics en soques clíniques i nivells de co-resistència en els transconjugants.

		ANTIBIÒTICS NO BETALACTÀMICS (% de soques resistents)[a]						
pACBL	**(nº)**	**NAL**	**CIP**	**SSS**	**TMP**	**SXT**	**TET**	**CHL**
CMY-2[b]	D_T (n=78)	77	47	73	44	40	81	59
	D_{TC} (n=66)	76	52	73	39	36	79	56
	Tc (n= 66)	0	0	50	3	3	47	42
DHA-1[c]	D_T (n=30)	77	60	47	47	37	43	17
	D_{TC} (n=26)	81	62	50	46	38	46	15
	Tc (n= 26)	62[e]	0	15	15	8	12	0
ACC-1	D_T (n=3)	2	2	2	1	0	2	0
	Tc (n= 3)	0	0	2	0	0	0	0
CMY-27	D_T (n=2)	2	2	0	1	0	2	0
	Tc (n= 2)	0	0	0	0	0	0	0
CMY-4 [d]	D_T (n=2)	2	2	2	2	2	2	1
CMY-25[d]	D_T (n=1)	0	0	0	0	0	0	0
CMY-40[d]	D_T (n=1)	1	1	0	1	0	0	0

D_T: soques clíniques; **D_{TC}:** soques clíniques que han conjugat; **Tc:** transconjugants

[a] Quan el nombre de soques és menor de 10, s'indica el nombre de soques resistents.
[b] Dues soques portaven a més a més una BLEA; una d'elles va ser conjugativa.
[c] Cinc soques portaven a més a més una BLEA; totes elles foren conjugatives.
[d] No s'obtingueren transconjugants de les soques portadores de CMY-4, CMY-25 i CMY-40.
[e] El 61,5% dels transconjugants (16/26) mostraren sensibilitat disminuïda a l'àcid nalidíxic (14-18 mm).

Els determinants de resistència transferits amb major freqüència en les soques productores de CMY-2 foren les sulfonamides (50% de les soques), les tetraciclines (47%) i el cloranfenicol (42%). En canvi, el determinant de resistència no betalactàmic majoritàriament transferit als transconjugants portadors de DHA-1 fou l'àcid nalidíxic, mostrant-se sensibilitat disminuïda a aquest antibiòtic en el 62% dels casos (Taula 36). Els transconjugants obtinguts per a les soques portadores d'ACC-1 i CMY-4 foren sensibles a la pràctica totalitat d'antibiòtics no betalactàmics avaluats, a excepció de dos transconjugants portadors d'ACC-1, que mostraren resistència a sulfonamides (Taula 36).

4.8. PREVALENÇA DE GENS *qnr*B EN SOQUES PORTADORES DE *bla*$_{\text{DHA-1}}$ (Annex VI)

El fet que el 62% dels transconjugants portadors de *bla*$_{\text{DHA-1}}$ presentaren sensibilitat disminuïda a l'àcid nalidíxic, va fer sospitar la possible presència de gens *qnr* en aquestes soques. L'amplificació per PCR dels gens *qnr*A, *qnr*B i *qnr*S es va dur a terme en les 30 soques de l'estudi portadores de DHA-1. Es varen detectar gens *qnr*B en la totalitat de les soques estudiades.

Amb l'objectiu de determinar si ambdós gens de resistència es trobaven al mateix plasmidi o en plasmidis diferents, les membranes transferides d'S1-PFGE hibridades anteriorment amb les sondes de *bla*$_{\text{DHA-1}}$ i dels replicons detectats per PBRT varen ser posteriorment hibridades amb sondes específiques de *qnr*. Dues de les soques d'estudi es varen lisar durant el S1-PFGE i no pogueren ser analitzades. Els resultats de la hibridació varen

demostrar la co-localització dels gens de resistència *bla*$_{DHA-1}$ i *qnr*B en el mateix plasmidi en les 28 soques testades (Taula 37).

Totes les soques portadores dels gens *qnr*B, *bla*$_{DHA-1}$ o *bla*$_{DHA-1}$ més *bla*$_{BLEA}$ portaven aquests gens en plasmidis IncL/M d'ampli rang d'hostatger, a excepció d'una soca que portava ambdós gens de resistència en un plasmidi tipus IncN (Taula 37).

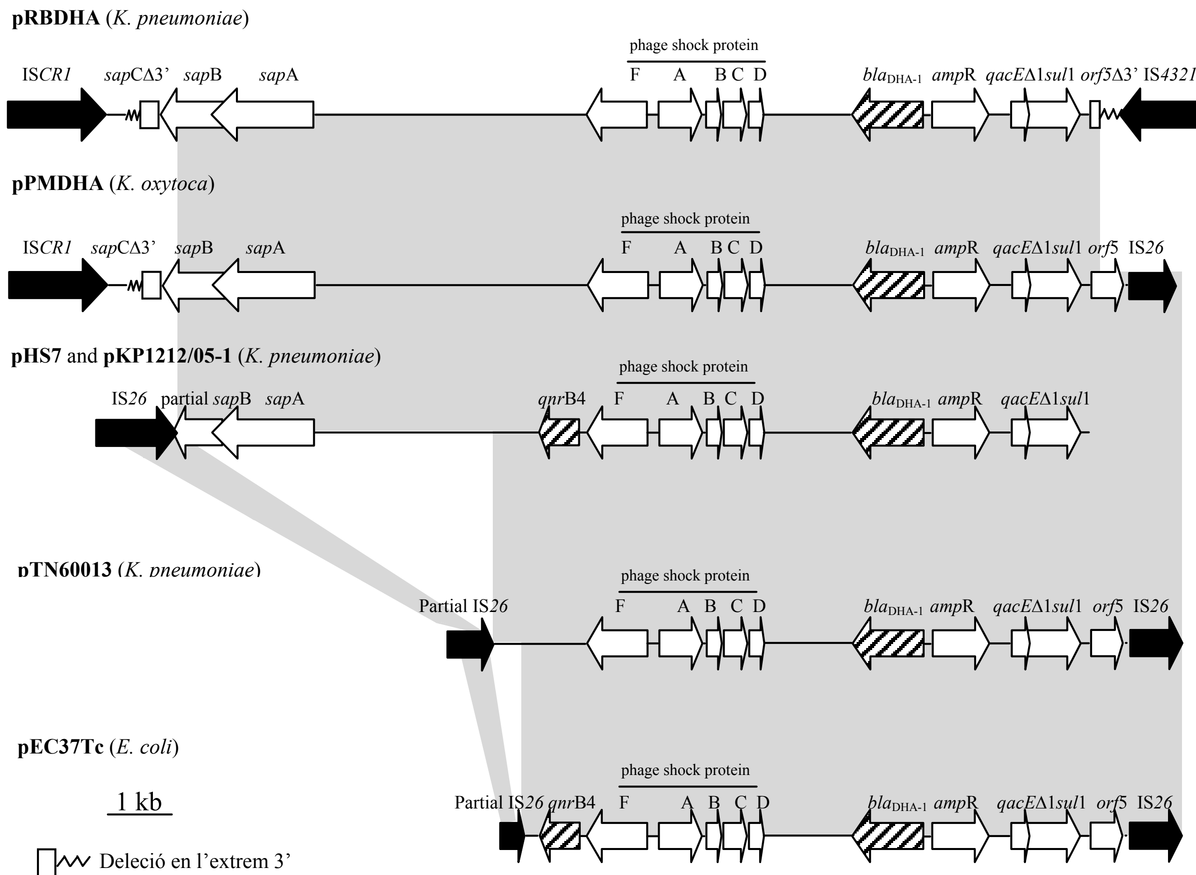
Tal i com es mostra en la Figura 42 de l'apartat 4.6.2, els gens *bla*$_{DHA-1}$ presentaren la mateixa organització genètica en el 93% (28/30) de les soques, trobant-se l'element IS*26* i una regió del cromosoma de *M. morganii upstream* i *downstream* del gen *amp*C respectivament. Per tal de localitzar el gen *qnr*B en les soques portadores de *bla*$_{DHA-1}$, es va realitzar l'extracció plasmídica d'una de les soques d'estudi. Els plasmidis aïllats foren seqüenciats per la casa comercial Eurofins MWG Operon. L'anàlisi parcial d'un dels plasmidis seqüenciats, anomenat pEC37Tc (Nº de GenBank: HQ700359), va mostrar que els gens *bla*$_{DHA-1}$ i *qnr*B4 estaven ambdós localitzats en un transposó IS*26* compost, presumiblement implicat en la seva mobilització. Altres regions trobades en aquesta estructura foren l'extrem 3'CS d'un integró de classe 1 i l'operó *psp* (*phage shock protein*) (Figura 46).

Taula 37. Patró de resistència a antibiòtics no betalactàmics en les soques salvatges i transconjugants, localització dels gens *bla* i *qnr*B i mida dels plasmidis.

SOQUES -D/Tc	ESPÈCIES	PATRÓ DE RESISTÈNCIA A ANTIBIÒTICS NO BETALACTÀMICS		REPLICONS/ ENZIMS DE RESISTÈNCIA/ MIDA PLASMIDI (kb)
		DONADORES	TRANSCONJUGANTS	
35-Tc	*E. coli*	SSS,SXT, TET, NAL, CIP	SSS	L/M+FIA$^{DHA-1,QnrB}$ (≈171)
36-Tc	*E. coli*	SSS, TET	NAL	L/M $^{DHA-1,QnrB}$ (≈209)
37-Tc	*E. coli*	SSS, TMP, SXT, TET, NAL, CIP	SSS, NAL	L/M $^{DHA-1,QnrB}$ (≈100 and 312)
38-Tc	*E. coli*	SSS, TMP, SXT, NAL, CIP	-	L/M $^{DHA-1,QnrB}$ (≈79)
62-Tc	*E. coli*	SSS, TMP, SXT, TET, CHL, NAL, CIP	SSS, NAL	L/M $^{DHA-1,QnrB}$ (≈74 and 164)
63-Tc	*E. coli*	NAL, CIP	NAL	L/M $^{DHA-1,QnrB}$ (≈293)
64-D	*E. coli*	-	-	F, FIB (soca lisada)
95-Tc	*E. coli*	NAL	NAL	L/M $^{DHA-1,QnrB}$ (≈236)
96-Tc	*E. coli*	SSS, TMP, SXT, NAL, CIP	NAL	L/M $^{DHA-1,QnrB}$ (≈96)
97-Tc	*E. coli*	SSS, TET, CHL, NAL, CIP	NAL	L/M $^{DHA-1,QnrB}$ (≈173)
98-Tc	*E. coli*	TET, NAL, CIP	TET, NAL	N $^{DHA-1,QnrB}$ (≈52)
99-Tc	*E. coli*	SSS, TMP, SXT, TET, NAL, CIP	NAL	L/M+FIA $^{DHA-1,QnrB}$ (≈155)
100-D	*E. coli*	SSS, TMP, SXT, TET, NAL, CIP	-	L/M, F, FIB (soca lisada)
101-Tc	*E. coli*	NAL	NAL	L/M $^{DHA-1,QnrB}$ (≈156), I1/Iγ $^{CTX-M-14}$ (≈88)
102-Tc	*E. coli*	SSS, TMP, SXT, TET, NAL, CIP	TMP, TET, NAL	L/M $^{DHA-1,QnrB}$ (≈93), F-FIB (≈148)
40-D	*K. pneumoniae*	-	-	L/M $^{DHA-1,QnrB}$ (≈79)
41-Tc	*K. pneumoniae*	NAL	NAL	L/M $^{DHA-1,QnrB}$ (≈202)
66-D	*K. pneumoniae*	TMP, CHL, NAL	-	L/M $^{DHA-1,QnrB}$ (≈72)
67-Tc	*K. pneumoniae*	-	-	L/M $^{DHA-1,QnrB}$ (≈72)
103-Tc	*K. pneumoniae*	TMP, NAL, CIP	TMP, SXT	L/M $^{DHA-1,QnrB,CTX-M-15}$ (≈168)
104-Tc	*K. pneumoniae*	TMP, NAL, CIP	TMP	L/M+FIC $^{DHA-1,QnrB,CTX-M-15}$ (≈153)
105-Tc	*K. pneumoniae*	SSS, TMP, SXT, TET, CHL, NAL, CIP	-	L/M $^{DHA-1,QnrB}$ (≈72)
106-Tc	*K. pneumoniae*	NAL	NAL	L/M $^{DHA-1,QnrB}$ (≈72)
107-Tc	*K. pneumoniae*	NAL, CIP	-	L/M $^{DHA-1,QnrB}$ (≈72)
108-Tc	*K. pneumoniae*	SSS, TMP, SXT, TET, CHL, NAL, CIP	-	L/M $^{DHA-1,QnrB}$ (≈70)
68-Tc	*K. oxytoca*	-	-	L/M $^{DHA-1,QnrB}$ (≈72)
69-Tc	*K. oxytoca*	SSS, TMP, SXT, TET	-	L/M $^{DHA-1,QnrB}$ (≈72)
109-Tc	*K. oxytoca*	SSS, TMP, SXT, TET, NAL, CIP	NAL	L/M+FIC $^{DHA-1,QnrB}$ (≈153)
110-Tc	*K. oxytoca*	NAL	NAL	L/M $^{DHA-1,QnrB}$ (≈112)
77-Tc	*P. mirabilis*	SSS, TMP, SXT, TET, NAL, CIP	SSS, TMP, SXT, TET, NAL	L/M+N$^{DHA-1,QnrB}$ (≈103)

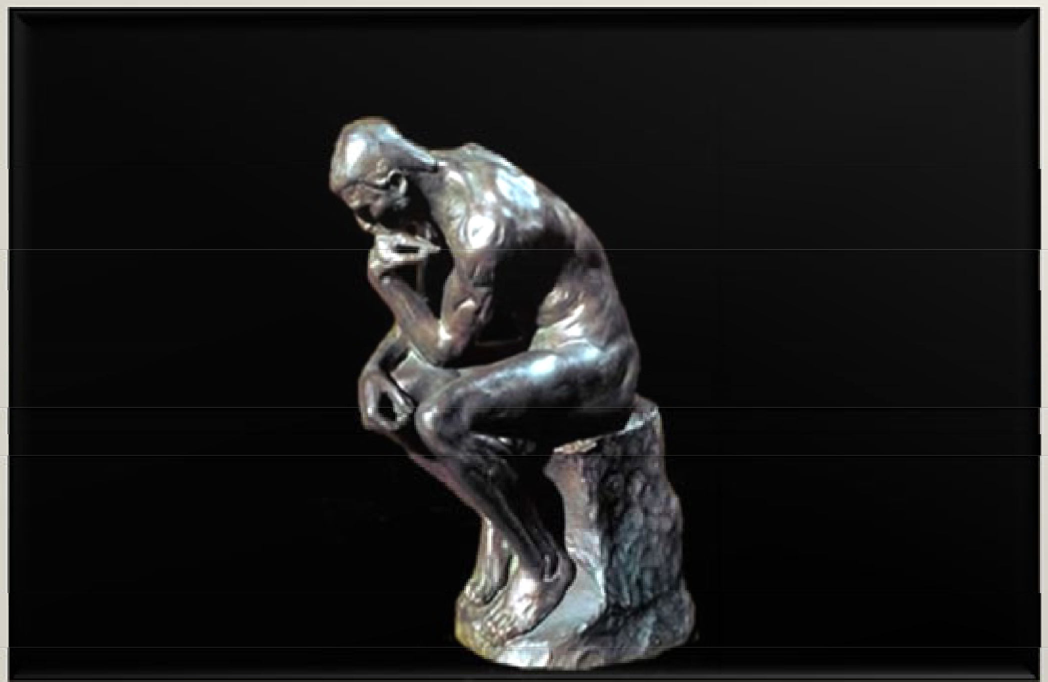
NAL: àcid nalidíxic, **CIP:** ciprofloxacina, **SSS:** sulfonamides, **TMP:** trimetoprim, **SXT:** trimetoprim-sulfametoxazol, **TET:** tetraciclina, **CHL:** cloranfenicol. Tots els transconjugants resistents a NAL varen mostrar sensibilitat reduïda (14-18 mm; categoria clínica intermèdia).

Figura 46. Organització genètica del plasmidi pEC37Tc en comparació amb altres regions comuns d'altres plasmidis portadors dels gens *bla*$_{DHA-1}$ i *qnr*B.



Els números d'accés al GenBank de pRBDHA, pMPDHA, pHS7, pKP1212/05-1, pTN60013 i pEC37Tc són AJ971343, AJ971344, EF683583, FJ943500, AJ971345 i HQ700359, respectivament. Els gens estan representats per fletxes. Els gens de resistència *qnr*B4 i *bla*$_{DHA-1}$ estan representats per fletxes ratllades. Les fletxes de color negre representen les seqüències d'inserció. Les regions marcades en gris representen regions amb 100% d'identitat amb les altres seqüències. Encara que els gens *qnr*B4 estan presents en tots els plasmidis, les fletxes per a pRBDHA, pMPDHA i pTN60013 no estan dibuixades perquè aquests gens no estan anotats en les seqüències dipositades al GenBank.

5. DISCUSSIÓ



DISCUSIÓ

La continua aparició de bacteris multiresistents, tant en l'àmbit hospitalari com en la comunitat, ha esdevingut un problema a nivell mundial. La causa més freqüent de multiresistència és la resistència adquirida, gràcies a la disseminació i l'intercanvi genètic entre bacteris.

Els membres de la família *Enterobacteriaceae* han esdevingut tradicionalment una font continua d'infecció en humans. Els múltiples esforços per fer front a aquestes infeccions, principalment mitjançant l'ús d'antibiòtics betalactàmics, s'han vist superats en molts casos per la capacitat d'aquests microorganismes d'adquirir nous mecanismes de resistència, principalment per la producció de betalactamases.

Aquest treball ha estat principalment enfocat en l'estudi de les pACBL en enterobacteris, que encara que presenten una menor prevalença que altres betalactamases adquirides com les BLEA, estan en continu augment i constitueixen una important font de resistència a antibiòtics betalactàmics.

La falta de mètodes fenotípics estandarditzats per a la detecció de soques productores de pACBL i el fet que aquestes betalactamases presenten un patró fenotípic indistingible al d'una hiperproducció d'una betalactamasa AmpC cromosòmica, planteja enormes dificultats a l'hora de detectar aquest tipus de resistències al laboratori (80, 129, 251). En aquest sentit, segons els resultats que es desprenen de l'estudi nacional del control de qualitat realitzat a 57 centres espanyols (annex I), s'observa que la seva capacitat per a detectar la producció d'enzims AmpC en soques de *K. pneumoniae* i *E. coli*, presenta

certes limitacions. Aquests resultats es deuen, a més a més d'una falta de marcadors específics, a la inexistència de recomanacions clares sobre com informar aquest tipus de soques. Els criteris aplicats pels centres foren molt variats, amb gran tendència a deixar com a única alternativa terapèutica dins dels betalactàmics als carbapenèmics, excloent també a la cefepima. Un dels principals motius de l'exclusió de la cefepima com a possible opció terapèutica fou la confusió del fenotip AmpC amb una BLEA. Aquest error fou també detectat en altres estudis previs. En un control de qualitat realitzat l'any 2003 a Espanya (48) on s'incloïa una soca d'*E. coli* hiperproductora de la seva AmpC cromosòmica amb impermeabilitat per pèrdua de porines, el 6% (3/52) dels centres la identificaren erròniament com una BLEA. D'igual manera en un estudi multicèntric realitzat el 1999 als EEUU (249), cap dels 38 centres avaluats va diferenciar la soca d'*E. coli* hiperproductora d'AmpC, confonent-la també amb una soca productora de BLEA.

En aquest estudi de qualitat, també es varen incloure soques de *K. pneumoniae* i *E. coli* productores de les BLEA més prevalents, incloent quatre soques productores de CTX-M. En els darrers anys s'ha anat observant un canvi molt ràpid en l'epidemiologia de les BLEE en aquestes espècies tant a Espanya com a altres països (47, 49, 50, 77). L'increment de BLEA tipus CTX-M i el fet que en estudis de qualitat anteriorment realitzats a l'Estat espanyol el 38,5% dels laboratoris varen fallar en la detecció d'aquest mecanisme de resistència en la única soca productora de CTX-M de l'estudi (48), varen ser els principals motius per a incloure aquest fenotip de resistència.

En el nostre estudi, el 91% dels laboratoris va detectar correctament la presència de BLEA en totes les soques enviades, evidenciant l'eficàcia dels laboratoris espanyols per a identificar de forma fiable les BLEA més prevalents entre els aïllats clínics d'*E. coli* i *K. pneumoniae*. En aquestes soques, la quantitat d'errors d'interpretació per a cefalosporines i aztreonam fou molt baixa. Els pocs VME detectats foren deguts principalment al fet de no haver detectat el fenotip BLEA, excepte en un cas on la categoria de la cefepima no es va canviar a resistent a pesar que el sistema expert utilitzat pel laboratori suggeria la presència de BLEA. El nombre de VME per a cefalosporines en aquest estudi va ser molt menor que el detectat en l'estudi de qualitat realitzat a Espanya prèviament esmentat (48). En el nostre estudi, dins de les cefalosporines i l'aztreonam, la cefepima va ser l'antibiòtic que va presentar la taxa d'errors més elevada. En aquests laboratoris, la cefepima es va informar com a sensible, sense modificar la categoria clínica a resistent. En un estudi similar realitzat a Itàlia, els problemes observats per a les cefalosporines en soques productores de BLEA foren superiors (161). La majoria de VME es varen detectar per a la soca de *P. mirabilis* productora de TEM-52, possiblement perquè en el moment en que es va realitzar l'estudi, l'espècie *P. mirabilis* no estava inclosa en les recomanacions del CLSI per a ser testada per a la presència de BLEA.

Les taxes d'errors més elevades en el nostre estudi per a les soques productores de BLEA es varen observar per a les combinacions de penicil·lines amb inhibidors de betalactamases. En les dues terceres parts dels casos, l'error es va produir en la categoria clínica interpretada, ja que els laboratoris

canviaven la categoria de sensible o intermedi a resistent quan es detectava el fenotip BLEA.

Encara que no existeixen mètodes fenotípics estandarditzats per a la detecció de pACBL, si que s'han desenvolupat alguns mètodes comercials, anteriorment esmentats, com els tests de sinèrgia de doble disc amb cloxacil·lina o àcid borònic entre d'altres (63, 80, 147, 167, 189). La problemàtica d'aquests mètodes radica en que no són vàlids per a detectar pACBL en microorganismes productors d'una AmpC cromosòmica natural. No obstant, la presència de colònies situades a la proximitat dels halos d'inhibició de la cefoxitina, cefotaxima, ceftazidima i aztreonam s'ha descrit com un possible indicador de la presència d'aquests enzims en soques d'*E. coli* (176). Aquest últim mètode va ser l'única tècnica fenotípica que ens va permetre sospitar la presència d'una pACBL en una soca productora d'una AmpC cromosòmica natural, aïllant-se per primer cop una soca de *S. marcescens* productora d'una pACBL, suggerint-se a més a més la transferència horitzontal *in vivo* d'un plasmidi portador dels gens $bla_{\text{DHA-1}}$ i *qnr*B entre aïllats de *S. marcescens* i d'*E. coli* (Annex II).

Encara que els nivells de prevalença d'aquests enzims són relativament baixos arreu del món, el nombre de soques productores de pACBL continua augmentant any darrere any (79, 84, 154, 160, 214, 237, 279, 281), fet que també s'ha constatat en aquesta tesi (Annex III). La prevalença global d'enterobacteris productors de pACBL a l'Hospital de la Santa Creu i Sant Pau durant 1999-2007 fou del 0,4%, observant-se un increment significatiu del 0,06% el 1999 a l'1,3% el 2007. Aquest increment de la prevalença ja s'havia constatat en un estudi previ dut a terme per dos hospitals espanyols (País

Basc i Catalunya), on s'analitzen tots els aïllats d'enterobacteris sense betalactamasa AmpC cromosòmica durant el període 1999-2000, observant-se un increment del 0,07% el 1999 a 0,41% l'any 2000 (191). En altres estudis realitzats a l'Estat espanyol s'observa un increment de la prevalença en soques d'*E. coli* productores de pACBL. En aquest sentit es registren valors de prevalença del 0,12% en soques aïllades durant 2002-2003 (39) i de l'1,8% en soques aïllades durant 2004-2007 (197). En aquest últim estudi s'observa que la prevalença de soques d'*E. coli* portadores pACBL incrementa un 38,9% l'últim any de l'estudi en comparació als dos primers anys. Recentment s'ha realitzat un estudi per a analitzar la prevalença d'enterobacteris productors de pACBL a nivell nacional, observant-se una prevalença global del 0,6%, sent les comunitats autònomes de Catalunya i Astúries les que presentaren els valors de prevalença més elevats, 0,91 i 0,85% respectivament (178).

El major increment de soques productores de pACBL a l'Hospital de la Santa Creu i Sant Pau es va produir principalment en els tres darrers anys d'estudi. Aquest increment podria justificar-se amb l'emergència de soques productores de DHA-1 (16 de 40 en 2007) i l'increment de soques de *P. mirabilis* productores de CMY-2 (18 soques en els tres darrers anys). L'augment de soques productores de DHA-1 s'ha vist especialment reflectit al continent asiàtic, principalment per l'emergència de soques de *K. pneumoniae* portadores de DHA-1, convertint-se en la pACBL més prevalent i de major importància clínica en aquesta zona (79, 144, 236, 237).

Aquest increment significatiu de la prevalença s'observa en la pràctica totalitat d'espècies estudiades, sent *P. mirabilis* l'espècie portadora de pACBL més prevalent (1%) (Annex III). Aquests resultats van en consonància amb els

resultats obtinguts en un estudi multicèntric realitzat a Polònia durant 2003-2004, on *P. mirabilis* fou també l'espècie portadora de pACBL més prevalent, amb valors del 20,5% (85). En altres estudis realitzats a Itàlia, s'observa un increment de la prevalença en soques de *P. mirabilis* portadores de CMY-16 del 0,3% el 2003 fins a valors del 4,6% el 2006 (160). Per contra, en altres estudis realitzats a Corea durant 2002-2004 no s'aïlla cap soca de *P. mirabilis* productora de pACBL (237), o s'aïllen en una menor proporció en comparació a altres espècies (2).

De la totalitat de pACBL presents, CMY-2 és la més prevalent i àmpliament distribuïda, sobretot en soques d'*E. coli*, *P. mirabilis* i *S. enterica* (11, 21, 76, 164, 188, 191, 207, 221, 224). En el present estudi va ser l'enzim predominant (67%), seguida de DHA-1, amb una representació del 26%. DHA-1 es va associar principalment a soques de *Klebsiella* spp., sent l'única pACBL detectada en *K. oxytoca*. Aquesta associació s'ha observat en molts altres estudis (79, 144, 236, 237, 279).

El continu augment de la prevalença d'aquests enzims es deu principalment a la disseminació dels gens *amp*C per transferència horitzontal. En aquest sentit, els gens *amp*C foren transferits per conjugació en el 83% de les soques testades en la present tesi (Annex IV), corroborant que els vectors que mobilitzen aquestes resistències tenen una gran capacitat per a disseminar-se i transmetre aquesta resistència possiblement tant intra- com inter-espècie.

A dia d'avui, encara existeix molt poca informació disponible sobre els vectors implicats en la disseminació dels gens *amp*C. Els pocs treballs presents actualment estan principalment basats en la caracterització de

plasmidis portadors de bla_{CMY-2}, mentre que els estudis que caracteritzen els vectors d'altres tipus de gens *amp*C són escassos o inexistents (52).

En el present estudi (Annex IV), el 81% (95/117) dels gens *amp*C foren localitzats en plasmidis de grups Inc/MOB coneguts. Els presents resultats mostren una estreta relació entre el tipus de gen *amp*C i el plasmidi implicat en la seva disseminació. Els plasmidis pertanyents als grups IncA/C, IncI1/Iγ s'han descrit com els principals vectors de mobilització dels gens bla_{CMY-2} (10, 52, 121, 173, 184, 188, 261). En el present estudi, els grups IncA/C i IncI1/Iγ foren també els plasmidis portadors de bla_{CMY-2} més prevalents, seguits per plasmidis IncK. Aquest últim grup Inc també s'ha trobat vehiculant els gens bla_{CMY-2} en altres estudis (20, 76, 172). No obstant, encara que els gens bla_{DHA-1} han estat principalment associats a plasmidis tipus IncFII en estudis previs (109, 121, 246), en la nostra col·lecció s'observa una clara associació entre aquests gens i els plasmidis IncL/M. Aquesta associació també s'ha observat recentment en una col·lecció de 26 soques de *K. pneumoniae* aïllades en un hospital de Granollers (78).

Els tres gens bla_{ACC-1} caracteritzats en aquesta tesi foren mobilitzats per un plasmidi tipus IncI1/Iγ en un dels casos i per plasmidis tipus MOB_{F11} en els dos casos restants. El fet que la PBRT no fóra capaç de detectar dos dels tres plasmidis portadors dels gens bla_{ACC-1} en la nostra col·lecció i que una d'aquestes relaxases MOB_{F11} fóra una nova relaxasa, suggereix que els gens bla_{ACC-1} podrien estar mobilitzats per nous plasmidis no contemplats per la tècnica del PBRT. A dia d'avui la informació existent sobre els plasmidis portadors de bla_{ACC-1} és escassa. En l'únic estudi on es va localitzar el gen bla_{ACC-1} en un plasmidi, aquest no es va poder caracteritzar per PBRT (76). Per

tant, aquest és el primer estudi on els plasmidis portadors de *bla*$_{ACC-1}$ s'han pogut caracteritzar.

Els resultats que inicialment s'esperaven de la caracterització dels plasmidis portadors dels nous gens *bla*$_{CMY-25,-27 \text{ i } -40}$ descrits en aquest estudi fou pensar que serien similars als plasmidis portadors dels gens *bla*$_{CMY-2}$, d'igual manera que s'ha descrit per a altres variants de *bla*$_{CMY-2}$ (59, 121). A diferència del que s'esperava, sols un gen *bla*$_{CMY-27}$ va ser mobilitzat per un plasmidi MOB$_{P12}$ (IncIα) identificat per la tipificació de la regió *mob* que codifica per a la relaxasa. Un dels gens de *bla*$_{CMY-4}$ i un dels gens de *bla*$_{CMY-27}$ es varen mobilitzar per plasmidis tipus IncF. Aquests tipus de plasmidis s'han vist menorment associats a gens *bla*$_{CMY-2}$ i en molts casos solen aparèixer en plasmidis amb més d'un replicó (121, 188). Pel que fa als plasmidis portadors dels gens *bla*$_{CMY-25}$ i *bla*$_{CMY-4}$ restants, no pogueren ser caracteritzats ni per la tècnica de PRBT ni per la caracterització de la regió *mob*, mentre que el gen *bla*$_{CMY-40}$ es va localitzar al cromosoma.

La tècnica del PBRT fou un mètode eficaç per a detectar la gran majoria de plasmidis portadors dels gens *amp*C (78%). No obstant, en les 25 soques on es varen estudiar totes les subfamílies de relaxases, aquesta tècnica ens va permetre observar una major diversitat plasmídica, sent capaç de detectar 20 plasmidis més que la tècnica del PBRT. Quatre d'aquests plasmidis, incloent 2 plasmidis de la subfamília MOB$_{F11}$, un MOB$_{P11}$ i un MOB$_{P12}$ foren portadors dels gens *amp*C.

A pesar de la bona correlació entre la tècnica de PBRT i la tipificació de la relaxasa, aquesta última tècnica no va ser capaç d'identificar 11 dels

replicons detectats per PBRT. Algunes raons podrien explicar aquest fracàs en la identificació de la regió *mob* corresponent. En primer lloc, s'han descrit molt pocs plasmidis amb més d'una relaxasa (100). En aquest sentit, cinc dels plasmidis, on la tècnica va fallar en la seva identificació, presentaven més d'un replicó. Una segona explicació és l'absència de gens *mob* en alguns plasmidis. Com que no varem obtenir transconjugants en 4 de les soques, l'absència de relaxosoma podria ser una explicació dels resultats de conjugació negatius per a aquestes soques. En dos dels casos, no es va trobar una possible explicació del perquè la tècnica de la relaxasa va fracassar en la identificació de dos plasmidis. En aquest cas potser ens trobem davant la presència de relaxases més divergents.

Sis soques portadores de pACBL de la present col·lecció mostraren múltiples plasmidis on tots ells hibridaven tant per al replicó com per al gen *amp*C en qüestió. Aquesta observació ha estat prèviament descrita per Dierikx *et al.* (76) en una soca de *Salmonella* portadora de $bla_{CTX\text{-}M\text{-}2}$. En aquest cas els gens $bla_{CTX\text{-}M\text{-}2}$ en varen localitzar en dos plasmidis de diferent mida i on ambdós plasmidis hibridaren tant per als replicons IncP com IncHI.

Quan es va aprofundir en l'estudi d'una de les soques de la col·lecció amb múltiples hibridacions i es va tornar a repetir l'anàlisi plasmídica, es va observar que el gen *amp*C es trobava localitzat en un plasmidi d'unes 70 kb en la soca salvatge, mentre que per al cas dels transconjugants s'observaren diferents patrons plasmídics on els gens *amp*C hibridaven en un o més plasmidis de diferents mides en funció del transconjugant específic. La posterior anàlisi cromosòmica va mostrar la presència d'una còpia del gen *amp*C al cromosoma bacterià tant de la soca donadora com de la resta de

transconjugants. El fet que s'obtingueren diferents patrons plasmídics procedents de múltiples transconjugants obtinguts a partir d'una mateixa soca donadora i que tots els transconjugants presentaren còpies del gen *amp*C al cromosoma, posa de manifest la complexitat i l'elevada quantitat de reorganitzacions de material genètic implicades en la disseminació d'aquests gens de resistència.

Les pACBL deriven de les betalactamases cromosòmiques de diferents membres de bacteris gramnegatius de la família *Enterobacteriaceae* i *Aeromonadaceae*, que el algun moment es varen transferir a plasmidis via elements mòbils com seqüències d'inserció i transposons entre d'altres (206). En aquest sentit alguns autors senyalen a l'IS*Ecp1* com el primer element mòbil responsable de la transferència del gen *amp*C cromosòmic de *C. freundii* a un plasmidi (261). Molts treballs on s'analitzen les regions adjacents al gen *bla*$_{CMY-2}$ indiquen que aquest gen es troba en un entorn genètic molt conservat (54, 68, 107, 114, 120, 184, 241), suggerint que la regió *amp*C-*blc*-*sug*E-*ecn*R del cromosoma de *C. freundii* es va mobilitzar tan sols un cop, sent l'element IS*Ecp1* el responsable de la mobilització de *bla*$_{CMY-2}$ a diferents tipus de plasmidis, independentment del seu replicó o de la seva estructura. El nostre estudi és consistent amb aquests treballs, ja que la totalitat de les soques portadores de *bla*$_{CMY-2,\ -4,\ -25,\ -27\ i\ -40}$ presentaren la mateixa estructura: IS*Ecp1*/ΔIS*Ecp1*-*amp*C-*blc*-*sug*E.

L'entorn genètic de *bla*$_{ACC-1}$ també fou molt conservat, sent possiblement també l'element IS*Ecp1* el relacionat en la transferència de la regió *amp*C-*gdha* del cromosoma d' *H. alvei* a un plasmidi. No obstant, encara que la seqüència d'inserció IS*26* s'ha relacionat amb la transmissió dels enzims ACC-1 i

normalment apareix interrompent IS*Ecp1* (83, 204), cap dels aïllats d'aquesta col·lecció presentà aquest element mòbil. L'organització genètica dels gens *bla*$_{DHA-1}$ fou més variada. La mobilització dels gens *bla*$_{DHA-1}$ s'ha associat principalment a integrons de classe 1 associats a IS*26* o IS*CR1* (253, 258, 260, 271). Es varen diferenciar tres estructures molt similars en les soques portadores de DHA-1, però l'element IS*CR1* no va estar present en cap d'elles. Les estructures I i II foren idèntiques a l'entorn genètic de *bla*$_{DHA-1}$ descrit prèviament en els plasmidis pTN60013 (260) i pT948 (271) respectivament. L'element IS*26* i els gens *qacEΔ1* i *sul1*, aquests dos últims pertanyents a l'extrem conservat 3'CS d'un integró de classe 1, estigueren presents en ambdues estructures. En l'únic aïllat mostrant l'estructura III, sols es varen trobar els gens *qacEΔ1* i *sul1*.

Encara que les pACBL s'han descrit principalment vehiculades per plasmidis, també s'han trobat pACBL de localització cromosòmica en algunes soques de *P. mirabilis* (38, 85, 156, 175) i d'*E. coli* (188). Recentment s'ha descrit per primer cop la mobilització de *bla*$_{CMY-2}$ per un ICE de la família SXT/R391 en una soca de *P. mirabilis* al Japó (116). En aquest sentit, set de les vuit soques de *P. mirabilis* d'aquesta col·lecció varen ser mobilitzades per aquest tipus d'elements (Annex V). L'organització genètica del gen *bla*$_{CMY-2}$ en aquestes soques va ser pràcticament idèntica a la mostrada per l'ICE*Pmi*Jap1, on el transposó Tn*10* podria ser el responsable de la mobilització de *bla*$_{CMY-2}$ a l'interior de l'ICE (116). El fet que un ICE de la família SXT/R391 sigui el responsable de la mobilització dels gens *bla*$_{CMY-2}$ en el 37% (7/19) de les soques de *P. mirabilis* portadores de CMY-2 en la nostra col·lecció i que aquests elements s'hagin descrit en dos parts del món tant distants mobilitzant *bla*$_{CMY-2}$

ens fa creure que no és un incident aïllat i que aquests elements juguen un paper molt important en la disseminació d'aquests gens de resistència. A més a més, aquestes set soques foren aïllades en els tres darrers anys de l'estudi, suggerint una tendència creixent en la mobilització de *bla*$_{CMY-2}$ per aquest vector.

De la mateixa manera que ocorre amb les soques portadores de BLEA, els plasmidis que vehiculen la resistència a pACBL solen contenir altres gens que confereixen resistència a altres famílies d'antibiòtics (7, 32, 129, 206, 288). La presència de determinants addicionals de resistència són una causa més de preocupació sobre el tractament apropiat de les soques productores d'AmpC tant en humans com animals. En aquest sentit, la majoria dels aïllats de l'estudi presentaren un patró de multiresistència, sent resistents a quinolones, tetraciclines, sulfonamides, cloranfenicol i diferents aminoglucòsids. No obstant, la transferència d'aquests gens de resistència va ser menor i diferencial en funció del tipus de pACBL implicada. A pesar que el 77% de les soques donadores productores de CMY-2 eren resistents a l'àcid nalidíxic, cap dels transconjugants va mostrar resistència a aquest antibiòtic. Els determinants de resistència antibiòtica transferits amb major freqüència foren les sulfonamides, les tetraciclines i el cloranfenicol. Aquests resultats van en concordança amb altres descrits anteriorment (109, 121, 172, 261), suggerint que aquestes resistències es localitzen pròximes a *bla*$_{CMY-2}$ en un entorn altament conservat, sent vehiculats per la mateixa estructura.

En canvi, la resistència a antibiòtics no betalactàmics majoritàriament transferida en els transconjugants portadors de DHA-1 fou a l'àcid nalidíxic, mostrant-se sensibilitat disminuïda a aquest antibiòtic en el 62% dels casos.

Des de fa uns anys s'ha constatat una clara associació entre els gens que codifiquen per a DHA-1 i els gens *qnr*, especialment *qnr*B4, trobant-se aquesta associació pràcticament a la totalitat de soques portadores de DHA-1 (78, 200, 203, 240, 246). Els resultats de la present tesi varen demostrar la co-localització dels gens de resistència *bla*$_{DHA-1}$ i *qnr*B en el mateix plasmidi conjugatiu en tots els aïllats analitzats (Annex VI). A excepció d'un cas, tots els plasmidis varen pertànyer a plasmidis tipus IncL/M d'ampli rang d'hostatger, mentre que *bla*$_{DHA-1}$ s'ha trobat tradicionalment en plasmis IncFII de curt rang d'hostatger (52, 109). La co-localització dels gens *bla*$_{DHA-1}$ i *qnr*B en un plasmidi IncL/M també es va detallar en l'Annex II, on es suggeria la transferència *in vivo* d'aquest plasmidi entre una soca d'*E. coli* i una de *S. marcescens*. La presència d'ambdós gens de resistència en el mateix plasmidi i el continu augment en la prevalença de gens *qnr* podria estar justificant l'augment de soques portadores de DHA-1 (203, 240, 246). La possibilitat que els gens *bla*$_{DHA-1}$ estiguen mobilitzats per un vector amb major capacitat de disseminació, com els plasmidis L/M, podria explicar la distribució generalitzada que s'ha anat observat en els darrers anys dels gens *bla*$_{DHA-1}$, arribant a ser la pACBL més prevalent en alguns països, sobretot en el continent asiàtic.

Els gens *bla*$_{DHA-1}$ i *qnr*B s'han localitzat formant part de la mateixa estructura, associats a integrons o transposons complexos (123, 136, 258). L'anàlisi detallada de l'entorn genètic d'una de les soques de l'estudi va revelar que ambdós gens es trobaven localitzats conjuntament en un transposó IS*26* compost, possiblement implicat en la seva mobilització. Altres gens trobats en aquesta estructura foren l'extrem conservat 3'CS d'un integró de classe 1 i

l'operó *psp*. Aquest operó s'indueix com a resposta a l'estrès. Sota una gran varietat de senyals ambientals, l'operó *psp* es transcriu àmpliament per a preservar la integritat cel·lular (126). Aquesta estructura genètica portadora de trets beneficiosos per al bacteri com gens de resistència antibiòtica i una àmplia resposta a factors ambientals, podria estar contribuint al manteniment d'aquest plasmidi en el bacteri, permetent així la seva difusió. L'associació de *bla*$_{DHA-1}$ i *qnr*B en estructures similars s'ha descrit anteriorment en soques de *Klebsiella* spp., principalment *K. pneumoniae* (123, 136, 258), però aquest és el primer estudi on es detalla aquesta organització genètica en una soca d'*E. coli*.

La presència de gens *qnr* s'ha vist associada a un increment en els valors de CIM de les quinolones i a fracassos terapèutics quan aquests antibiòtics són utilitzats en el tractament d'enterobacteris amb valors de CIM dins del rang de sensibilitat per a les quinolones (170). Tenint en compte que la prevalença de plasmidis portadors de *bla*$_{DHA-1}$ i *qnr*B és tan elevada i que aquests plasmidis presenten una distribució mundial, seria recomanable evitar l'ús de quinolones per al tractament d'infeccions causades per enterobacteris productors de DHA-1.

6. CONCLUSIONS

CONCLUSIONS

1. La majoria dels laboratoris espanyols de microbiologia clínica identifiquen de forma fiable les BLEA més prevalents a Espanya entre els aïllats clínics d'*E. coli* i *K. pneumoniae*. Per contra, la seva capacitat per a detectar la producció de pACBL en aquestes dues espècies, o la hiperproducció de la betalactamasa AmpC cromosòmica en el cas d'*E. coli* és limitada.

2. Les taxes d'errors més elevades per a les soques productores de BLEA es varen observar per a les combinacions de penicil·lines amb inhibidors de betalactamases. En les soques amb fenotip AmpC, les majors taxes d'errors s'observaren per a les cefalosporines. En aquest cas, la principal tendència dels laboratoris va ser deixar com a única alternativa terapèutica dins dels betalactàmics als carbapenèmics, excloent la cefepima.

3. S'ha suggerit la transferència horitzontal *in vivo* d'un plasmidi IncL/M de 70 kb portador dels gens bla_{DHA-1} i *qnr*B entre aïllats de *S. marcescens* i *E. coli*, descrivint-se per primer cop una pACBL en una soca de *S. marcescens*.

4. L'observació de colònies situades a la proximitat dels halos d'inhibició de la cefoxitina, cefotaxima, ceftazidima i aztreonam ha sigut l'única tècnica fenotípica que ha permès sospitar la presència d'una pACBL en una soca productora d'AmpC cromosòmica natural.

5. La prevalença global d'enterobacteris productors de pACBL a l'Hospital de la Santa Creu i Sant Pau durant 1999-2007 és del 0,4%, incrementant-se significativament del 0,06% el 1999 a l'1,3% el 2007. Aquest increment s'ha produït principalment en els tres darrers anys d'estudi.

Aquesta circumstància podria ser deguda a l'emergència de soques productores de DHA-1 i a l'increment de soques de *P. mirabilis* productores de CMY-2.

6. El 6% de les soques productores de pACBL són també productores d'una BLEA, recolzant la noció del capitalisme genètic, on s'incideix amb el fet que els bacteris resistents tenen major facilitat que els sensibles per a adquirir nous mecanismes de resistència.

7. CMY-2 ha sigut la pACBL aïllada amb major freqüència en aquest estudi, seguida de DHA-1. Altres pACBL aïllades amb menor freqüència són ACC-1, CMY-4, CMY-25, CMY-27 i CMY-40, sent les tres últimes pACBL descrites per primer cop.

8. La distribució d'aquests enzims varia en funció de la espècie estudiada. CMY-2 s'ha detectat majoritàriament en *E. coli*, *P. mirabilis* i *S. enterica*, mentre que DHA-1 s'ha associat principalment a *K. pneumoniae* i *K. oxytoca*.

9. S'observa una gran diversitat clonal entre les soques productores de pACBL, detectant-se tan sols petits *clusters* en soques d'*E. coli*, *K. pneumoniae*, *P. mirabilis* i *K. oxytoca*.

10. El 81% dels gens *amp*C s'han localitzat en plasmidis Inc/MOB coneguts. Aquests gens *amp*C ha sigut capaços de mobilitzar-se per conjugació en el 93% dels casos, amb freqüències d'autotransferència entre 10^{-2} i 10^{-8}. Els resultats obtinguts mostren una estreta relació entre el gen *amp*C i el plasmidi implicat, constatant a més a més la gran mobilitat dels plasmidis portadors de pACBL.

11. Els plasmidis portadors de *bla*$_{CMY-2}$ més prevalents en aquest estudi són del grup IncA/C, IncI1/Iγ i en menor proporció del grup IncK. En el 17% dels casos, els gens *bla*$_{CMY-2}$ es localitzen en plasmidis amb més d'un replicó.

12. Tots els plasmidis portadors de *bla*$_{DHA-1}$, a excepció d'un cas, pertanyen al grup IncL/M. En el 19% dels casos els gens *bla*$_{DHA-1}$ es localitzen en plasmidis amb més d'un replicó.

13. Els tres gens *bla*$_{ACC-1}$ han estat mobilitzats per plasmidis IncI1/Iγ (n=1) i MOB$_{F11}$ (n=2). La tècnica de PBRT va fracassar en la detecció de dos dels tres plasmidis analitzats i una de les relaxases MOB$_{F11}$ detectades va ser una nova relaxasa no descrita anteriorment. Aquests resultats suggereixen que els gens *bla*$_{ACC-1}$ podrien estar vehiculats per nous plasmidis no contemplats per la tècnica de PBRT. Aquest és el primer estudi on els plasmidis portadors de *bla*$_{ACC-1}$ s'han pogut caracteritzar.

14. Algunes de les variants de *bla*$_{CMY-2}$ del present estudi no s'han pogut caracteritzar per cap de les tècniques de tipificació emprades (*bla*$_{CMY-4}$ i *bla*$_{CMY-25}$), altres es troben localitzades al cromosoma (*bla*$_{CMY-40}$) o mobilitzades per plasmidis F (*bla*$_{CMY-4}$ i *bla*$_{CMY-27}$) i MOB$_{F12}$ (*bla*$_{CMY-27}$).

15. Els entorns genètics dels gens *bla*$_{CMY-2,-4,-25,-27\ i\ -40}$ i *bla*$_{ACC-1}$ han sigut molt conservats, relacionant-se en tots els casos amb l'element mòbil IS*Ecp1*.

16. S'ha observat una major variabilitat en l'entorn genètic dels gens *bla*$_{DHA-1}$, relacionant-se principalment amb els gens conservats de l'extrem 3'CS d'un integró de classe 1, *qacE*Δ*1* i *sul1*, i l'element mòbil IS*26*.

17. Els gens *bla*$_{CMY-2}$ presenten una localització cromosòmica en el 40% de les soques de *P. mirabilis*. En set soques aïllades en els tres darrers anys d'estudi, els gens *bla*$_{CMY-2}$ foren mobilitzats per un ICE de la família SXT/R391, suggerint que aquests elements juguen un paper molt important en la disseminació de *bla*$_{CMY-2}$, almenys en aquesta espècie i sobretot en els darrers anys.

18. L'entorn genètic d'aquests ICE va ser pràcticament idèntic al descrit en una soca de *P. mirabilis* aïllada al Japó. El transposó Tn*10* sembla ser el responsable de la mobilització de *bla*$_{CMY-2}$ a l'interior de l'ICE.

19. Les soques clíniques portadores de pACBL mostren un elevat grau de resistència a la majoria d'antibiòtics no betalactàmics testats. No obstant, la transferència dels determinants de resistència via conjugació és molt menor, suggerint que aquestes resistències podrien estar localitzades al cromosoma bacterià o en diferents plasmidis que no han conjugat. Els determinants de resistència transferits amb major freqüència en les soques portadores de CMY-2 han sigut les sulfonamides, les tetraciclines i el cloranfenicol. La resistència a l'àcid nalidíxic ha sigut transferida majoritàriament en les soques productores de DHA-1.

20. S'han trobat gens *qnr*B en tots els enterobacteris portadors de *bla*$_{DHA-1}$, trobant-se ambdós gens vehiculats per plasmidis d'ampli rang d'hostatger en totes les soques estudiades. Aquests resultats posen de manifest la possible co-selecció dels gens *bla*$_{DHA-1}$ amb l'ús de quinolones, justificant l'augment de les soques productores de DHA-1, sobretot en els darrers anys.

21. Aquest és el primer estudi on es descriu la co-localització dels gens *qnr*B4 i *bla*$_{DHA-1}$ a l'interior d'un transposó IS*26* compost en una soca d'*E. coli*.

CONCLUSIONS

1. Most clinical microbiology laboratories reliably identified the most prevalent ESBL among clinical isolates of *E. coli* and *K. pneumoniae* in Spain. However, their ability to detect and report pACBL-producers of the two species and *E. coli* hyperproducing AmpC was limited.

2. The highest error rates in the clinical microbiology laboratories for ESBL-producing strains occurred when reporting combinations of penicillins plus β-lactamase inhibitors. In AmpC-producing strains, the highest error rates were observed when reporting cephalosporins, mainly because the AmpC phenotype was misidentified as an ESBL phenotype. The criteria that laboratories often used in this case were to leave carbapenems as the only therapeutic alternatives within the β-lactams, and exclude cefepime.

3. The *in vivo* horizontal transfer of a 70 kb IncL/M plasmid coharbouring *bla*$_{DHA-1}$ and *qnr*B resistance genes between *S. marcescens* and *E. coli* isolates was suggested. Moreover, this is the first report of an isolate of *S. marcescens* harbouring a pACBL.

4. The observation of scattered colonies near the edge of the inhibition zones of cefoxitin, cefotaxime, ceftazidime and aztreonam has been the only phenotypic method that led us to suspect the presence of a pACBL in a chromosomal AmpC producer.

5. The overall prevalence of *Enterobacteriaceae* carrying pACBL at Hospital de la Santa Creu i Sant Pau from 1999 to 2007 was 0.4%, increasing significantly over this period from 0.06% to 1.3%. The largest increase in pACBL-

producing strains occurred mainly in the last three years of the study. This increase could have been due to the emergence of DHA-1-producing strains and the increase of CMY-2-producing *P. mirabilis*.

6. Six percent of the pACBL-producing strains were also ESBL producers, reinforcing the idea of genetic capitalism. This notion suggests that resistant bacteria have greater ability to acquire new resistance mechanisms than non-resistant bacteria.

7. In our study, CMY-2 was the most prevalent AmpC enzyme, followed by DHA-1. Less commonly found enzymes were ACC-1, CMY-4, CMY-25, CMY-27 and CMY-40. These last three mentioned enzymes are described for the first time.

8. Distribution of pACBL depends on the species studied. CMY-2 was mainly detected in *E. coli*, *P. mirabilis* and *S. enterica*, while DHA-1 is mainly associated with *K. pneumoniae* and *K. oxytoca*.

9. Great clonal diversity was observed among pACBL producing strains. Only small clusters were detected in strains of *E. coli*, *K. pneumoniae*, *P. mirabilis* and *K. oxytoca*.

10. Eighty-one percent of *amp*C genes were located on plasmids of known Inc/MOB groups. These *amp*C genes were able to mobilise by conjugation in 93% of cases, with conjugation frequencies ranging between 10^{-2} and 10^{-8}. The results show a close relationship between the *amp*C gene and the plasmid involved, and they also demonstrate the high mobility of plasmids carrying pACBL.

11. A/C, I1/Iγ and the less frequently found IncK groups were the most prevalent replicons in plasmids carrying *bla*$_{CMY-2}$. In 17% of cases, plasmids carrying these genes displayed more than one replicon.

12. All but one plasmid carrying *bla*$_{DHA-1}$ belonged to the IncL/M group. In 19% of cases, plasmids carrying these genes displayed more than one replicon.

13. The three *bla*$_{ACC-1}$ genes were carried by IncI1/Iγ (n=1) and MOB$_{F11}$ (n=2) plasmids. The PBRT failed to detect two out of three *bla*$_{ACC-1}$-carrying plasmids, and one of the MOB$_{F11}$ relaxases found was a novel relaxase that has not been previously reported. These results suggest that *bla*$_{ACC-1}$ genes could be carried by new backbones uncovered by PBRT. This is the first time that plasmids carrying *bla*$_{ACC-1}$ have been typed.

14. Some of the newly-described *bla*$_{CMY-2}$ variants could not be typed by any of the methods used (*bla*$_{CMY-4}$ and *bla*$_{CMY-25}$). Others were found on the chromosome (*bla*$_{CMY-40}$) or mobilized by F (*bla*$_{CMY-4}$ and *bla*$_{CMY-27}$) and MOB$_{F12}$ (*bla*$_{CMY-27}$) plasmids.

15. The regions surrounding *bla*$_{CMY-2,-4,-25,-27,-40}$ and *bla*$_{ACC-1}$ were highly conserved. In all cases they were associated with the IS*Ecp1* mobile element.

16. The regions surrounding *bla*$_{DHA-1}$ were more variable. They were mainly associated with the conserved genes of the 3'CS of class 1 integrons, *qacE*Δ*1* and *sul1*, and the IS*26* mobile element.

17. The *bla*$_{CMY-2}$ genes had a chromosomal location in 40% of *P. mirabilis* isolates. In seven *P. mirabilis* isolated in the last three years of study, *bla*$_{CMY-2}$ were mobilised by the SXT/R391 ICE family, suggesting that these elements play an important role in the dissemination of *bla*$_{CMY-2}$, at least in this species and especially in recent years.

18. The regions surrounding these ICE were almost identical to those described in a *P. mirabilis* strain isolated in Japan. The Tn*10* transposon seemed to be responsible for mobilising *bla*$_{CMY-2}$ inside the ICE.

19. pACBL-producing clinical isolates displayed a high level of resistance to most non-β-lactam agents tested. However, the transfer of resistance determinants via conjugation was much lower. This suggests that resistance may be located on the bacterial chromosome or present on other plasmids that have no conjugated. The resistance determinants most frequently transferred in CMY-2-producing isolates were sulfonamides, tetracyclines and chloramphenicol. The nalidixic acid determinant was transferred mostly in DHA-1-producing isolates.

20. *qnr*B genes were found in all DHA-1-producing *Enterobacteriaceae*. *bla*$_{DHA-1}$ and *qnr*B genes were both mobilised by the same broad-host-range plasmids in all the studied isolates. These results suggest the possible co-selection of the *bla*$_{DHA-1}$ genes when using quinolones, justifying the increase of DHA-1-producing isolates, especially in recent years.

21. This is the first study to describe co-localization of *qnr*B4 and *bla*$_{DHA-1}$ genes inside an IS*26* composite transposon in an *E. coli* strain.

7. BIBLIOGRAFIA

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1. **Abraham, E. i Chain, E.** 1988. An enzyme from bacteria able to destroy penicillin (1940). Rev Infect Dis **10:**677-8.

2. **Adler, H., Fenner, P., Hohler, D., Schultheiss, E., Oezcan, S. i Frei, R.** 2007. Plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes: prevalence at a Swiss university hospital and occurrence of the different molecular types in Switzerland. J Antimicrob Chemother**:**457-8.

3. **Ahmed, A., Motoi, Y., Sato, M., Maruyama, A., Watanabe, H., Fukumoto, Y. i Shimamoto, T.** 2007. Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. Appl Environ Microbiol **73:**6686-90.

4. **Ahmed, A. i Shimamoto, T.** 2008. Emergence of a cefepime- and cefpirome-resistant *Citrobacter freundii* clinical isolate harbouring a novel chromosomally encoded AmpC β-lactamase, CMY-37. Int J Antimicrob Agents **32:**256-61.

5. **Alvarado, A., Garcillán-Barcia, M. i de la Cruz, F.** 2008. A PCR-based method for classification of conjugative plasmids based on relaxase sequences. International Society for Plasmid Biology (ISPB). International Plasmid Biology Conference 2008, p. 134. Saleem A. Khan, Gdansk, Poland.

6. **Alvarez, M., Tran, J., Chow, N. i Jacoby, G.** 2004. Epidemiology of conjugative plasmid-mediated AmpC β-lactamases in the United States. Antimicrob Agents Chemother **48:**533-7.

7. **Allen, K. i Poppe, C.** 2002. Occurrence and characterization of resistance to extended-spectrum cephalosporins mediated by β-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. Can J Vet Res **66:**137-44.

8. **Ambler, R.** 1980. The structure of β-lactamases. Philos Trans R Soc Lond B Biol Sci **289:**321-31.

9. **Ambler, R., Coulson, A., Frere, J., Ghuysen, J., Joris, B., Forsman, M., Levesque, R., Tiraby, G. i Waley, S.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem J **276:**269-70.

10. **Antunes, P., Coque, T. i Peixe, L.** 2010. Emergence of an IncIγ plasmid encoding CMY-2 β-lactamase associated with the international ST19 OXA-30-producing β-lactamase *Salmonella* Typhimurium multidrug-resistant clone. J Antimicrob Chemother **65:**2097-100.

11. **Aragón, L., Mirelis, B., Miró, E., Mata, C., Gómez, L., Rivera, A., Coll, P. i Navarro, F.** 2008. Increase in β-lactam-resistant *Proteus mirabilis* strains due to CTX-M and CMY-type as well as new VEB- and inhibitor-resistant TEM-type β-lactamases. J Antimicrob Chemother **61:**1029-32.

12. **Arduino, S., Roy, P., Jacoby, G., Orman, B., Pineiro, S. i Centron, D.** 2002. $bla_{CTX-M-2}$ is located in an unusual class 1 integron (In35) which includes Orf513. Antimicrob Agents Chemother **46:**2303-6.

13. **Averhoff, B. i Friedrich, A.** 2003. Type IV pili-related natural transformation systems: DNA transport in mesophilic and thermophilic bacteria. Arch Microbiol **180:**385-93.

14. **Avison, M., Niumsup, P., Walsh, T. i Bennett, P.** 2000. *Aeromonas hydrophila* AmpH and CepH β-lactamases: derepressed expression in mutants of *Escherichia coli* lacking *cre*B. J Antimicrob Chemother **46:**695-02.

15. **Barlow, M. i Hall, B.** 2002. Origin and evolution of the AmpC β-lactamases of *Citrobacter freundii*. Antimicrob Agents Chemother **46:**1190-8.

16. **Barnaud, G., Arlet, G., Verdet, C., Gaillot, O., Lagrange, P. i Philippon, A.** 1998. *Salmonella enteritidis*: AmpC plasmid-mediated inducible β-lactamase (DHA-1) with an *amp*R gene from *Morganella morganii*. Antimicrob Agents Chemother **42:**2352-8.

17. **Barnaud, G., Labia, R., Raskine, L., Sanson-Le Pors, M., Philippon, A. i Arlet, G.** 2001. Extension of resistance to cefepime and cefpirome associated to a six amino acid deletion in the H-10 helix of the cephalosporinase of an *Enterobacter cloacae* clinical isolate. FEMS Microbiol Lett **195:**185-90.

18. **Barton, B., Harding, G. i Zuccarelli, A.** 1995. A general method for detecting and sizing large plasmids. Anal Biochem **226:**235-40.

19. **Batchelor, M., Hopkins, K., Threlfall, E., Clifton-Hadley, F., Stallwood, A., Davies, R. i Liebana, E.** 2005. Characterization of AmpC-mediated resistance in clinical *Salmonella* isolates recovered from humans during the period 1992 to 2003 in England and Wales. J Clin Microbiol **43:**2261-5.

20. **Baudry, P., Mataseje, L., Zhanel, G., Hoban, D. i Mulvey, M.** 2009. Characterization of plasmids encoding CMY-2 AmpC β-lactamases from *Escherichia coli* in Canadian intensive care units. Diagn Microbiol Infect Dis **65:**379-83.

21. **Baudry, P., Nichol, K., DeCorby, M., Mataseje, L., Mulvey, M., Hoban, D. i Zhanel, G.** 2008. Comparison of antimicrobial resistance profiles among extended-spectrum-β-lactamase-producing and acquired AmpC β-lactamase-producing *Escherichia coli* isolates from Canadian intensive care units. Antimicrob Agents Chemother **52:**1846-9.

22. **Bauernfeind, A., Chong, Y. i Schweighart, S.** 1989. Extended broad spectrum β-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection **17:**316-21.

23. **Bauernfeind, A., Schneider, I., Jungwirth, R., Sahly, H. i Ullmann, U.** 1999. A novel type of AmpC β-lactamase ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrob Agents Chemother **43:**1924-31.

24. **Bauernfeind, A., Stemplinger, I., Jungwirth, R. i Giamarellou, H.** 1996. Characterization of the plasmidic β-lactamase CMY-2, which is responsible for cephamycin resistance. Antimicrob Agents Chemother **40:**221-4.

25. **Bauernfeind, A., Stemplinger, I., Jungwirth, R., Wilhelm, R. i Chong, Y.** 1996. Comparative characterization of the cephamycinase bla_{CMY-1} gene and its relationship with other β-lactamase genes. Antimicrob Agents Chemother **40:**1926-30.

26. **Bauernfeind, A., Wagner, S., Jungwirth, R., Schneider, I. i Meyer, D.** 1997. A novel class C β-lactamase (FOX-2) in *Escherichia coli* conferring resistance to cephamycins. Antimicrob Agents Chemother **41:**2041-6.

27. **Ben Slama, K., Jouini, A., Ben Sallem, R., Somalo, S., Sáenz, Y., Estepa, V., Boudabous, A. i Torres, C.** 2010. Prevalence of broad-spectrum cephalosporin-resistant *Escherichia coli* isolates in food samples in Tunisia, and characterization of integrons and antimicrobial resistance mechanisms implicated. Int J Food Microbiol **137:**281-6.

28. **Bennett, P. i Chopra, I.** 1993. Molecular basis of β-lactamase induction in bacteria. Antimicrob Agents Chemother **37:**153-8.

29. **Bergstrom, C., Lipsitch, M. i Levin, B.** 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics **155:**1505-19.

30. **Bergstrom, S., Olsson, O. i Normark, S.** 1982. Common evolutionary origin of chromosomal β-lactamase genes in enterobacteria. J Bacteriol **150:**528-34.

31. **Beveridge, T.** 1999. Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol **181:**4725-33.

32. **Blanc, V., Mesa, R., Saco, M., Lavilla, S., Prats, G., Miró, E., Navarro, F., Cortés, P. i Llagostera, M.** 2006. ESBL- and plasmidic class C β-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. Vet Microbiol **118:**299-04.

33. **Bobrowski, M., Matthew, M., Barth, P., Datta, N., Grinter, N., Jacob, A., Kontomichalou, P., Dale, J. i Smith, J.** 1976. Plasmid-determined β-lactamase indistinguishable from the chromosomal β-lactamase of *Escherichia coli*. J Bacteriol **125:**149-57.

34. **Bou, G., Oliver, A., Ojeda, M., Monzón, C. i Martínez-Beltran, J.** 2000. Molecular characterization of FOX-4, a new AmpC-type plasmid-mediated β-lactamase from an *Escherichia coli* strain isolated in Spain. Antimicrob Agents Chemother **44:**2549-53.

35. **Bradford, P., Urban, C., Mariano, N., Projan, S., Rahal, J. i Bush, K.** 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. Antimicrob Agents Chemother **41:**563-9.

36. **Brendler, T., Reaves, L. i Austin, S.** 2004. Interplay between plasmid partition and postsegregational killing systems. J Bacteriol **186:**2504-7.

37. **Brenner, D.** 1984. *Enterobacteriaceae*. A: N. Krieg i J. Holt (ed.). Bergey's Manual of Systematic Bacteriology. Primera edició. Williams and Wilkins Co, Baltimore. **1:**408-420.

38. **Bret, L., Chanal-Claris, C., Sirot, D., Chaibi, E., Labia, R. i Sirot, J.** 1998. Chromosomally encoded AmpC-type β-lactamase in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother **42:**1110-4.

39. **Briñas, L., Lantero, M., de Diego, I., Álvarez, M., Zarazaga, M. i Torres, C.** 2005. Mechanisms of resistance to expanded-spectrum cephalosporins in *Escherichia coli* isolates recovered in a Spanish hospital. J Antimicrob Chemother **56:**1107-10.

40. **Briñas, L., Zaragoza, M., Sáenz, Y., Ruiz-Larrea, F. i Torres, C.** 2002. β-Lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob Agents Chemother **46:**3156-63.

41. **Burrus, V., Marrero, J. i Waldor, M.** 2006. The current ICE age: Biology and evolution of SXT-related integrating conjugative elements. Plasmid **55:**173-83.

42. **Burrus, V., Pavlovic, G., Decaris, B. i Guedon, G.** 2002. Conjugative transposons: the tip of the iceberg. Mol Microbiol **46:**601-10.

43. **Bush, K.** 1989. Characterization of β-lactamases. Antimicrob Agents Chemother **33:**259-63.

44. **Bush, K. i Jacoby, G.** 2010. Updated functional classification of β-lactamases. Antimicrob Agents Chemother **54:**969-76.

45. **Bush, K., Jacoby, G. i Medeiros, A.** 1995. A functional classification scheme for β–lactamases and its correlation with molecular structure. Antimicrob Agents Chemother **39:**1211-33.

46. **Campos, J., Pérez-Vázquez, M. i Oteo, J.** 2010. Las estrategias internacionales y las campañas para promover el uso prudente de los antibióticos en los profesionales y los usuarios. Enferm Infecc Microbiol Clin **28 (Supl 4):**50-4.

47. **Cantón, R. i Coque, M.** 2006. The CTX-M β-lactamase pandemic. Curr Opin Microbiol **9:**466-75.

48. **Cantón, R., Loza, E., Conejo, M., Baquero, F. i Martínez-Martínez, L.** 2003. Quality control for β-lactam susceptibility testing with a well-defined collection of *Enterobacteriaceae* and *Pseudomonas aeruginosa* strains in Spain. J Clin Microbiol **41:**1912-8.

49. **Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L., Baquero, F. i Coque, T.** 2008. Prevalence and spread of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in Europe. Clin Microb Infect **14 (Suppl. 1):**144-53.

50. **Cantón, R., Valverde, A., Novais, A., Baquero, F. i Coque, T.** 2007. Evolución y panorama actual de las BLEE. Enferm Infecc Microbiol Clin **25 (Supl. 2):**2-10.

51. **Cao, V., Arlet, G., Ericsson, B., Tammelin, A., Courvalin, P. i Lambert, T.** 2000. Emergence of imipenem resistance in *Klebsiella pneumoniae* owing to combination of plasmid-mediated CMY-4 and permeability alteration. J Antimicrob Chemother **46:**895-00.

52. **Carattoli, A.** 2009. Resistance plasmid families in *Enterobacteriaceae*. Antimicrob Agents Chemother **53:**2227-38.

53. **Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. i Threlfall, E.** 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods **63:**219-28.

54. **Carattoli, A., Tosini, F., Giles, W., Rupp, M., Hinrichs, S., Angulo, F., Barrett, T. i Fey, P.** 2002. Characterization of plasmids carrying CMY-2 from expanded-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrob Agents Chemother **46:**1269-72.

55. **Clewell, D.** 1993. Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*. A: Clewell, D. (ed). Bacterial Conjugation. New York: Plenum Press, pp.349-67.

56. **Clinical and Laboratory Standards Institute (CLSI).** 2007. Performance standards for antimicrobial susceptibility testing. 17th informational supplement. M100-S17. CLSI: Wayne, PA, USA.

57. **Coetzee, J., Datta, N. i Hedges, R.** 1972. R factors from *Proteus rettgeri*. J Gen Microbiol **72:**543-52.

58. **Cohen, S.** 1976. Transposable genetic elements and plasmid evolution. Nature **263:**731-8.

59. **Colinon, C., Miriagou, V., Carattoli, A., Luzzaro, F. i Rossolini, G.** 2007. Characterization of the IncA/C plasmid pCC416 encoding VIM-4 and CMY-4 β-lactamases. J Antimicrob Chemother **60:**258-62.

60. **Collis, C. i Hall, R.** 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob Agents Chemother **39:**155-62.

61. **Corvec, S., Cremet, L., Leprince, C., Dauvergne, S., Reynaud, A., Lepelletier, D. i Caroff, N.** 2010. Epidemiology of *Escherichia coli* clinical isolates producing AmpC plasmidic β-lactamase during a 5-year period in a French teaching Hospital. Diagn Microbiol Infect Dis **67:**277-81.

62. **Coudron, P., Hanson, N. i Climo, M.** 2003. Occurrence of extended-spectrum and AmpC β-lactamases in bloodstream isolates of *Klebsiella pneumoniae*: isolates harbor plasmid-mediated FOX-5 and ACT-1 AmpC β-lactamases. J Clin Microbiol **41:**772-7.

63. **Coudron, P., Moland i Thomson, K.** 2000. Occurrence and Detection of AmpC β-lactamases among *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Isolates at a Veterans Medical Center. J Clin Microbiol **38:**1791-6.

64. **Couturier, M., Bex, F., Bergquist, P. i Maas, W.** 1988. Identification and classification of bacterial plasmids. Microbiol Rev **52:**375-95.

65. **Craig, N.** 1997. Target site selection in transposition. Annu Rev Biochem **66:**437-74.

66. **Chahboune, A., Decaffmeyer, M., Brasseur, R. i Joris, B.** 2005. Membrane topology of the *Escherichia coli* AmpG permease required for recycling of cell wall anhydromuropeptides and AmpC β-lactamase induction. Antimicrob Agents Chemother **49:**1145-9.

67. **Chen, Y., Cheng, J., Wang, Q., Ye, Y., Li, J. i Zhang, X.** 2009. ACT-3, a novel plasmid-encoded class C β-lactamase in a *Klebsiella pneumoniae* isolate from China. Int J Antimicrob Agents **33:**95-6.

68. **Chiu, C., Su, L., Chu, C., Chia, J., Wu, T., Lin, T., Lee, Y. i Ou, J.** 2004. Isolation of *Salmonella enterica* serotype choleraesuis resistant to ceftriaxone and ciprofloxacin. Lancet **363:**1285-6.

69. **D'Andrea, M., Nucleo, E., Luzzaro, F., Giani, T., Migliavacca, R., Vailati, F., Kroumova, V., Pagani, L. i Rossolini, G.** 2006. CMY-16, a novel acquired AmpC-type β-Lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from Northern Italy. Antimicrob Agents Chemother **50:**618-24.

70. **Damjanova, I., Tóth, Á., Kardos, G., Szántai, P., Orosi, P. i Böröcz, K.** 2009. **P1218**. First detection of plasmid-mediated AmpC-type ACC-4 β-lactamase from *Proteus mirabilis* in Hungary. 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Helsinki (Finland).

71. **Datta, N. i Hedges, R.** 1971. Compatibility groups among fi - R factors. Nature **234:**222-3.

72. **Datta, N. i Kontomichalou, P.** 1965. Penicillinase synthesis controlled by infectious R factors in *Enterobacteriaceae*. Nature **208:**239-41.

73. **Decré, D., Verdet, C., Raskine, L., Blanchard, H., Bughoffer, B., Philippon, A., Sanson-Le-Pors, M., Petit, J. i Arlet, G.** 2002. Characterization of CMY-type β-lactamases in clinical strains of *Proteus mirabilis* and *Klebsiella pneumoniae* isolated infour hospitals in the Paris area. J Antimicrob Chemother **50:**681-8.

74. **Del Solar, G., Giraldo, R., Ruiz-Echevarría, J., Espinosa, M. i Díaz-Orejas, R.** 1998. Replication and control of circular bacterial plasmids. Microbiol Mol Biol Rev **62:**434-64.

75. **Deshpande, L., Jones, R., Fritsche, T. i Sader, H.** 2006. Occurrence of plasmidic AmpC type β-lactamase-mediated resistance in *Escherichia coli*: report from the SENTRY Antimicrobial Surveillance Program (North America, 2004). Int J Antimicrob Agents **28:**578-81.

76. **Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H. i Mevius, D.** 2010. Increased detection of extended spectrum β-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet Microbiol **145:**273-8.

77. **Diestra, K., Juan, C., Curiao, T., Moyà, B., Miró, E., Oteo, J., Coque, T., Pérez-Vázquez, M., Campos, J., Cantón, R., Oliver, A., Navarro, F. i Red Española de Investigación en Patología Infecciosa.** 2009. Characterization of plasmids encoding bla_{ESBL} and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. J Antimicrob Chemother **63:**60-6.

78. **Diestra, K., Miró, E., Martí, C., Navarro, D., Cuquet, J., Coll, P. i Navarro, F.** 2010. Multiclonal epidemic of *Klebsiella pneumoniae* isolates producing DHA-1 in a Spanish hospital. Clin Microbiol Infect. Doi: 10.1111/j.1469-0691.2010.03319.x.

79. **Ding, H., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., Wang, A., Deng, Q., Zhang, H., Wang, C., Liu, L., Xu, X., Wang, L. i Shen, X.** 2008. The prevalence of plasmid-mediated AmpC β-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from five children's hospitals in China. Eur J Clin Microbiol Infect Dis **27:**915-21.

80. **Doi, Y. i Paterson, D.** 2007. Detection of plasmid-mediated class C β-lactamases. Int J Infect Dis **11:**191-7.

81. **Doi, Y., Paterson, D., Adams-Haduch, J., Sidjabat, H., O'Keefe, A., Endimiani, A. i Bonomo, R.** 2009. Reduced susceptibility to cefepime among *Escherichia coli* clinical isolates producing novel variants of CMY-2 β-lactamase. Antimicrob Agents Chemother **53:**3159-61.

82. **Doi, Y., Shibata, N., Shibayama, K., Kamachi, K., Kurokawa, H., Yokoyama, K., Yagi, T. i Arakawa, Y.** 2002. Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother **46:**2427-34.

83. **Doloy, A., Verdet, C., Gautier, V., Decré, D., Ronco, E., Hammami, A., Philippon, A. i Arlet, G.** 2006. Genetic environment of acquired bla_{ACC-1} β-lactamase gene in *Enterobacteriaceae* isolates. Antimicrob Agents Chemother **50:**4177-81.

84. **Dunne, E., Fey, P., Kludt, P., Reporter, R., Mostashari, F., Shillam, P., Wicklund, J., Miller, C., Holland, B., Stamey, K., Barrett, T., Rasheed, J., Tenover, F., Ribot, E. i Angulo, F.** 2000. Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC β-lactamase. JAMA **284:**3151-6.

85. **Empel, J., Baraniak, A., Literacka, E., Mrówka, A., Fiett, J., Sadowy, E., Hryniewicz, W., Gniadkowski, M. i Beta-PL Study Group.** 2008. Molecular survey of β-lactamases conferring resistance to newer β-lactams in *Enterobacteriaceae* isolates from Polish hospitals. Antimicrob Agents Chemother **52:**2449-54.

86. **Empel, J., Hrabak, J., Kozinska, A., Bergerova, T., Urbaskova, P., Kern-Zdanowicz, I. i Gniadkowski, M.** 2010. DHA-1-Producing *Klebsiella pneumoniae* in a Teaching Hospital in the Czech Republic. Microb Drug Resist **16:**291-5.

87. **Ferreira, S., Paradela, A., Velez, J., Ramalheira, E., Walsh, T. i Mendo, S.** 2009. **P1217**. Prevalence of *amp*C β-lactamase genes in *Enterobacteriaceae* clinical isolates from Aveiro, Portugal. 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Helsinki (Finland).

88. **Fleming, A.** 1929. On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. B J Experiment Pathol **10:**226-36.

89. **Fluit, A. i Schmitz, F.** 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur J Clin Microbiol Infect Dis **18:**761-70.

90. **Fortineau, N., Poirel, L. i Nordmann, P.** 2001. Plasmid-mediated and inducible cephalosporinase DHA-2 from *Klebsiella pneumoniae*. J Antimicrob Chemother **47:**207-10.

91. **Fosse, T., Giraud-Morin, C., Madinier, I. i Labia, R.** 2003. Sequence analysis and biochemical characterisation of chromosomal CAV-1 (*Aeromonas caviae*), the parental cephalosporinase of plasmid-mediated AmpC 'FOX' cluster. FEMS Microbiol Lett **222:**93-8.

92. **Francia, M., Varsaki, A., Garcillán-Barcia, M., Latorre, A., Drainas, C. i De la Cruz, F.** 2004. A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol Rev **28:**79-100.

93. **Frost, L., Leplae, R., Summers, A. i Toussaint, A.** 2005. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol **3:**722-32.

94. **Gaillot, O., Clement, C., Simonet, M. i Philippon, A.** 1997. Novel transferable β-lactam resistance with cephalosporinase characteristics in *Salmonella enteritidis*. J Antimicrob Chemother **39:**85-7.

95. **Galani, I., Souli, M., Koratzanis, E., Chryssouli, Z. i Giamarellou, H.** 2006. Molecular characterization of an *Escherichia coli* clinical isolate that produces both metallo-β-lactamase VIM-2 and extended-spectrum β-lactamase GES-7: identification of the In8 integron carrying the bla_{VIM-2} gene. J Antimicrob Chemother **58:**432-3.

96. **Galleni, M., Lamotte-Brasseur, J., Rossolini, G., Spencer, J., Dideberg, O. i Frere, J.** 2001. Standard numbering scheme for class B β-lactamases. Antimicrob Agents Chemother **45:**660-3.

97. **Garau, G., García-Sáez, I., Bebrone, C., Anne, C., Mercuri, P., Galleni, M., Frere, J. i Dideberg, O.** 2004. Update of the standard numbering scheme for class B β-lactamases. Antimicrob Agents Chemother **48:**2347-9.

98. **García-Fernández, A., Fortini, D., Veldman, K., Mevius, D. i Carattoli, A.** 2009. Characterization of plasmids harbouring *qnr*S1, *qnr*B2 and *qnr*B19 genes in *Salmonella*. J Antimicrob Chemother **63:**274-81.

99. **García, A., Rebollo, M. i Navarro, F.** 2004. Difusió de la resistència als antimicrobians. Diferents camins amb un mateix final. A: J. Ruiz (ed.). Antimicrobians. Societat Catalana de Biologia. Barcelona. **55:**29-48.

100. **Garcillán-Barcia, M., Francia, M. i De la Cruz, F.** 2009. The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol Rev **33:**657-87.

101. **Garriss, G., Waldor, M. i Burrus, V.** 2009. Mobile antibiotic resistance encoding elements promote their own diversity. PLoS Genet **5:**e1000775.

102. **Gazouli, M., Tzouvelekis, L., Prinarakis, E., Miriagou, V. i Tzelepi, E.** 1996. Transferable cefoxitin resistance in enterobacteria from Greek hospitals and characterization of a plasmid-mediated group 1 β-lactamase (LAT-2). Antimicrob Agents Chemother **40:**1736–40.

103. **Gazouli, M., Tzouvelekis, L., Vatopoulos, C. i Tzelepi, E.** 1998. Transferable class C β-lactamases in *Escherichia coli* strains isolated in Greek hospitals and characterization of two enzyme variants (LAT-3 and LAT-4) closely related to *Citrobacter freundii* AmpC β-lactamase. J Antimicrob Chemother **42:**419-25.

104. **Georgopapadakou, N.** 1993. Penicillin-binding proteins and bacterial resistance to β-lactams. Antimicrob Agents Chemother **37:**2045-53.

105. **Gerdes, K., Moller-Jensen, J. i Jensen, R.** 2000. Plasmid and chromosome partitioning: surprises from phylogeny. Mol Microbiol **37:**455-66.

106. **Giakkoupi, P., Tambic-Andrasevic, A., Vourli, S., Skrlin, J., Sestan-Crnek, S., Tzouvelekis, L. i Vatopoulos, A.** 2006. Transferable DHA-1 cephalosporinase in *Escherichia coli*. Int J Antimicrob Agents **27:**77-80.

107. **Giles, W., Benson, A., Olson, M., Hutkins, R., Whichard, J., Winokur, P. i Fey, P.** 2004. DNA sequence analysis of regions surrounding bla_{CMY-2} from multiple *Salmonella* plasmid backbones. Antimicrob Agents Chemother **48:**2845-52.

108. **Girlich, D., Naas, T., Bellais, S., Poirel, L., Karim, A. i Nordmann, P.** 2000. Biochemical-genetic characterization and regulation of expression of an ACC-1-like chromosome-borne cephalosporinase from *Hafnia alvei*. Antimicrob Agents Chemother **44:**1470-8.

109. **González-Sanz, R., Herrera-León, S., de la Fuente, M., Arroyo, M. i Echeita, M.** 2009. Emergence of extended-spectrum β-lactamases and AmpC-type β-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. J Antimicrob Chemother **64:**1181-6.

110. **González Leiza, M., Pérez-Díaz, J., Ayala, J., Casellas, J., Martínez-Beltrán, J., Bush, K. i Baquero, F.** 1994. Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated beta-lactamase with two molecular variants. Antimicrob Agents Chemother **38:**2150-7.

111. **Gray, J., Hungerford, L., Fedorka-Cray, P. i Headrick, M.** 2004. Extended-spectrum-cephalosporin resistance in *Salmonella enterica* isolates of animal origin. Antimicrob Agents Chemother **48:**3179-81.

112. **Grindley, N., Whiteson, K. i Rice, P.** 2006. Mechanisms of site-specific recombination. Annu Rev Biochem **75:**567-605.

113. **Haeggman, S., Lofdahl, S., Paauw, A., Verhoef, J. i Brisse, S.** 2004. Diversity and evolution of the class A chromosomal β-lactamase gene in *Klebsiella pneumoniae*. Antimicrob Agents Chemother **48:**2400-8.

114. **Haldorsen, B., Aasnaes, B., Dahl, K., Hanssen, A., Simonsen, G., Walsh, T., Sundsfjord, A. i Lundblad, E.** 2008. The AmpC phenotype in Norwegian clinical isolates of *Escherichia coli* is associated with an acquired ISEcp1-like *amp*C element or hyperproduction of the endogenous *amp*C. J Antimicrob Chemother **62:**694-702.

115. **Hammad, A., Ishida, Y. i Shimamoto, T.** 2009. Prevalence and molecular characterization of ampicillin-resistant *Enterobacteriaceae* isolated from traditional Egyptian Domiati cheese. J Food Prot **72:**624-30.

116. **Harada, S., Ishii, Y., Saga, T., Tateda, K. i Yamaguchi, K.** 2010. Chromosomally encoded bla_{CMY-2} located on a novel SXT/R391-related Integrating Conjugative Element in a *Proteus mirabilis* clinical isolate. Antimicrob Agents Chemother **54:**3545-50.

117. **Hayes, F.** 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science **301:**1496-9.

118. **Hochhut, B. i Waldor, M.** 1999. Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. Mol Microbiol **32:**99-110.

119. **Hodge, W., Ciak, J. i Tramont, E.** 1978. Simple method for detection of penicillinase-producing *Neisseria gonorrhoeae*. J Clin Microbiol **7:**102-3.

120. **Hopkins, K., Batchelor, M., Liebana, E., Deheer-Graham, A. i Threlfall, E.** 2006. Characterisation of CTX-M and AmpC genes in human isolates of *Escherichia coli* identified between 1995 and 2003 in England and Wales. Int J Antimicrob Agents **28:**180-92.

121. **Hopkins, K., Liebana, E., Villa, L., Batchelor, M., Threlfall, E. i Carattoli, A.** 2006. Replicon typing of plasmids carrying CTX-M or CMY β-lactamases circulating among *Salmonella* and *Escherichia coli* isolates. Antimicrob Agents Chemother **50:**3203-6.

122. **Horii, T., Arakawa, Y., Ohta, M., Ichiyama, S., Wacharotayankun, R. i Kato, N.** 1993. Plasmid-mediated AmpC-type β-lactamase isolated from *Klebsiella pneumoniae* confers resistance to broad-spectrum β-lactams, including moxalactam. Antimicrob Agents Chemother **37:**984-90.

123. **Hu, F., Xu, X., Zhu, D. i Wang, M.** 2008. Coexistence of *qnr*B4 and *qnr*S1 in a clinical strain of *Klebsiella pneumoniae*. Acta Pharmacol Sin **29:**320-4.

124. **Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R. i Philippon, A.** 2002. β-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother **46:**3045-9.

125. **Huovinen, P., Huovinen, S. i Jacoby, G.** 1988. Sequence of PSE-2 β-lactamase. Antimicrob Agents Chemother **32:**134-6.

126. **Huvet, M., Toni, T., Sheng, X., Thorne, T., Jovanovic, G., Engl, C., Buck, M., Pinney, J. i Stumpf, M.** 2010. The evolution of the Phage shock protein (Psp) response system: interplay between protein function, genomic organization and system function. Mol Biol Evol **28:**1141-55.

127. **Iabadene, H., Messai, Y., Ammari, H., Alouache, S., Verdet, C., Bakour, R. i Arlet, G.** 2009. Prevalence of plasmid-mediated AmpC β-lactamases among *Enterobacteriaceae* in Algiers hospitals. Int J Antimicrob Agents **34:**340-2.

128. **Jacobs, C., Joris, B., Jamin, M., Klarsov, K., Van Beeumen, J., Mengin-Lecreulx, D., van Heijenoort, J., Park, J., Normark, S. i Frere, J.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. Mol Microbiol **15:**553-9.

129. **Jacoby, G.** 2009. AmpC β-Lactamases. Clin Microbiol Rev **22:**161-82.

130. **Jacoby, G.** 2006. β-lactamase nomenclature. Antimicrob Agents Chemother **50:**1123-9.

131. **Jacoby, G. i Tran, J.** 1999. Sequence of the MIR-1 β-lactamase gene. Antimicrob Agents Chemother **43:**1759-60.

132. **Janda, J. i Abbot, S.** 2006. The enterobacteria. 2nd edition. Janda, J i Abbot S. (ed.). ASM press. Whashington DC. USA.

133. **Jaurin, B. i Grundstrom, T.** 1981. AmpC cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β-lactamases of the penicillinase type. Proc Natl Acad Sci USA **78:**4897-01.

134. **Jaurin, B., Grundstrom, T. i Normark, S.** 1982. Sequence elements determining *amp*C promoter strength in *E. coli*. EMBO J **1:**875-81.

135. **Jenks, P., Hu, Y., Danel, F., Mehtar, S. i Livermore, D.** 1995. Plasmid-mediated production of class I (AmpC) β-lactamase by two *Klebsiella pneumoniae* isolates from the UK. J Antimicrob Chemother **35:**235-6.

136. **Jiang, Y., Yu, D., Wei, Z., Shen, P., Zhou, Z. i Yu, Y.** 2010. Complete nucleotide sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKP048, carrying bla_{KPC-2}, bla_{DHA-1}, *qnr*B4, and *arm*A. Antimicrob Agents Chemother **54:**3967-9.

137. **Jorgensen, R., Nielsen, J., Friis-Moller, A., Fjeldsoe-Nielsen, H. i Schonning, K.** 2010. Prevalence and molecular characterization of clinical isolates of *Escherichia coli* expressing an AmpC phenotype. J Antimicrob Chemother **65:**460-4.

138. **Kahan, J., Kahan, F., Goegelman, R., Currie, S., Jackson, M., Stapley, E., Miller, T., Miller, A., Hendlin, D., Mochales, S., Hernández, S., Woodruff, H. i Birnbaum, J.** 1979. Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J Antibiot (Tokyo) **32:**1-12.

139. **Kang, M., Besser, T. i Call, D.** 2006. Variability in the region downstream of the bla_{CMY-2} β-lactamase gene in *Escherichia coli* and *Salmonella enterica* plasmids. Antimicrob Agents Chemother **50:**1590-3.

140. **Kelly, J., Dideberg, O., Charlier, P., Wery, J., Libert, M., Moews, P.C., Knox, J., Duez, C., Fraipont, C., Joris, B., Dusart, J., Frère, J. i Ghuysen, J.** 1986. On the origin of bacterial resistance to penicillin: comparison of a β-lactamase and a penicillin target. Science **231:**1429-31.

141. **Kim, J., Kang, H. i Lee, Y.** 2008. The identification of CTX-M-14, TEM-52, and CMY-1 enzymes in *Escherichia coli* isolated from the Han River in Korea. J Microbiol **46:**478-81.

142. **Kirby, W.** 1944. Extraction of a highly potent penicillin inactivator from penicillin resistant Staphylococci. Science **99:**452-3.

143. **Knudsen, E., Rolinson, G. i Sutherland, R.** 1967. Carbenicillin: a new semisynthetic penicillin active against *Pseudomonas pyocyanea*. Br Med J **3:**75-8.

144. **Koh, T., Sng, L., Wang, G., Hsu, L., Lin, R. i Tee, N.** 2007. Emerging problems with plasmid-mediated DHA and CMY AmpC β-lactamases in *Enterobacteriaceae* in Singapore. Int J Antimicrob Agents **30:**278-80.

145. **Ktari, S., Arlet, G., Verdet, C., Jaoua, S., Kachrid, A., Ben Redjeb, S., Mahjoubi-Rhimi, F. i Hammami, A.** 2009. Molecular epidemiology and genetic environment of acquired bla_{ACC-1} in *Salmonella enterica* serotype Livingstone causing a large nosocomial outbreak in Tunisia. Microb Drug Resist **15:**279-86.

146. **Lederberg, J. i Tatum, E.** 1953. Sex in bacteria; genetic studies, 1945-1952. Science **118:**169-75.

147. **Lee, K., Hong, S., Park, Y., Lee, H., Song, W., Jeong, J., Yong, D. i Chong, Y.** 2005. Evaluation of phenotypic screening methods for detecting plasmid-mediated AmpC beta-lactamases-producing isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Diagn Microbiol Infect Dis **53:**319-23.

148. **Lee, K., Lee, M., Shin, J., Lee, M., Kang, S., Park, A., Yong, D. i Chong, Y.** 2006. Prevalence of plasmid-mediated AmpC β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Korea. Microb Drug Resist **12:**44-9.

149. **Lee, S., Jeong, S. i Park, Y.** 2003. Characterization of bla_{CMY-10} a novel, plasmid-encoded AmpC-type β-lactamase gene in a clinical isolate of *Enterobacter aerogenes*. J Appl Microbiol **95:**744-52.

150. **Lee, S., Kim, J., Lee, G., Cheon, S., An, Y., Jeong, S. i Lee, K.** 2002. Characterization of bla_{CMY-11}, an AmpC-type plasmid-mediated β-lactamase gene in a Korean clinical isolate of *Escherichia coli*. J Antimicrob Chemother **49:**269-73.

151. **Leverstein-van Hall, M., Blok, H., Donders, A., Paauw, A., Fluit, A. i Verhoef, J.** 2003. Multidrug resistance among *Enterobacteriaceae* is strongly associated with the presence of integrons and is independent of species or isolate origin. J Infect Dis **187:**251-9.

152. **Levy, S. i Marshall, B.** 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med **10:**S122-9.

153. **Li, X. i Nikaido, H.** 2009. Efflux-mediated drug resistance in bacteria: an update. Drugs **69:**1555-623.

154. **Li, Y., Li, Q., Du, Y., Jiang, X., Tang, J., Wang, J., Li, G.L. i Hang, Y.** 2008. Prevalence of plasmid-mediated AmpC β-lactamases in a chinese university hospital from 2003 to 2005: First report of CMY-2-type AmpC β-lactamase resistance in China. J Clin Microbiol **46:**1317-21.

155. **Lin, C., Hsu, S., Chen, C., Huang, J. i Lo, H.** 2010. Genotypic detection and molecular epidemiology of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a regional hospital in central Taiwan. J Med Microbiol **59:**665-71.

156. **Literacka, E., Empel, J., Bariniak, A., Sadowy, E., Hryniewicz, W. i Gniadkowski, M.** 2004. Four variants of the *Citrobacter freundii* AmpC-type cephalosporinases, including novel enzymes CMY-14 and CMY-15, in a *Proteus mirabilis* clone widespread in Poland. Antimicrob Agents Chemother **48:**4136-43.

157. **Liu, S., Hessel, A. i Sanderson, K.** 1993. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. Proc Natl Acad Sci USA **15:**6874-8.

158. **Livermore, D.** 1995. β-lactamases in laboratory and clinical resistance. Clin Microbiol Rev **8:** 557-84.

159. **Livermore, D., Winstanley, T. i Shannon, K.** 2001. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. J Antimicrob Chemother **48 Suppl 1:**87-102.

160. **Luzzaro, F., Brigante, G., D'Andrea, M., Pini, B., Giani, T., Mantengoli, E., Rossolini, G. i Toniolo, A.** 2009. Spread of multidrug-resistant *Proteus mirabilis* isolates producing an AmpC-type β-lactamase: epidemiology and clinical management. Int J Antimicrob Agents **33:**328-33.

161. **Luzzaro, F., Gesu, G., Endimiani, A., Ortisi, G., Malandrin, S., Pagani, L., Rossolini, G. i AMCLI.** 2006. Performance in detection and reporting β-lactam resistance phenotypes in *Enterobacteriaceae*: a nationwide proficiency study in Italian laboratories. Diagn Microbiol Infect Dis **55:**311-8.

162. **Llosa, M., Gomis-Rüth, F., Coll, M. i De la Cruz, F.** 2002. Bacterial conjugation: a two-step mechanism for DNA transport. Mol Microbiol **45:**1-8.

163. **Mahillon, J. i Chandler, M.** 1998. Insertion sequences. Microbiol Mol Biol Rev **62:**725-74.

164. **Mammeri, H., Eb, F., Berkani, A. i Nordmann, P.** 2008. Molecular characterization of AmpC-producing *Escherichia coli* clinical isolates recovered in a French hospital. J Antimicrob Chemother **61:**498-03.

165. **Mammeri, H., Poirel, L., Bemer, P., Drugeon, H. i Nordmann, P.** 2004. Resistance to cefepime and cefpirome due to a 4-amino-acid deletion in the chromosome-encoded AmpC β-lactamase of a *Serratia marcescens* clinical isolate. Antimicrob Agents Chemother **48:**716-20.

166. **Mammeri, H., Poirel, L. i Nordmann, P.** 2007. Extension of the hydrolysis spectrum of AmpC β-lactamase of *Escherichia coli* due to amino acid insertion in the H-10 helix. J Antimicrob Chemother **60:**490-4.

167. **Manchanda, V. i Sing, N.** 2002. Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. J Antimicrob Chemother **51:**415-8.

168. **Marchese, A., Arlet, G., Schito, G., Lagrange, P. i Philippon, A.** 1998. Characterization of FOX-3, an AmpC-type plasmid-mediated β-lactamase from an Italian isolate of *Klebsiella oxytoca*. Antimicrob Agents Chemother **42:**464-7.

169. **Marín, M. i Gudiol, F.** 2003. Antibióticos betalactámicos. Enferm Infecc Microbiol Clin **21:**42-3.

170. **Martínez-Martínez, L. i Calvo, J.** 2010. El problema creciente de la resistencia antibiotica en bacilos gramnegativos: situacion actual. Enferm Infecc Microbiol Clin **28 Suppl 2:**25-31.

171. **Massova, I. i Mobashery, S.** 1998. Kinship and diversification of bacterial penicillin-binding proteins and β-lactamases. Antimicrob Agents Chemother **42:**1-17.

172. **Mataseje, L., Neumann, N., Crago, B., Baudry, P., Zhanel, G., Louie, M. i Mulvey, M.** 2009. Characterization of cefoxitin-resistant *Escherichia coli* isolated from recreational beaches and private drinking water in Canada between 2004 and 2006. Antimicrob Agents Chemother **53:**3126-30.

173. **Mataseje, L., Xiao, J., Kost, S., Ng, L., Dore, K. i Mulvey, M.** 2009. Characterization of Canadian cefoxitin-resistant non-typhoidal *Salmonella* isolates, 2005-06. J Antimicrob Chemother **64:**723-30.

174. **Messai, Y., Benhassine, T., Naim, M., Paul, G. i Bakour, R.** 2006. Prevalence of β-lactams resistance among *Escherichia coli* clinical isolates from a hospital in Algiers. Rev Esp Quimioter **19:**144-51.

175. **Migliavacca, R., Nucleo, E., D'Andrea, M., Spalla, M., Giani, T. i Pagani, L.** 2007. Acquired AmpC type β-lactamases: an emerging problem in Italian long-term care and rehabilitation facilities. New Microbiol **30:**295-8.

176. **Mirelis, B., Rivera, A., Miró, E., Mesa, R., Navarro, F. i Coll, P.** 2006. A simple phenotypic method for differentiation between acquired and chromosomal AmpC β-lactamases in *Escherichia coli*. Enferm Infecc Microbiol Clin **24:**370-2.

177. **Miriagou, V., Tzouvelekis, L., Villa, L., Lebessi, E., Vatopoulos, C., Carattoli, A. i Tzelepi, E.** 2004. CMY-13, a novel inducible cephalosporinase encoded by an *Escherichia coli* plasmid. Antimicrob Agents Chemother **48:**3172-4.

178. **Miró, E., Agüero, J., Bartolomé, R., Fernández, A., Conejo, C., Bou, G., González, J.J., Lara, N., Martínez-Martínez, L., Mirelis, B., Oliver, A., Oteo, J., Pascual, A., Rodríguez-Baño, J., Zamorano, L. i Navarro, F.** 2009. **P706**. Estudio de la prevalencia de β-lactamasas AmpC plasmídicas y carbapenemasas en enterobacterias en España. XIV Congreso Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC), Barcelona.

179. **Miró, E., Rivera, A. i Mesa, R.** 2004. Els betalactàmics. A: J. Ruiz (ed.). Antimicrobians. Treballs de la Societat Catalana de Biologia. Barcelona. **55:**91-106.

180. **Mocktar, C., Govindena, U., Sturmb, A. i Essacka, S.** 2008. CMY-20, a novel AmpC-type β-lactamase from South African clinical *Escherichia coli* isolates. Diagn Microbiol Infect Dis **60:**405-8.

181. **Moland, E., Black, J., Ourada, J., Reisbig, M., Hanson, N. i Thomson, K.** 2002. Occurrence of newer β-lactamases in *Klebsiella pneumoniae* isolates from 24 U.S. hospitals. Antimicrob Agents Chemother **46:**3837-42.

182. **Moland, E., Hanson, N., Black, J., Hossain, A., Song, W. i Thomson, K.** 2006. Prevalence of newer β-lactamases in gram-negative clinical isolates collected in the United States from 2001 to 2002. J Clin Microbiol **44:**3318-24.

183. **Montagnani, F., Arena, F., Giani, T., Zanchi, A., D'Andrea, M., Cresti, S., Zanelli, G., Cellesi, C. i Rossolini, G.** 2009. **P1219.** A complex outbreak of multiresistant *Enterobacteriaceae* producing the acquired AmpC-type β-lactamase FOX-7 in a neonatal intensive care unit setting. 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Helsinki (Finland).

184. **Mulvey, M., Bryce, E., Boyd, D., Ofner-Agostini, M., Land, A., Simor, A., Paton, S., Canadian Hospital Epidemiology Committee, Canadian Nosocomial Infection Surveillance Program i Health Canada.** 2005. Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. Antimicrob Agents Chemother **49:**358-65.

185. **Muratani, T., Kobayashi, T. i Matsumoto, T.** 2006. Emergence and prevalence of β-lactamase-producing *Klebsiella pneumoniae* resistant to cephems in Japan. Int J Antimicrob Agents **27:**491-9.

186. **Murphy, C., Reid-Smith, R., Boerlin, P., Weese, J., Prescott, J., Janecko, N., Hassard, L. i McEwen, S.** 2010. *Escherichia coli* and selected veterinary and zoonotic pathogens isolated from environmental sites in companion animal veterinary hospitals in southern Ontario. Can Vet J **51:**963-72.

187. **Nakano, R., Okamoto, R., Nakano, Y., Kaneko, K., Okitsu, N., Hosaka, Y. i Inoue, M.** 2004. CFE-1, a novel plasmid-encoded AmpC β-lactamase with an *amp*R gene originating from *Citrobacter freundii.* Antimicrob Agents Chemother **48:**1151-8.

188. **Naseer, U., Haldorsen, B., Simonsen, G. i Sundsfjord, A.** 2009. Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. Clin Microbiol Infect **16:**171-8.

189. **Nasim, K., Elsayed, S., Pitout, J., Conly, J., Church, D. i Gregson, D.** 2004. New Method for Laboratory Detection of AmpC β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. J Clin Microbiol **42:**4799-02.

190. **Navarro, F., Miró, E. i Mirelis, B.** 2002. Lectura interpretada del antibiograma de enterobacterias. Enferm Infecc Microbiol Clin **20:**225-34.

191. **Navarro, F., Pérez-Trallero, E., Marimón, J., Aliaga, R. i Gomariz, M.** 2001. CMY-2-producing *Salmonella enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis* and *Escherichia coli* strains isolated in Spain (October 1999-December 2000). J Antimicrob Chemother **48:**383-9.

192. **Newton, G. i Abraham, E.** 1955. Cephalosporin C, a new antibiotic containing sulphur and D-α-aminoadipic acid. Nature **175:**548.

193. **Nikaido, H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev **67:**593-656.

194. **Nikaido, H.** 2009. Multidrug resistance in bacteria. Annu Rev Biochem **78:**119-46.

195. **Novick, R.** 1963. Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. J Gen Microbiol **33:**121-36.

196. **Olson, M.** 1989. Separation of large DNA molecules by pulsed-field gel electrophoresis. A review of the basic phenomenology. J Chromatogr **470:**377-83.

197. **Oteo, J., Cercenado, E., Cuevas, O., Bautista, V., Delgado-Iribarren, A., Orden, B., Pérez-Vázquez, M., García-Cobos, S. i Campos, J.** 2010. AmpC β-lactamases in *Escherichia coli*: emergence of CMY-2-producing virulent phylogroup D isolates belonging mainly to STs 57, 115, 354, 393, and 420, and phylogroup B2 isolates belonging to the international clone O25b-ST131. Diagn Microbiol Infect Dis **67:**270-6.

198. **Ouellette, M., Bissonnette, L. i Roy, P.** 1987. Precise insertion of antibiotic resistance determinants into Tn21-like transposons: nucleotide sequence of the OXA-1 β-lactamase gene. Proc Natl Acad Sci USA **84:**7378-82.

199. **Pai, H., Kang, C., Byeon, J., Lee, K., Park, W., Kim, H., Kim, E., Oh, M. i Choe, K.** 2004. Epidemiology and clinical features of bloodstream infections caused by AmpC-type-β-lactamase-producing *Klebsiella pneumoniae*. Antimicrob Agents Chemother **48:**3720-8.

200. **Pai, H., Seo, M. i Choi, T.** 2007. Association of *qnr*B determinants and production of extended-spectrum beta-lactamases or plasmid-mediated AmpC β-lactamases in clinical isolates of *Klebsiella pneumoniae*. Antimicrob Agents Chemother **51:**366-8.

201. **Papagiannitsis, C., Tzouvelekis, L., Tzelepi, E. i Miriagou, V.** 2007. Plasmid-encoded ACC-4, an extended-spectrum cephalosporinase variant from *Escherichia coli*. Antimicrob Agents Chemother **51:**3763-7.

202. **Papanicolaou, G., Medeiros, A. i Jacoby, G.** 1990. Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and α-methoxy β-lactams in clinical isolates of *Kebsiella pneumoniae*. Antimicrob Agents Chemother **34:**2200-9.

203. **Park, Y., Yu, J., Lee, S., Oh, E. i Woo, G.** 2007. Prevalence and diversity of *qnr* alleles in AmpC-producing *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Serratia marcescens*: a multicentre study from Korea. J Antimicrob Chemother **60:**868-71.

204. **Partridge, S.** 2007. Genetic environment of IS*Ecp1* and bla_{ACC-1}. Antimicrob Agents Chemother **51:**2658-9.

205. **Pérez-Pérez, F. i Hanson, N.** 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol **40:**2153-62.

206. **Philippon, A., Arlet, G. i Jacoby, G.** 2002. Plasmid-Determined AmpC-type β–lactamases. Antimicrob Agents Chemother **46:**1-11.

207. **Pitout, J., Gregson, D., Church, D. i Laupland, K.** 2007. Population-based laboratory surveillance for AmpC β-lactamase-producing *Escherichia coli*, Calgary. Emerg Infect Dis **13:**443-8.

208. **Poirel, L., Kampfer, P. i Nordmann, P.** 2002. Chromosome-encoded Ambler class A β-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases. Antimicrob Agents Chemother **46:**4038-40.

209. **Pope, C., Carter, P. i Heffernan, H.** 2009. CMY-29 and CMY-30, two novel plasmid-mediated AmpC β-lactamases. Antimicrob Agents Chemother **53:**3178.

210. **Popham, D. i Young, K.** 2003. Role of penicillin-binding proteins in bacterial cell morphogenesis. Curr Opin Microbiol **6:**594-9.

211. **Prats, G.** 2006. Microbiología Clínica, Editorial Médica Panamericana. Madrid.

212. **Prats, G. i Mirelis, B.** 1992. Enterobacteriaceae. A: E. Perea (ed.). Enfermedades Infecciosas y Microbiología Clínica. Doyma, Barcelona. pp 624-46.

213. **Price, K. i McGregor, D.** 1984. Basic design of β-lactam antibiotics-cephalosporins. Scand J Infect Dis Suppl **42:**50-63.

214. **Qin, X., Zerr, D., Weissman, S., Englund, J., Denno, D., Klein, E., Tarr, P., Kwong, J., Stapp, J., Tulloch, L. i Galanakis, E.** 2008. Prevalence and mechanisms of broad-spectrum β-lactam resistance in *Enterobacteriaceae*: a Children's Hospital Experience. Antimicrob Agents Chemother **52:**3909-14.

215. **Queenan, A., Jenkins, S. i Bush, K.** 2001. Cloning and biochemical characterization of FOX-5, an AmpC-type plasmid-encoded β-lactamase from a New York City *Klebsiella pneumoniae* clinical isolate. Antimicrob Agents Chemother **45:**3189-94.

216. **Raskine, L., Borrel, I., Barnaud, G., Boyer, S., Hanau-Bercot, B., Gravisse, J., Labia, R., Arlet, G. i Sanson-Le-Pors, M.** 2002. Novel plasmid-encoded class C β-lactamase (MOX-2) in *Klebsiella pneumoniae* from Greece. Antimicrob Agents Chemother **46:**2262-5.

217. **Reading, C. i Cole, M.** 1977. Clavulanic acid: a β-lactamase-inhibiting β-lactam from *Streptomyces clavuligerus*. Antimicrob Agents Chemother **11:**852-7.

218. **Reisbig, M. i Hanson, N.** 2004. Promoter sequences necessary for high-level expression of the plasmid-associated *amp*C β-lactamase gene $bla_{\text{MIR-1}}$. Antimicrob Agents Chemother **48:**4177-82.

219. **Reisbig, M., Hossain, A. i Hanson, N.** 2003. Factors influencing gene expression and resistance for Gram-negative organisms expressing plasmid-encoded *amp*C genes of *Enterobacter* origin. J Antimicrob Chemother **51:**1141-51.

220. **Richmond, M. i Sykes, R.** 1973. The betalactamases of gram negative bacteria and their possible physiological role. Adv Microb Physiol **9:**31-88.

221. **Robberts, F., Kohner, P. i Patel, R.** 2009. Unreliable extended-spectrum β-lactamase detection in the presence of plasmid-mediated AmpC in *Escherichia coli* clinical isolates. J Clin Microbiol **47:**358-61.

222. **Robicsek, A., Strahilevitz, J., Sahm, D., Jacoby, G. i Hooper, D.** 2006. *qnr* Prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob Agents Chemother **50:**2872-4.

223. **Roche, C., Boo, T., Walsh, F. i Crowley, B.** 2008. Detection and molecular characterisation of plasmidic AmpC β-lactamases in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Dublin, Ireland. Clin Microbiol Infect **14:**616-8.

224. **Rodríguez, I., Barownick, W., Helmuth, R., Mendoza, M., Rodicio, M., Schroeter, A. i Guerra, B.** 2009. Extended-spectrum β-lactamases and AmpC β-lactamases in ceftiofur-resistant *Salmonella enterica* isolates from food and livestock obtained in Germany during 2003-07. J Antimicrob Chemother **64:**301-9.

225. **Rolinson, G.** 1986. β-lactam antibiotics. J Antimicrob Chemother **17:**5-36.

226. **Rolinson, G. i Stevens, S.** 1961. Microbiological studies on a new broad-spectrum penicilin, "Penbritin". Br Med J **2:**191-6.

227. **Rolinson, G., Stevens, S., Batchelor, F., Wood, J. i Chain, E.** 1960. Bacteriological studies on a new penicillin-BRL.1241. Lancet **2:**564-7.

228. **Rottman, M., Benzerara, Y., Hanau-Bercot, B., Bizet, C., Philippon, A. i Arlet, G.** 2002. Chromosomal *amp*C genes in *Enterobacter* species other than *Enterobacter cloacae*, and ancestral association of the ACT-1 plasmid-encoded cephalosporinase to *Enterobacter asburiae*. FEMS Microbiol Lett **210:**87-92.

229. **Rowe-Magnus, D. i Mazel, D.** 2002. The role of integrons in antibiotic resistance gene capture. Int J Med Microbiol **292:**115-25.

230. **Sabaté, M. i Prats, G.** 2002. Estructura y función de los integrones. Enferm Infecc Microbiol Clin **20:**341-5.

231. **Scott, J. i Churchward, G.** 1995. Conjugative transposition. Annu Rev Microbiol **49:**367-97.

232. **Schmitz, F., Hafner, D., Geisel, R., Fallmann, P., Kirschke, C., Verhoef, J., Köhrer, K. i Fluit, A.** 2001. Increased prevalence of class I integrons in *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species isolates over a 7-Year period in a German University Hospital. J Clin Microbiol **39:**3724-6.

233. **Schwartz, D. i Cantor, C.** 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell **37:**67-75.

234. **Shahid, M., Ensor, V. i Hawkey, P.** 2009. Emergence and dissemination of *Enterobacteriaceae* with plasmid-mediated CMY-6 and CTX-M-15 β-lactamases in community in North-India. World J Microbiol Biotechnol **25:**1439-46.

235. **Smith, M. i Thorpe, H.** 2002. Diversity in the serine recombinases. Mol Microbiol **44:**299-07.

236. **Song, W., Kim, J., Kim, H., Jeong, S., Yong, D. i Lee, K.** 2006. Emergence of *Escherichia coli* isolates producing conjugative plasmid-mediated DHA-1 β-lactamase in a Korean university hospital. J Hosp Infect **63:**459-64.

237. **Song, W., Kim, J., Kim, H., Yong, D., Jeong, S., Park, M. i Lee, K.** 2006. Increasing trend in the prevalence of plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking chromosomal *amp*C gene at a Korean university hospital from 2002 to 2004. Diagn Microbiol Infect Dis **55:**219-24.

238. **Stapley, E., Jackson, M., Hernández, S., Zimmerman, S., Currie, S., Mochales, S., Mata, J., Woodruff, H. i Hendlin, D.** 1972. Cephamycins, a new family of β-lactam antibiotics. I. Production by Actinomycetes, including *Streptomyces lactamdurans*. Antimicrob Agents Chemother **2:**122-31.

239. **Stokes, H. i Hall, R.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol Microbiol **3:**1669-83.

240. **Strahilevitz, J., Jacoby, G., Hooper, D. i Robicsek, A.** 2009. Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat. Clin Microbiol Rev **22:**664-89.

241. **Su, L., Chen, H., Chia, J., Liu, S., Chu, C., Wu, T. i Chiu, C.** 2006. Distribution of a transposon-like element carrying bla_{CMY-2} among *Salmonella* and other *Enterobacteriaceae*. J Antimicrob Chemother **57:**424-29.

242. **Suárez, A., Güttler, A., Strätz, M., Staendner, L., Timmis, K. i Guzmán, C.** 1997. Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. Gene **196:**69-74.

243. **Suárez, C. i Gudiol, F.** 2009. Antibióticos β-lactámicos. Enferm Infecc Microbiol Clin **27:**116-29.

244. **Sykes, R. i Bonner, D.** 1985. Discovery and development of the monobactams. Rev Infect Dis **7 Suppl 4:**S579-93.

245. **Sykes, R., Bonner, D., Bush, K., Georgopapadakou, N. i Wells, J.** 1981. Monobactams-monocyclic β-lactam antibiotics produced by bacteria. J Antimicrob Chemother **8 Suppl E:**1-16.

246. **Tamang, M., Seol, S., Oh, J., Kang, H., Lee, J., Lee, Y., Cho, D. i Kim, J.** 2008. Plasmid-mediated quinolone resistance determinants *qnr*A, *qnr*B, and *qnr*S among clinical isolates of *Enterobacteriaceae* in a Korean Hospital. Antimicrob Agents Chemother **52:**4159-62.

247. **Tarnberg, M., Nilsson, L. i Monstein, H.** 2009. Molecular identification of bla_{SHV}, bla_{LEN} and bla_{OKP} β-lactamase genes in *Klebsiella pneumoniae* by bi-directional sequencing of universal SP6- and T7-sequence-tagged bla_{SHV}-PCR amplicons. Mol Cell Probes **23:**195-200.

248. **Tenover, F., Arbeit, R., Goering, R., Mickelsen, P., Murray, B., Persing, D. i Swaminathan, B.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol **33:**2233-9.

249. **Tenover, F., Mohammed, M., Gorton, T. i Dembek, Z.** 1999. Detection and reporting of organisms producing extended-spectrum β-lactamases: survey of laboratories in Connecticut. J Clin Microbiol **37:**4065-70.

250. **Thomas, C. i Nielsen, K.** 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol **3:**711-21.

251. **Thomson, K. i Sanders, C.** 1992. Detection of extended-spectrum β-lactamases in members of the family *Enterobacteriaceae*: comparison of the double-disk and three-dimensional tests. Antimicrob Agents Chemother **36:**1877-82.

252. **Tipper, D. i Strominger, J.** 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci U S A **54:**1133-41.

253. **Toleman, M., Bennet, P. i Walsh, T.** 2006. ISCR elements: Novel gene-capturing systems of the 21st century? Microbiol Mol Biol Rev **70:**296-16.

254. **Tomasz, A.** 1979. The mechanism of the irreversible antimicrobial effects of penicillins: how the β-lactam antibiotics kill and lyse bacteria. Annu Rev Microbiol **33:**113-37.

255. **Tracz, D., Boyd, D., Bryden, L., Hizon, R., Giercke, S., Van Caeseele, P. i Mulvey, M.** 2005. Increase in *amp*C promoter strength due to mutations and deletion of the attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT-PCR. J Antimicrob Chemother **55:**768-72.

256. **Tzouvelekis, L., Tzelepi, E. i Mentis, A.** 1994. Nucleotide sequence of a plasmid-mediated cephalosporinase gene $bla_{\text{LAT-1}}$ found in *Klebsiella pneumoniae*. Antimicrob Agents Chemother **38:**2207-9.

257. **Vanwynsberghe, T., Verhamme, K., Raymaekers, M., Cartuyvels, R., Boel, A. i De Beenhouwer, H.** 2007. Outbreak of *Klebsiella pneumoniae* strain harbouring an AmpC (DHA-1) and a $bla_{\text{SHV-11}}$ in a Belgian hospital, August-December 2006. Euro Surveill **12:**E070201 3.

258. **Verdet, C., Arlet, G., Barnaud, G., Lagrange, P. i Philippon, A.** 2000. A novel integron in *Salmonella enterica* serovar enteritidis, carrying the $bla_{\text{DHA-1}}$ gene and its regulator gene *amp*R, originated from *Morganella morganii.* Antimicrob Agents Chemother **44:**222-25.

259. **Verdet, C., Arlet, G., Ben Redjeb, S., Ben Hassen, A., Lagrange, P. i Philippon, A.** 1998. Characterisation of CMY-4, an AmpC-type plasmid-mediated β-lactamase in a Tunisian clinical isolate of *Proteus mirabilis.* FEMS Microbiol Lett **169:**235-40.

260. **Verdet, C., Benzerara, Y., Gautier, V., Adam, O., Ould-Hocine, Z. i Arlet, G.** 2006. Emergence of DHA-1-producing *Klebsiella* spp. in the Parisian region: Genetic organization of the *ampC* and *ampR* genes originating from *Morganella morganii*. Antimicrob Agents Chemother **50:**607-17.

261. **Verdet, C., Gautier, V., Chachaty, E., Ronco, E., Hidri, N., Decre, D. i Arlet, G.** 2009. Genetic context of plasmid-carried $bla_{\text{CMY-2-like}}$ genes in *Enterobacteriaceae*. Antimicrob Agents Chemother **53:**4002-6.

262. **Versalovic, J., Koeuth, T. i Lupski, J.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res **19:**6823-31.

263. **Wachino, J., Kurokawa, H., Suzuki, S., Yamane, K., Shibata, N., Kimura, K., Ike, Y. i Arakawa, Y.** 2006. Horizontal transfer of bla_{CMY}-bearing plasmids among clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates and emergence of cefepime-hydrolyzing CMY-19. Antimicrob Agents Chemother **50:**534-41.

264. **Waldor, M., Tschape, H. i Mekalanos, J.** 1996. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J Bacteriol **178:**4157-65.

265. **Walsh, T., Hall, L., MacGowan, A. i Bennett, P.** 1995. Sequence analysis of two chromosomally mediated inducible β-lactamases from *Aeromonas sobria*, strain 163a, one a class D penicillinase, the other an AmpC cephalosporinase. J Antimicrob Chemother **36:**41-52.

266. **Walther-Rasmussen, J. i Hoiby, N.** 2002. Plasmid-borne AmpC β-lactamases. Can J Microbiol **48:**479-93.

267. **Wang, H., Roberts, A., Lyras, D., Rood, J., Wilks, M. i Mullany, P.** 2000. Characterization of the ends and target sites of the novel conjugative transposon Tn*5397* from *Clostridium difficile*: excision and circularization is mediated by the large resolvase, TndX. J Bacteriol **182:**3775-83.

268. **Wang, H., Roberts, A.P. i Mullany, P.** 2000. DNA sequence of the insertional hot spot of Tn*916* in the *Clostridium difficile* genome and discovery of a Tn*916*-like element in an environmental isolate integrated in the same hot spot. FEMS Microbiol Lett **192:**15-20.

269. **Wang, Q., Cheng, J., Chen, Y., Ye, Y., Li, J. i Zhang, X.** 2008. Characterization of a novel AmpC-type plasmid-mediated β-lactamase from an *Escherichia coli* strain isolated in China. Curr Microbiol **57:**558-63.

270. **Watanabe, T.** 1963. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. VI. High-frequency resistance transfer system in *Escherichia coli*. J Bacteriol **85:**788-94.

271. **Wei, Z., Chen, Y., Yu, Y., Lu, W. i Li, L.** 2005. Nosocomial spread of multi-resistant *Klebsiella pneumoniae* containing a plasmid encoding multiple β-lactamases. J Med Microbiol **54:**885-8.

272. **Whichard, J., Gay, K., Stevenson, J., Joyce, K., Cooper, K., Omondi, M., Medalla, F., Jacoby, G. i Barrett, T.** 2007. Human *Salmonella* and concurrent decreased susceptibility to quinolones and extended-spectrum cephalosporins. Emerg Infect Dis **13:**1681-8.

273. **Whichard, J., Joyce, K., Fey, P., Nelson, J., Angulo, F. i Barrett, T.** 2005. β-lactam resistance and *Enterobacteriaceae*, United States. Emerg Infect Dis **11:**1464-6.

274. **Woodford, N., Payne, D., Johnson, A., Weinbren, M., Perinpanayagam, R., George, R., Cookson, B. i Amyes, S.** 1990. Transferable cephalosporin resistance not inhibited by clavulanate in *Escherichia coli*. Lancet **336:**253.

275. **Woodford, N., Reddy, S., Fagan, E., Hill, R., Hopkins, K., Kaufmann, M., Kistler, J., Palepou, M., Pike, R., Ward, M., Cheesbrough, J. i Livermore, D.** 2007. Wide geographic spread of diverse acquired AmpC β-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. J Antimicrob Chemother **59:**102-5.

276. **Wozniak, R., Fouts, D., Spagnoletti, M., Colombo, M., Ceccarelli, D., Garriss, G., Dery, C., Burrus, V. i Waldor, M.** 2009. Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. PLoS Genet **5:**e1000786.

277. **Wu, L., Hung, S., Chuang, Y., Chen, H., Jones, R. i Yu, W.** 2005. Identification of a novel cephalosporinase (DHA-3) in *Klebsiella pneumoniae* isolated in Taiwan. Clin Microbiol Infect **11:**893-7.

278. **Wu, S., Dornbusch, K., Kronvall, G. i Norgren, M.** 1999. Characterization and nucleotide sequence of a *Klebsiella oxytoca* cryptic plasmid encoding a CMY-type β-lactamase: confirmation that the plasmid-mediated cephamycinase originated from the *Citrobacter freundii* AmpC β-lactamase. Antimicrob Agents Chemother **43:**1350-7.

279. **Yamasaki, K., Komatsu, M., Abe, N., Fukuda, S., Miyamoto, Y., Higuchi, T., Ono, T., Nishio, H., Sueyoshi, N., Kida, K., Satoh, K., Toyokawa, M., Nishi, I., Sakamoto, M., Akagi, M., Nakai, I., Kofuku, T., Orita, T., Wada, Y., Zikimoto, T., Kinoshita, S., Miyamoto, K., Hirai, I. i Yamamoto, Y.** 2010. Laboratory surveillance for prospective plasmid-mediated AmpC β-lactamases in the Kinki region of Japan. J Clin Microbiol **48:**3267-73.

280. **Yan, J., Hsueh, P., Lu, J., Chang, F., Shyr, J., Wan, J., Liu, Y., Chuang, Y., Yang, Y., Tsao, S., Wu, H., Wang, L., Lin, T., Wu, H., Chen, H. i Wu, J.** 2006. Extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of *Escherichia coli* and *Kebsiella pneumoniae* from seven medical centers in Taiwan. Antimicrob Agents Chemother **50:**1861-4.

281. **Yan, J., Ko, W., Chiu, C., Tsai, S., Wu, H. i Wu, J.** 2003. Emergence of ceftriaxone-resistant *Salmonella* isolates and rapid spread of plasmid-encoded CMY-2-like cephalosporinase, Taiwan. Emerg Infect Dis **9:**323-8.

282. **Yan, J., Ko, W., Wu, H., Tsai, S., Chuang, C. i Wu, J.** 2004. Complexity of *Klebsiella pneumoniae* isolates resistant to both cephamycins and extended-spectrum cephalosporins at a teaching hospital in Taiwan. J Clin Microbiol **42:**5337-40.

283. **Yan, J., Wu, S., Tsai, S., Wu, J. i Su, I.** 2000. Prevalence of SHV-12 among clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β-lactamases and identification of a novel AmpC enzyme (CMY-8) in Southern Taiwan. Antimicrob Agents Chemother **44:**1438-42.

284. **Yang, B., Qu, D., Zhang, X., Shen, J., Cui, S., Shi, Y., Xi, M., Sheng, M., Zhi, S. i Meng, J.** 2010. Prevalence and characterization of *Salmonella* serovars in retail meats of marketplace in Shaanxi, China. Int J Food Microbiol **141:**63-72.

285. **Ye, Y., Xu, X. i Li, J.** 2010. Emergence of CTX-M-3, TEM-1 and a new plasmid-mediated MOX-4 AmpC in a multiresistant *Aeromonas caviae* isolate from a patient with pneumonia. J Med Microbiol **59:**843-7.

286. **Yoo, J., Byeon, J., Yang, J., Yoo, J., Chung, G. i Lee, Y.** 2010. High prevalence of extended-spectrum β-lactamases and plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* isolated from long-term care facilities in Korea. Diagn Microbiol Infect Dis **67:**261-5.

287. **Yum, J., Kim, S., Lee, H., Yong, D., Lee, K., Cho, S. i Chong, Y.** 2005. Emergence and wide dissemination of CTX-M-type ESBLs, and CMY-2- and DHA-1-type AmpC β-lactamases in Korean respiratory isolates of *Klebsiella pneumoniae*. J Korean Med Sci **20:**961-5.

288. **Zaidi, M., León, V., Canche, C., Pérez, C., Zhao, S., Hubert, S., Abbott, J., Blickenstaff, K. i McDermott, F.** 2007. Rapid and widespread dissemination of multidrug-resistant bla_{CMY-2} *Salmonella* Typhimurium in Mexico. J Antimicrob Chemother **60:**398-01.

289. **Zhao, S., White, D., McDermott, P., Friedman, S., English, L., Ayers, S., Meng, J., Maurer, J., Holland, R. i Walker, R.** 2001. Identification and expression of cephamycinase *bla*$_{CMY}$ genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob Agents Chemother **45:**3647-50.

290. **Zinder, N. i Lederberg, J.** 1952. Genetic exchange in *Salmonella*. J Bacteriol **64:**679-99.

291. **Zioga, A., Whichard, J., Kotsakis, S., Tzouvelekis, L., Tzelepi, E. i Miriagou, V.** 2009. CMY-31 and CMY-36 cephalosporinases encoded by ColE1-like plasmids. Antimicrob Agents Chemother **53:**1256-9.

8. ANNEXOS

ANNEX I

8.1. Detection and reporting β-lactam resistance phenotypes in *Escherichia coli* and *Klebsiella pneumoniae*: a multicenter proficiency study in Spain.

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Detection and reporting β-lactam resistance phenotypes in *Escherichia coli* and *Klebsiella pneumoniae*: a multicenter proficiency study in Spain

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Abstract

The ability of 57 Spanish microbiology laboratories in detecting and reporting β-lactam resistance phenotypes in *Escherichia coli* and *Klebsiella pneumoniae* was evaluated. Laboratories received 6 well-characterized isolates expressing the most widespread extended-spectrum β-lactamases (ESBLs) in Spain (4 CTX-M type, 1 TEM type, and 1 SHV type), 3 isolates producing AmpC-type enzymes (2 plasmid mediated and 1 *E. coli* hyperproducing its chromosomal AmpC), and 3 quality control strains. Ninety-one percent of laboratories recognized all ESBL producers correctly, and therefore, low error rates were observed when testing cephalosporins and aztreonam. The highest error rates were observed with combinations of penicillin plus β-lactamase inhibitor, although more than 60% of cases were due to the interpretation made by the microbiologists. Correct recognition of all AmpC β-lactamase–producing strains occurred in only 47.4% of laboratories. These isolates were wrongly reported as ESBL producers and penicillinase hyperproducers in 7.6 % and 5.8% of cases, respectively. Detection of the AmpC-type phenotype by Spanish laboratories needs to be improved.
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Keywords: β-Lactamase; ESBL; AST; Quality control

1. Introduction

Antimicrobial susceptibility testing is one of the most important tasks in the clinical microbiology laboratory. Most laboratories use standardized methods that allow from in vitro results to determine the most suitable antimicrobial treatment. These results may be affected by methodology, interpretative criteria, and changes in bacterial resistance mechanisms. Quality assurance of antimicrobial susceptibility testing is commonly carried out using internal quality control programs to evaluate methods, reagents, and staff capabilities, with additional external proficiency testing for quality assurance of antimicrobial susceptibility testing methods. In Spain, most of the clinical microbiology laboratories are part of an official quality control program developed by the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC), which includes identification and antimicrobial susceptibility testing of 4 to 6 unknown enterobacterial pathogens every year. The application of this program at national level has increased the quality of Spanish laboratories, although it is insufficient in evaluating how well laboratories are able to routinely detect specific antimicrobial resistance phenotypes. The

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Table 1
Strains used for proficiency testing and expected susceptibility results for test drugs

Strain	Species and characteristics	MIC (mg/L) and susceptibility categories according to CLSI									
		AMC	PTZ	CRO	CTX	CAZ	FEP	AZT	IPM	MEM	ERT
CCG01	*E. coli* 33-22 producing the CTX-M-14 ESBL	16/8 (I)	2/4 (S)	>64 (R)	>64 (R)	1 (R)	8–16 (R)	8 (R)	0.125 (S)	≤0.03 (S)	≤0.03 (S)
CCG02	*E. coli* 10-7 producing the CTX-M-9 ESBL	4/2–8/4 (S)	2/4 (S)	>64 (R)	16–32 (R)	0.25 (R)	2 (R)	1 (R)	0.125 (S)	≤0.03 (S)	≤0.03 (S)
CCG03	*E. coli* 23-9 producing the CTX-M-10 ESBL	4/2–8/4 (S)	2/4 (S)	>64 (R)	32–64 (R)	1 (R)	16 (R)	4 (R)	0.125 (S)	≤0.03 (S)	≤0.03 (S)
CCG04	*E. coli* 22- 3 producing the SHV-12 ESBL	4/2–8/4 (S)	4/4 (S)	16 (R)	4–8 (R)	>64 (R)	1 (R)	>64 (R)	0.125 (S)	≤0.03 (S)	≤0.03 (S)
CCG05	*K. pneumoniae* 16-3 producing the TEM-4 ESBL	8/4 (S)	32/4 (I)	4–8 (R)	4–8 (R)	>64 (R)	0.5 (R)	>64 (R)	0.06 (S)	≤0.03 (S)	≤0.03 (S)
CCG06	*K. pneumoniae* 25-2 producing the SHV-1 + CTX-M-10 ESBL	8/4 (S)	4/4 (S)	>64 (R)	>64 (R)	4 (R)	4 (R)	16 (R)	0.125 (S)	≤0.03 (S)	0.03–0.06 (S)
CCG07	*K. pneumoniae* 1960 producing the FOX-5 plasmid AmpC-type β-lactamase	32/16 (R)	16/4 (S)	4 (S)	4 (S)	16 (I)	0.25 (S)	1 (S)	0.125 (S)	≤0.03 (S)	0.06 (S)
CCG08	*E. coli* TG31 hyperproducing its chromosomal AmpC β-lactamase	64/32 (R)	16/4 (S)	2–4 (S)	4 (S)	16 (I)	0.125 (S)	8 (S)	0.125 (S)	≤0.03 (S)	≤0.03 (S)
CCG09	*E. coli* TG22 producing the CMY-2 plasmid AmpC-type β-lactamase	32/16 (R)	8/4 (S)	32 (I)	16 (I)	32 (R)	0.5 (S)	8 (S)	0.125 (S)	≤0.03 (S)	1–2 (S)
CCG10	*E. coli* ATCC 25922 QC for susceptibility testing	4/2 (S)	2/4 (S)	0.06 (S)	0.06 (S)	0.25 (S)	≤0.03 (S)	0.125 (S)	0.06 (S)	≤0.03 (S)	≤0.03 (S)
CCG11	*E. coli* ATCC 35218 QC for susceptibility testing	4/2–8/4 (S)	1/4 (S)	≤0.03 (S)	≤0.03 (S)	0.125 (S)	≤0.03 (S)	≤0.03 (S)	0.125 (S)	≤0.03 (S)	≤0.03 (S)
CCG12	*K. pneumoniae* ATCC 700603 producing the SHV-18 ESBL	4/2–8/4 (S)	16/4 (S)	4 (R)	2–4 (R)	32 (R)	1 (R)	32 (R)	0.125 (S)	≤0.03 (S)	0.06 (S)

AMC = amoxicillin–clavulanic acid; PTZ = piperacillin–tazobactam; CRO = ceftriaxone; CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; AZT = aztreonam; IPM = imipenem; MEM = meropenem; ERT = ertapenem; I = intermediate; S = susceptible; R = resistant.

epidemiology of antimicrobial resistance mechanisms in Gram-negative rods is changing very rapidly. The worldwide spread of extended-spectrum β-lactamases (ESBLs) and the emergence of plasmid-mediated AmpC β-lactamases (pAmpCs) in members of the Enterobacteriaceae family are examples of such changes that call for a revision of the systems used to test and report susceptibility to broad-spectrum β-lactams. In 2001, a nationwide quality control study of β-lactam susceptibility testing was performed in Spain (Cantón et al., 2003), which included, according to the epidemiology of antimicrobial resistance in Spain at the time, only 1 CTX-M–producing strain and no pAmpC producers. Categoric errors in the evaluation of the CTX-M-9–producing strain occurred in 38.5% of laboratories. Since then, the epidemiology of β-lactam resistance in Enterobacteriaceae in Spain has changed. In the 1st nationwide study of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* in Spain, the most prevalent ESBLs were CTX-M-9, SHV-12, and CTX-M-14 for *E. coli* and TEM-3 and TEM-4 for *K. pneumoniae* (Hernández et al., 2005). Isolates expressing pAmpC are becoming increasingly common (Mirelis et al., 2006).

The purpose of this study was to evaluate the proficiency of Spanish laboratories in detecting the most prevalent ESBL and pAmpC phenotypes in clinical isolates of *E. coli* and *K. pneumoniae*. Moreover, susceptibility to β-lactams and interpretation of them were also evaluated.

2. Materials and methods

2.1. Bacterial strains

Twelve well-characterized strains were selected: 7 ESBL producers, which included 6 clinical isolates expressing the most commonly found ESBLs in Spain and *K. pneumoniae* ATCC 700603 as a positive quality control strain recommended by the Clinical and Laboratory Standards Institute (CLSI, 2007); 3 AmpC β-lactamase–producing strains, comprising 1 *E. coli* strain, which overexpressed its chromosomal AmpC cephalosporinase because of mutations into their promoter sequence, and 2 pAmpC-producing strains; and finally, 2 ATCC strains recommended by the CLSI as quality control strains for susceptibility testing assays (CLSI, 2007). A detailed description of the strains is reported in Table 1. Identification at species level, antimicrobial susceptibility testing, and confirmation of resistance phenotypes to β-lactams were independently evaluated by 2 reference laboratories (Departamento de Microbiología, Facultad de Medicina, Sevilla, Spain, and Servei de Microbiologia, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain). Bacterial identification was performed using API 20E strips (bioMérieux, Marcy l'Étoile, France). Antimicrobial susceptibility testing was performed by both disk diffusion and microdilution methods, according to CLSI guidelines. β-Lactamases produced by selected strains were characterized by conventional methods, which included isoelectric focusing, substrate profile determination by bioassay, and molecular characterization based on polymerase chain reaction and DNA sequencing (Miró et al., 2005).

2.2. Study design

The study was designed as a nationwide proficiency study, under the scientific auspices of the SEIMC's official quality control program. In March 2007, the 12 test strains, numbered as in Table 1, were sent to all participating laboratories as blood culture isolates. Each laboratory was requested to use routine methods for the identification and antimicrobial susceptibility testing (AST) of the strains. An electronic form was issued for reporting identification and AST results, which included both quantitative (inhibition zone diameter or MIC) and qualitative results (susceptible, intermediate, or resistant according to the breakpoints used). Laboratories were also requested to report qualitative results to clinicians in their normal manner and to include additional informative notes concerning resistance phenotypes. Information about the AST method and the criteria used to interpret AST results was also requested (Table 2).

2.3. Evaluation of results

Discrepancies between AST results and reference values were classified as follows: very major errors (VMEs), when the laboratory reported a resistant strain as susceptible (VME percentages were determined for resistant isolates only); major errors (MaEs), when the laboratory reported a susceptible strain as resistant (the percentages of MaE were determined for susceptible isolates only); or minor errors (MiEs), when the laboratory reported an intermediate strain as resistant or susceptible, or a resistant or susceptible strain as intermediate (Cantón et al., 2003).

Detection of ESBL expression was evaluated on the basis of the specific notes reported on the electronic form. For these isolates, changes in the interpretation of AST results were assessed when the laboratory provided a quantitative result, which was lower than the resistance breakpoint and reported the interpretative category as resistant.

Table 2
Information requested of participant centers

Specify the bacterial identification method used (type [manual, automated, or semiautomated], galleries, panels, or cards used) and the identification results
Specify the AST method used (disk diffusion assay, agar or broth dilution methods, type of tool used, and galleries, panels, or cards used)
AST quantitative results obtained (inhibition zone diameter in millimeters or MIC in milligrams per liter)
Qualitative results (S, I, R) according to the breakpoints used
Interpretative results (S, I, R) and criteria used (CLSI, MENSURA, EUCAST, others)
Additional test performed and results obtained
Additional informative notes included in your routine reports

AST = antimicrobial susceptibility testing; S = susceptible; I = intermediate; R = resistant.

Detection of AmpC expression was only evaluated on the basis of the specific notes reported on the electronic form.

3. Results

3.1. Participation rate and characteristics of participating laboratories

Fifty-seven clinical microbiology laboratories representing every region in Spain (including the Balearic Islands and Canary Islands) agreed to take part in this study and provided the data required. Most centers (61.4%) used automated methods for bacterial identification and AST, which included MicroScan WalkAway (*n* = 25; Dade Behring MicroScan, West Sacramento, CA), Vitek 2 (*n* = 8; bioMérieux), and Phoenix (*n* = 2; Becton Dickinson Diagnostic Systems, Sparks, MD). The remaining 38.6% used semiautomated systems, including Wider (*n* = 16; Francisco Soria Melguizo, Madrid, Spain), MicroScan AutoScan 4 (*n* = 2; Dade Behring MicroScan), or manual systems, including API 20E (*n* = 2; bioMérieux), BBL enterotube (*n* = 1; Becton Dickinson), and unspecified (*n* = 1). All laboratories using automated or semiautomated methods for identification procedures used the same ones for AST, except for 2 centers (Vitek 2) that used disk diffusion assays. The center using manual identification by BBL used disk diffusion for AST, and the 2 centers using API 20E used Vitek 2 and Sensititre (TREK Diagnostic Systems, West Sussex, UK), respectively. Every laboratory in the survey reported using the CLSI criteria to interpret AST results (CLSI, 2007), except for one (which adopted the MENSURA guidelines) (Baquero et al., 1997).

3.2. Proficiency in bacterial identification

Bacterial identification at genus and species level was correct in 99.3% and 99.1% of cases, respectively. *E. coli* CCG04 was identified as *Klebsiella ozaenae* in 2 centers and as *Klebsiella* spp. in 1 center. *K. pneumoniae* CCG06 was identified as *Enterobacter aerogenes* in 1 center, *K. pneumoniae* CCG07 as *Klebsiella oxytoca* in 1 center, and *K. pneumoniae* CCG12 (control strain) as *E. coli* in 1 center. There were 2 misidentifications where the MicroScan WalkAway was used, 2 in centers using the MicroScan AutoScan 4, and 2 using manual methods (1 with BBL enterotube and the other with API 20E).

3.3. Proficiency in AST

Because the range of antimicrobial concentrations varied depending on the method used and some centers used the disk diffusion assay, interpretative categories were used to analyze AST proficiency. The interpretative criteria were those proposed by the CLSI with supplementary comments. Because there are no specific recommendations for reporting susceptibility to several drugs in ESBL- and AmpC-producing *E. coli* and *K. pneumoniae*, and bearing in mind the potential risk of therapeutic failure of these agents in complicated infections, the criteria established in Table 3 were applied (Livermore et al., 2008; Paterson, 2006; Paterson et al., 2004).

The distributions of discrepancies by type of categoric error, by organism, and by antimicrobial agent are shown in Table 4 (for cephalosporins and aztreonam) and in Table 5 (for carbapenems and combinations of penicillins plus β-lactamase inhibitors).

In general, there were low error rates for cephalosporins and aztreonam in ESBL-producing strains, probably because a large number of centers had detected the ESBL phenotypes (see below). For cephalosporins, all but 1 VME were due to failure to detect ESBL production. In 1 case, the cefepime category was not changed to resistant, despite the fact that ESBL production had been suggested. One VME for aztreonam was due to failure to detect ESBL production and the other 2 due to the fact that the center did not change the category for this agent when ESBL production had in fact been detected.

The highest error rates in ESBL-producing strains were observed for combinations of penicillins plus β-lactamase inhibitors. Nevertheless, 69.5% of errors with amoxicillin–clavulanic acid and 64.4% with piperacillin–tazobactam were due to the interpretative criteria used by the laboratories in changing the category from susceptible or intermediate to resistant when ESBL production was detected. There were no discrepancies for carbapenems in ESBL-producing strains.

For AmpC-producing strains, most errors detected for cephalosporins, aztreonam, or piperacillin–tazobactam were, again, not due to the methodology but to the interpretative criteria applied in the laboratory, and depending on the encountered phenotype. In 13 cases, AmpC production was interpreted as ESBL production, inducing a change to the resistant category for cephalosporins, aztreonam, and even piperacillin–tazobactam (35.1%) as reported for ESBL-producing strains. In other laboratories, although the AmpC phenotype was identified, the lack of clear interpretative criteria for cephalosporins and piperacillin–tazobactam

Table 3
Supplementary comments established to interpret ESBL- and AmpC-producing *E. coli* and *K. pneumoniae*

Drug and resistance mechanism	Qualitative clinical category	Interpretative clinical category
ESBL producers		
Penicillin plus β-lactamase inhibitor combinations: AMC, PTZ	S	S/I
AmpC producers		
Penicillin plus β-lactamase inhibitors combinations: PTZ	S	S/I
Third generation cephalosporins and aztreonam: CRO, CTX, CAZ, AZT	S I	S/I I/R

AMC = amoxicillin–clavulanic acid; PTZ = piperacillin–tazobactam; CRO = ceftriaxone; CTX = cefotaxime; CAZ = ceftazidime; AZT = aztreonam.

Table 4
Distribution of interpretative discrepancies and categoric errors rates for cephalosporins and aztreonam

Strain	Species/β-lactamase	Ceftriaxone				Cefotaxime				Ceftazidime				Cefepime				Aztreonam			
		No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]		
			MiE	MaE	VME		MiE	MaE	VME		MiE	MaE	VME		MiE	MaE	VME		MiE	MaE	VME
CCG01	*E. coli*/CTX-M-14	9	0 (0.0)	NA[c]	0 (0.0)	53	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	38	0 (0.0)	NA	1 (2.6)
CCG02	*E. coli*/CTX-M-9	9	0 (0.0)	NA	0 (0.0)	52	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	1 (1.8)	57	0 (0.0)	NA	1 (1.8)	38	0 (0.0)	NA	2 (5.3)
CCG03	*E. coli*/CTX-M-10	9	0 (0.0)	NA	0 (0.0)	53	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	38	0 (0.0)	NA	0 (0.0)
CCG04	*E. coli*/SHV-12	9	0 (0.0)	NA	0 (0.0)	53	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	38	0 (0.0)	NA	0 (0.0)
CCG05	*K. pneumoniae*/TEM-4	9	0 (0.0)	NA	1 (11.1)	53	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	2 (3.5)	38	0 (0.0)	NA	0 (0.0)
CCG06	*K. pneumoniae*/CTX-M-10	8	0 (0.0)	NA	0 (0.0)	49	0 (0.0)	NA	0 (0.0)	52	0 (0.0)	NA	0 (0.0)	52	0 (0.0)	NA	0 (0.0)	35	0 (0.0)	NA	0 (0.0)
CCG12	*K. pneumoniae*/SHV-18	9	0 (0.0)	NA	0 (0.0)	53	0 (0.0)	NA	1 (1.9)	57	0 (0.0)	NA	0 (0.0)	57	0 (0.0)	NA	3 (5.3)	38	0 (0.0)	NA	0 (0.0)
Total	ESBL-positive strains	62	0 (0.0)	NA	1 (1.6)	366	0 (0.0)	NA	1 (0.3)	394	0 (0.0)	NA	1 (0.3)	394	0 (0.0)	NA	6 (1.5)	263	0 (0.0)	NA	3 (1.1)
CCG07	*K. pneumoniae*/FOX-5	9	6 (66.7)	0 (0.0)	NA	53	32 (60.4)	0 (0.0)	NA	57	4 (7.0)	NA	NA	56	2 (3.6)	9 (16.1)	NA	38	22 (57.9)	0 (0.0)	NA

(continued on next page)

Table 5
Distribution of interpretative discrepancies and categoric errors rates for carbapenems and combinations of penicillins plus β-lactamase inhibitors

Strain	Species/β-lactamase	Imipenem				Meropenem				Ertapenem				Amoxicillin–clavulanic acid				Piperacillin–tazobactam			
		No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]			No.[a]	Errors (%)[b]		
			MiE	MaE	VME		MiE	MaE	VME		MiE	MaE	VME		MiE	MaE	VME		MiE	MaE	VME
CCG01	*E. coli*/CTX-M-14	54	0 (0.0)	0 (0.0)	NA[c]	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	37 (66.1)	NA	NA	56	15 (26.8)	0 (0.0)	NA
CCG02	*E. coli*/CTX-M-9	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	19 (33.9)	0 (0.0)	NA	56	15 (26.8)	0 (0.0)	NA
CCG03	*E. coli*/CTX-M-10	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	19 (33.9)	0 (0.0)	NA	56	15 (26.8)	0 (0.0)	NA
CCG04	*E. coli*/SHV-12	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	18 (32.1)	0 (0.0)	NA	56	14 (25.0)	0 (0.0)	NA
CCG05	*K. pneumoniae*/TEM-4	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	22 (39.3)	0 (0.0)	NA	56	47 (83.9)	NA	NA
CCG06	*K. pneumoniae*/CTX-M-10	49	0 (0.0)	0 (0.0)	NA	48	0 (0.0)	0 (0.0)	NA	25	0 (0.0)	0 (0.0)	NA	51	16 (31.4)	0 (0.0)	NA	51	11 (21.6)	0 (0.0)	NA
CCG12	*K. pneumoniae*/SHV-18	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	20 (35.7)	0 (0.0)	NA	56	15 (26.8)	0 (0.0)	NA
Total	ESBL-positive strains	373	0 (0.0)	0 (0.0)	NA	366	0 (0.0)	0 (0.0)	NA	193	0 (0.0)	0 (0.0)	NA	387	151 (39.0)	0 (0.0)	NA	387	132 (34.1)	0 (0.0)	NA
CCG07	*K. pneumoniae*/FOX-5	53	0 (0.0)	0 (0.0)	NA	52	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	6 (10.7)	NA	0 (0.0)	55	20 (36.4)	0 (0.0)	NA
CCG08	*E. coli*/AmpC	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	56	9 (16.1)	NA	0 (0.0)	56	17 (30.4)	0 (0.0)	NA
CCG09	*E. coli*/CMY-2	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	1 (3.6)	1 (3.6)	NA	56	8 (14.3)	NA	2 (3.6)	56	22 (39.3)	0 (0.0)	NA
Total	AmpC-positive strains	161	0 (0.0)	0 (0.0)	NA	158	0 (0.0)	0 (0.0)	NA	84	1 (1.2)	1 (1.2)	NA	168	23 (13.7)	NA	2 (1.2)	167	59 (35.3)	0 (0.0)	NA
CCG10	*E. coli*	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	28	0 (0.0)	0 (0.0)	NA	55	0 (0.0)	0 (0.0)	NA	56	0 (0.0)	0 (0.0)	NA
CCG11	*E. coli*/TEM-1	54	0 (0.0)	0 (0.0)	NA	53	0 (0.0)	0 (0.0)	NA	29	0 (0.0)	0 (0.0)	NA	56	2 (3.6)	0 (0.0)	NA	56	1 (1.8)	0 (0.0)	NA
Total	ESBL-negative. AmpC-negative strains	108	0 (0.0)	0 (0.0)	NA	106	0 (0.0)	0 (0.0)	NA	57	0 (0.0)	0 (0.0)	NA	111	2 (1.8)	0 (0.0)	NA	112	1 (0.9)	0 (0.0)	NA

[a] Number of susceptibility testing determinations carried out for this organism–antimicrobial combination.
[b] Percentage rates were calculated considering the number of susceptibility testing determinations for the antibiotic as the denominator.
[c] Not applicable.

yielded different interpretations with a noticeable tendency to consider carbapenems and non–β-lactam antimicrobials as the only therapeutic options. In this way, most of the 28 centers that identified any of the 3 AmpC producers reported most of the cephalosporins, aztreonam, and piperacillin–tazobactam as resistant, irrespective of their MIC values. It is worth mentioning that most of these centers also corrected cefepime to the resistant (7 centers) or intermediate (1 center) category in those strains susceptible in terms of MIC.

The only 2 discrepancies for carbapenems were observed in the CMY-2–producing strain. In 1 case, an MIC value of 3 mg/L was obtained by E-test, and ertapenem was reported as intermediate. In the other case, an MIC of 4 mg/L (intermediate) was obtained using MicroScan WalkAway, although the microbiologist changed the category to resistant.

There were no significant discrepancies with the quality control strains recommended by CLSI (CCG10 and CCG11). An exception was CCG11 (control strain for combinations of penicillins plus β-lactamase inhibitors) and piperacillin–tazobactam. In 1 case, this was due to an error determining the MIC; in 2 other cases, however, it was due to an error in the interpretative criterion applied where the centers involved considered that TEM-1 production required a change in category from susceptible to resistant.

3.4. Performance in detecting ESBL producers

Twenty of 53 laboratories using automated or semiautomatic methods reported to use ESBL confirmatory tests (50% disk diffusion and 50% E-test) when an ESBL phenotype was detected. All ESBL-producing strains were identified correctly in 86% of the centers (49 of 57). Although 3 centers did not report an ESBL phenotype, they made the correct interpretation and modified the clinical category for cephalosporins and aztreonam, thereby increasing the success rate from 86% to 91.2%. Only 5 centers misidentified an ESBL-producing strain: The CTX-M-9–producing strain was misidentified in 1 center, probably the result of using Vitek 2 cards without cefotaxime or ceftriaxone, and the TEM-4–producing strain was misidentified in 2 centers; in 1 center, the error was due to a negative synergy with clavulanic acid by disk diffusion assay, the strain being interpreted as a TEM-1 hyperproducer; and the last one did not recognize the ESBL phenotype, despite the Wider system having obtained MIC values for oxyimino-cephalosporin and aztreonam in the resistant category. The same center failed to detect the ESBL phenotype in strain CCG12 (the quality control strain for the ESBL phenotype). Curiously, there were 2 other errors detecting the ESBL in this strain. In 1 case (MicroScan AutoScan 4), the microbiologist or technician did not suspect ESBL production, despite high MIC values in some cephalosporins and aztreonam. In the other case (Wider), the expert system suggested the presence of an ESBL associated with decreased permeability or the presence of an AmpC β-lactamase, although no further test was performed to differentiate them.

3.5. Performance in the detection of AmpC β-lactamase

This resistance phenotype is difficult to detect because there is no reliable routine method for detecting this kind of enzymes. The presence of pAmpC (in the FOX-5- and CMY-2–producing strains) was indicated by 50.9% and 59.9% of participants, respectively; hyperproduction of chromosomal AmpC in strain CCG08 was suggested by 52.6%, and only 47.4% of centers correctly identified the 3 AmpC-producing strains. More than 30% of centers did not specify the resistance phenotype of the strains, whereas 10% did so, but incorrectly. The most frequent error was misidentifying it as an ESBL phenotype (7.6%), followed by penicillinase hyperproduction plus permeability defect (5.8%).

In general, no remarkable differences in identification or AST were observed between the methods used by participating laboratories (data not shown).

4. Discussion

ESBL-producing Enterobacteriaceae are becoming more prevalent throughout the world. In the 1st national study developed in Spain (2002), the frequency of ESBL-producing *E. coli* and *K. pneumoniae* was 0.5% and 2.7%, respectively (Hernández et al., 2005). A 2nd national study, still under development, using the same methodology increased these values up to 4.0% and 5.0%, respectively (Cantón et al., 2007). In these studies, as has been observed in most European countries, the most prevalent ESBLs in *E. coli* belonged to the CTX-M family. A major challenge for microbiologists is the detection and reporting of ESBL production in Enterobacteriaceae. Following the CLSI criteria, ESBL producers should be reported as resistant to all penicillins, cephalosporins, and aztreonam, independently of their MIC values. Nevertheless, recent EUCAST expert rules (http://www.eucast.org) recommend reporting as intermediate any result of susceptible to all penicillins, cephalosporins, and aztreonam and to change it to resistant if it is in the intermediate range.

Furthermore, an increasing prevalence of pAmpC-producing enterobacteria has been described (Alvarez et al., 2004). These enzymes are not inactivated by β-lactamase inhibitors and confer a spectrum of activity greater than has been observed in ESBLs. Taking into account the difficulty of detecting pAmpCs, the real prevalence of these enzymes is probably underestimated in several countries (Philippon et al., 2002). Some local studies suggest that the prevalence of pAmpC-producing strains are increasing in Spain (Mirelis et al., 2006). This fact leads us to highlight the importance of developing standard recommendations to deal with AmpC producers.

Various multicenter studies have been performed to evaluate the ability of clinical microbiology laboratories to

detect specific resistance phenotypes of clinical concern. These studies are usually designed as proficiency quality control studies, in which resistant strains are distributed from a central laboratory to participating centers. In 2001, a quality control survey was carried out in Spain for β-lactam susceptibility testing with a well-defined collection of Enterobacteriaceae and *Pseudomonas aeruginosa* strains (Cantón et al., 2003). In this study, using a broad panel of strains expressing different resistance mechanisms, only 1 CTX-M–producing strain was included and no pAmpC producers. The present study was developed for 3 major reasons: a) 38.5% of laboratories taking part in the previously mentioned study failed to detect ESBL production in the only CTX-M–producing strain tested; b) the epidemiology of ESBLs has changed very rapidly in Spain, as in other countries, and those ESBL-producing *E. coli* and *K. pneumoniae*, which are the most widespread, on the basis of national studies, have been selected for inclusion in this study; and c) for the 1st time in a study of this kind in Spain, pAmpC-producing strains have been included.

With quality control strains (ATCC 29922, ATCC 700603, and ATCC 35218), laboratories exhibited a high overall performance. Very few problems were observed for ESBL producers when testing cephalosporins and aztreonam. The few VMEs detected were mainly due to a failure to identify the ESBL phenotype, except in 1 case where the cefepime category was not changed to resistant, despite the fact that the system had suggested an ESBL. The number of VME with cephalosporins in this study was much lower than that reported in the previous proficiency study in Spain (Cantón et al., 2003). In this study, cefepime was the antimicrobial tested, which had the highest error rates. Every VME arose with ESBL-producing strains. In these laboratories, cefepime was reported as susceptible, and no categoric modification was performed. In a similar study performed in Italy (Luzzaro et al., 2006), major problems were observed for ESBL producers when testing cephalosporins. Most VMEs were detected with a TEM-52–producing *Proteus mirabilis* strain, probably because, at the time of the survey, *P. mirabilis* was not included among the species to be tested for ESBL production by the CLSI. The highest error rates for ESBL-producing strains were observed with combinations of penicillins plus β-lactamase inhibitors. Nevertheless, two-thirds of errors were due to the interpretative criteria because the category was changed from susceptible or intermediate to resistant when the ESBL phenotype was detected. Although therapeutic failures have been reported when combinations of penicillin plus β-lactamase inhibitors have been used, there is some controversy about their utility in the treatment of infections caused by ESBL-producing Enterobacteriaceae (Peterson, 2008). Accordingly, specific recommendations are desirable to avoid such discrepancies in reporting the susceptibilities of these combinations.

In AmpC-producing strains, there was a high rate of MiE for cephalosporins (except cefepime). Thirteen cases were due to identifying them as ESBL-producing strains, which led to a change of category to resistant. More often though, even when the AmpC phenotype had been established, it was the absence of specific recommendations that produced a great variety of results, the most common being to leave carbapenems and non–β-lactams as the only therapeutic alternatives. In some centers, even cefepime was categorized as intermediate when the MIC value was in the susceptibility range. Luzzaro et al. (2006) described a large number of VME for piperacillin–tazobactam and *E. coli* hyperproducing AmpC, mentioning differences in intrinsic activity between amoxicillin–clavulanic acid and piperacillin–tazobactam against these strains as a possible explanation. Because the AmpC-producing strains used in our study were susceptible to piperacillin–tazobactam, these differences could not be evaluated. However, we observed no important differences in terms of errors between the 2 combinations. The only 2 discrepancies with carbapenems were observed with ertapenem and CMY-2–producing *E. coli*. In 2 centers, the MIC value was in the intermediate range and reported as resistant in one of them. Susceptibility test results for imipenem and meropenem were correct in all the participating centers. Similar results were obtained in other proficiency studies (Cantón et al., 2003; Luzzaro et al., 2006).

In conclusion, Spanish laboratories carried out the AST of the most prevalent ESBL-producing strains in Spain very efficiently, with correct recognition of the ESBL phenotype in more than 90% of the laboratories. It is now necessary to develop specific interpretative criteria for combinations of penicillins plus β-lactamase inhibitors, based on both in vitro and clinical experience. More difficulties were observed for AmpC-producing strains. A search for reliable screening methods in detecting pAmpCs and chromosomal AmpC hyperproduction is desirable. Furthermore, clinical microbiologists should be alert to the detection of such microorganisms because some local studies suggest that they may have increased in recent years (Mirelis et al., 2006).

Because the epidemiology of resistance in Enterobacteriaceae changes very rapidly, prevalence studies should be routinely accompanied by proficiency studies to evaluate the proficiency of clinical microbiology laboratories in detecting and reporting the most prevalent phenotypes of antimicrobial resistance. This information could be very useful to clinicians in choosing adequate therapeutic options and effective infection control measures.

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References

Alvarez M, Tran JH, Chow N, Jacoby GA (2004) Epidemiology of conjugative plasmid-mediated AmpC β-lactamases in the United States. *Antimicrob Agents Chemother* 48:533–537.

Baquero F, Martínez Beltrán J, Cantón R (1997) MENSURA break-points. Spanish criteria for determination of susceptibility to antibiotics. *Rev Esp Quimioter* 10:303–313.

Cantón R, Loza E, Conejo MC, Baquero F, Martínez-Martínez L (2003) Quality control for β-lactam susceptibility testing with a well-defined collection of Enterobacteriaceae and *Pseudomonas aeruginosa* strains in Spain. *J Clin Microbiol* 41:1912–1918.

Cantón R, Valverde A, Novais A, Baquero F, Coque T (2007) Evolution and current situation of ESBL. *Enferm Infecc Microbiol Clin* 25(Suppl 2): S2–S10.

Clinical and Laboratory Standards Institute (2007) Performance standards for antimicrobial susceptibility testing, document M100-S17. Seventeenth informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute.

Hernández JR, Martínez-Martínez L, Cantón R, Coque TM, Pascual A (2005) Nationwide study of *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. *Antimicrob Agents Chemother* 49:2122–2125.

Livermore DM, Hope R, Mushtaq S, Warner M (2008) Orthodox and unorthodox clavulanate combinations against extended-spectrum β-lactamase producers. *Clin Microbiol Infect* 14(Suppl 1):189–193.

Luzzaro F, Gesu G, Endimiani A, Ortisi G, Malandrin S, Pagani L, Rossolini GM (2006) Performance in detection and reporting β-lactam resistance phenotypes in Enterobacteriaceae: a nationwide proficiency study in Italian laboratories. *Diagn Microbiol Infect Dis* 55:311–318.

Mirelis B, Rivera A, Miró E, Mesa RJ, Navarro F, Coll P (2006) A simple phenotypic method for differentiation between acquired chromosomal AmpC β-lactamases in *Escherichia coli*. *Enferm Infecc Microbiol Clin* 24:370–372.

Miró E, Mirelis B, Navarro F, Rivera A, Mesa RJ, Roig MC, Gómez L, Coll P (2005) Surveillance of extended-spectrum β-lactamases from clinical samples and faecal carriers in Barcelona, Spain. *J Antimicrob Chemother* 56:1152–1155.

Paterson DL (2006) Resistance in gram-negative bacteria: Enterobacteriaceae. *Am J Med* 119(Suppl 6A):S20–S28.

Paterson DL, Ko WC, Von Gottberg A, Mohapatra S, Casellas JM, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice LB, Wagener MM, McCormack JG, Yu VL (2004) Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of extended-spectrum β-lactamases. *Clin Infect Dis* 39:31–39.

Peterson LR (2008) Antibiotic policy and prescribing strategies for therapy of extended spectrum β-lactamase–producing Enterobacteriaceae: the role of piperacillin–tazobactam. *Clin Microbiol Infect* 14(Suppl 1): 181–184.

Philippon A, Arlet G, Jacoby GA (2002) Plasmid-determined AmpC-type β-lactamases. *Antimicrob Agents Chemother* 46:1–11.

ANNEX II

8.2. *In vivo* transmission of a plasmid coharbouring *bla*$_{DHA-1}$ and *qnr*B genes between *Escherichia coli* and *Serratia marcescens*.

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RESEARCH LETTER

In vivo transmission of a plasmid coharbouring bla_{DHA-1} and *qnr*B genes between *Escherichia coli* and *Serratia marcescens*

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plasmid-mediated β-lactamases; plasmid-mediated quinolone resistance; incompatibility groups; relaxases; *Enterobacteriaceae*.

Abstract

We report a *Serratia marcescens* and an *Escherichia coli* isolate simultaneously detected in the same patient. Both isolates showed susceptibility patterns suggestive of harbouring a plasmid-mediated AmpC β-lactamase (pACBL) and a plasmid-encoded quinolone resistance (PMQR). PCR-based replicon, MOB typing, plasmid profile and Southern hybridization analyses revealed that both isolates coharboured bla_{DHA-1} and *qnr*B genes on the same IncL/M-MOB_{P13} plasmid approximately 70 kb in size. Together with the fact that both plasmids were conjugative in the laboratory, these results strongly suggest that a horizontal transfer event could take place *in vivo*. This is the first report of an isolate of *S. marcescens* harbouring a pACBL. The only phenotypic method that suggests the presence of a pACBL in an isolate harbouring an inducible chromosomal AmpC enzyme is the observation of scattered colonies near the edge of the inhibition zones of some β-lactams. The presence of both resistance genes on the same plasmid and the reported increase in PMQR could perhaps explain the widespread distribution of bla_{DHA-1} genes.

Introduction

Serratia marcescens is an opportunistic pathogen that is mainly involved in nosocomial infections and especially affects immune-suppressed patients. It sometimes shows high-level resistance to β-lactam antibiotics. This phenomenon occurs mainly in two ways: by derepression of its natural chromosomally encoded AmpC β-lactamase or by acquisition of new genes (Naumiuk *et al.*, 2004). The plasmid-mediated acquisition of β-lactamases such as extended-spectrum β-lactamases (TEM, SHV and CTX-M type) or carbapenemases (KPC, GES, IMP and VIM type) is well known (Naumiuk *et al.*, 2004; Walther-Rasmussen & Hoiby, 2007; Pitout, 2008). Although plasmid-mediated AmpC β-lactamases (pACBLs) have been reported in other *Enterobacteriaceae* (Pérez-Pérez & Hanson, 2002; Mirelis *et al.*, 2006; Park *et al.*, 2007; Pitout, 2008; Tamang *et al.*, 2008; Carattoli, 2009; Strahilevitz *et al.*, 2009; Mata *et al.*, 2010), to our knowledge, pACBLs have not been reported in *S. marcescens*. pACBLs confer resistance to all β-lactams, including cephamycins, except cefepime and carbapenems, and they are not inhibited by commercialized β-lactamase inhibitors. Acquired *amp*C genes derive from the chromosomal *amp*C genes of several bacterial species and are traditionally classified into six groups (CIT, DHA, ACC, EBC, FOX and MOX) (Pérez-Pérez & Hanson, 2002; Mirelis *et al.*, 2006; Mata *et al.*, 2010). Plasmids carrying these genes often carry multiple other resistances.

Several reports have recently described cotransmission between bla_{DHA-1} and *qnr* genes. *qnr* genes are plasmid-mediated and confer low resistance to quinolones. There are four major families of *qnr* determinants: *qnr*A, *qnr*B, *qnr*S and *qnr*C, all of which are present in clinical isolates of the family *Enterobacteriaceae* (Robicsek *et al.*, 2006; Park *et al.*, 2007; Tamang *et al.*, 2008; Carattoli, 2009; Strahilevitz *et al.*, 2009).

The aim of this study was to demonstrate the expression of an inducible acquired pACBL in *S. marcescens* and *Escherichia coli* isolates from the same patient. Moreover, as the *E. coli* isolate showed reduced susceptibility to quinolones, plasmid-encoded quinolone resistance (PMQR) were also screened.

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Materials and methods

Clinical sample

Bacterial isolates were recovered from a urine specimen collected during nephrostomy in a 68-year-old patient who had initially undergone BCG instillation therapy and was later treated surgically by radical cyst-prostatectomy for a vesicle and ureteral transitional cell carcinoma. This patient carried an ileal conduit.

Conjugation experiments

Conjugation assays were performed using the broth mating method at 37 °C. *Escherichia coli* and *S. marcescens* isolates suspected to harbour pACBL were used as donor strains. As a recipient strain, we used the *E. coli* HB101 (UA6190), which expresses a green fluorescent protein marker and is resistant to rifampin, gentamicin and kanamycin. Briefly, donor and recipient cells from exponentially growing cultures [3 h at 37 °C with agitation in Luria–Bertani (LB) media] were mixed with a donor/recipient ratio of 1 : 1 and incubated overnight at 37 °C. Transconjugants were selected on LB agar supplemented with ceftazidime ($10\,\mu g\,mL^{-1}$) and rifampin ($100\,\mu g\,mL^{-1}$) and were exposed to UV illumination.

Bacterial identification and antimicrobial susceptibility

Isolates were identified using the API System 20E (bioMérieux, Marcy l'Étoile, France). The disc diffusion susceptibility test was performed on both donor and transconjugant strains, according to Clinical Laboratory Standards Institute guidelines, using commercially available discs (Neo-Sensitabs, Rosco Diagnostica S/A, Taastrup, Denmark). The antimicrobial agents included were ampicillin, piperacillin, cephalotin, cefuroxime, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, cefoxitin, amoxicillin–clavulanic acid, piperacillin–tazobactam, nalidixic acid, ciprofloxin, sulphonamides, trimethoprim, trimethoprim–sulphamethoxazole, chloramphenicol, rifampin, tetracycline, gentamicin, kanamycin, tobramycin, amikacin and streptomycin. The inducible AmpC β-lactamase was suspected when antagonism between oxyimino-β-lactams and imipenem or cefoxitin was observed on primary antibiogram plates. The presence of scattered colonies in the inhibition halo of cefoxitin, cefotaxime, ceftazidime and aztreonam was also examined (Mirelis *et al.*, 2006).

Characterization of antimicrobial resistance genes

Antimicrobial resistance genes present in donor and transconjugant strains were studied. *amp*C genes were characterized using a previously described multiplex PCR (Pérez-Pérez & Hanson, 2002). Specific primers used to obtain the complete bla_{DHA-1} gene sequence were: DHA-1A 5′-CTG ATG AAA AAA TCG TTA TC-3′ and DHA-1B 5′-ATT CCA GTG CAC TCC AAA ATA-3′. PCR conditions were one cycle of denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min. The amplification was ended with an extension cycle at 72 °C for 7 min.

Screening of *qnr* genes was carried out by multiplex PCR amplification of *qnr*A, *qnr*B and *qnr*S genes as described (Robicsek *et al.*, 2006).

All amplicons obtained were purified using the Wizard® SV Gel and PCR clean-up system kit (Promega Corporations, Madison, WI). DNA sequencing of purified PCR products was performed by Macrogen (Macrogen Inc., Seoul, Korea). Nucleotide and amino acid sequences were analysed using MEGA-BLAST and PSI-BLAST, respectively (www.ncbi.nlm.nih.gov).

PCR-based replicon typing

PCR-based Inc/rep typing was performed to identify the major incompatibility groups of the plasmids present in parental and transconjugant strains (Carattoli *et al.*, 2005). Template DNA was prepared by extraction of total DNA using the GenElute™ Bacterial Genomic DNA commercial kit (Sigma). The PCR products obtained were then purified and sequenced as mentioned above.

Characterization of the relaxase MOB family

To identify the relaxase MOB family of the plasmids present in parental and transconjugant strains, a PCR-based MOB amplification method was performed (Alvarado *et al.*, 2008). The primers used to amplify the MOB_{P13} subfamily were MOB_{P13} forward (5′-AAC CCA CGC TGC AAR GAY CCV GT-3′) and MOB_{P13} reverse (5′-AGC GAT GTG GAT GTG AAG GTT RTC NGT RTC-3′). PCR conditions were one cycle of denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 15 s and a final extension at 72 °C for 5 min. The amplified DNA fragments were then purified and sequenced using primers MOB_{P13} forward and MOB_{P13} reverse clamp (5′-AGC GAT GTG GAT GTG AAG-3′).

Plasmid profiles and Southern blot analysis

From each parental and transconjugant strain, plasmid profiles were visualized after DNA linearization with the S1 enzyme, followed by pulsed-field gel electrophoresis (PFGE) as described previously (Barton *et al.*, 1995). Plasmid sizes were estimated using FINGERPRINTING II INFORMATIX™ software. S1-PFGE was then transferred onto a nylon-

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membrane by Southern blotting. Purified DNA products obtained from the PCR of bla_{DHA-1}, *qnr*B genes and the replicon IncL/M were used as probes for hybridization of the S1-PFGE blots. These probes were labelled using the commercial kit Amersham ECL Direct Nucleic Acid Labelling and Detection Systems, as recommended by the manufacturer (GE Healthcare).

Results and discussion

An *S. marcescens* and an *E. coli* with an inducible AmpC-β-lactamase phenotype were isolated from a urine sample together with an *E. coli* with its natural susceptible pattern, a meticillin-resistant *Staphylococcus aureus*, an *Enterococcus faecalis* and a *Morganella morganii*.

Primary antibiogram plates of *S. marcescens* and the resistant *E. coli* isolate showed oxyimino-β-lactams antagonism with imipenem or cefoxitin. Moreover, we observed scattered colonies located near the edge of cefoxitin, cefotaxime, ceftazidime and aztreonam. This pattern of susceptibility was compatible with the presence of a pACBL (Mirelis *et al*., 2006). Both isolates showed the same antimicrobial susceptibility pattern for all β-lactam and non-β-lactam antibiotics, except for cefotaxime and nalidixic acid. Cefotaxime showed reduced susceptibility in *S. marcescens* (14 mm), whereas *E. coli* remained susceptible (25 mm). Nalidixic acid showed reduced susceptibility in *E. coli* (15 mm), whereas *S. marcescens* remained susceptible (21 mm) (Table 1). The two transconjugants showed the same antimicrobial susceptibility pattern. The acquired reduced susceptibility to nalidixic acid in the *S. marcescens* transconjugant should be noted (Table 1).

The presence of bla_{DHA-1} and *qnr*B genes was confirmed by PCR and amplicon sequencing in both isolates and their respective transconjugants. DNA sequencing of the amplicons obtained for *qnr*B genes (429 bp) revealed 100% identity to the *qnr*B4 gene. These results were in complete agreement with other reports that found a close association between *qnr*B4 and bla_{DHA-1} determinants in isolates of the family *Enterobacteriaceae* (Park *et al*., 2007; Tamang *et al*., 2008; Strahilevitz *et al*., 2009).

Although pACBLs have been described in *Enterobacteriaceae* with a natural chromosomal AmpC enzyme (Park *et al*., 2007; Tamang *et al*., 2008; Mata *et al*., 2010), to our knowledge, this is the first time that a pACBL is reported in an *S. marcescens* isolate.

The observation of scattered colonies near the edge of the inhibition zones was the only phenotypic method to suspect the presence of a pACBL in an isolate harbouring an inducible chromosomal AmpC enzyme. Although this method proved to be effective in *Enterobacteriaceae* lacking inducible chromosomal AmpC β-lactamase (Mirelis *et al*., 2006; Mata *et al*., 2010), more phenotypic tests are needed to detect pACBLs in chromosomal AmpC producers. The lack of standardized phenotypic methods could be the main cause of failure in the detection of these acquired resistances in many clinical laboratories, especially in chromosomal AmpC producers.

Although more than one plasmid was observed by S1-PFGE in donor strains (Fig. 1), the results of PCR-based replicon typing and relaxase characterization only revealed a single replicon (IncL/M) and a single relaxase family (MOB_{P13}), respectively (Fig. 1). Nucleotide sequences of the amplicons obtained for IncL/M replicons (681 bp) from *S. marcescens* and *E. coli* were identical, as were their transconjugants. These nucleotide sequences were 96% homologous with the IncL/M plasmids pEL60 (AY422214), pCTX-M3 (AF550415) and pCTXM360 (EU938349). Nucleotide sequences of the amplicons obtained by relaxase gene amplification (177 bp) both from donor and transconjugant isolates were identical. They showed 86% homology with the same IncL/M-MOB_{P13} enterobacterial plasmids pEL60, pCTX-M3 and pCTXM360 mentioned above. In *Enterobacteriaceae*, plasmids showing identical rep and mob genes, components of the plasmid core, usually share the major part of their genetic backbone. It can therefore be expected that plasmids from *S. marcescens* and *E. coli* isolates are highly similar to each other.

The narrow-host-range IncF-like plasmids (Carattoli, 2009) have been described as the most prevalent plasmids harbouring bla_{DHA-1} genes. Nevertheless, it is worth noting

Table 1. Phenotypic and genotypic characteristics of *Serratia marcescens* and *Escherichia coli* isolates and their transconjugants all carrying bla_{DHA-1} and *qnr*B genes

Clinical isolates and transconjugants	Antibiotic resistant enzymes	Antimicrobial resistance pattern	Replicon type of plasmid	MOB family
N4112 (*S. marcescens*)	DHA-1, qnrB4	AMP, PIP, AMC, CEF, CXM, FOX, CAZ, CTX	L/M	MOB_{P13}
N4112 Tc (*E. coli* transconjugant)	DHA-1, qnrB4	AMP, PIP, AMC, CEF, CXM, FOX, CAZ, CTX, NAL*	L/M	MOB_{P13}
N4114 (*E. coli*)	DHA-1, qnrB4	AMP, PIP, AMC, CEF, CXM, FOX, CAZ, NAL*	L/M	MOB_{P13}
N4114 Tc (*E. coli* tranconjugant)	DHA-1, qnrB4	AMP, PIP, AMC, CEF, CXM, FOX, CAZ, CTX, NAL*	L/M	MOB_{P13}

*Nalidixic acid (NAL) with reduced susceptibility (14–15 mm). All the isolates were susceptible for the remaining antibiotic agents tested as mentioned in Materials and methods.

AMP, ampicillin; PIP, piperacillin; CEF, cephalotin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; AMC,amoxicillin–clavulanic acid; RIF, rifampin.

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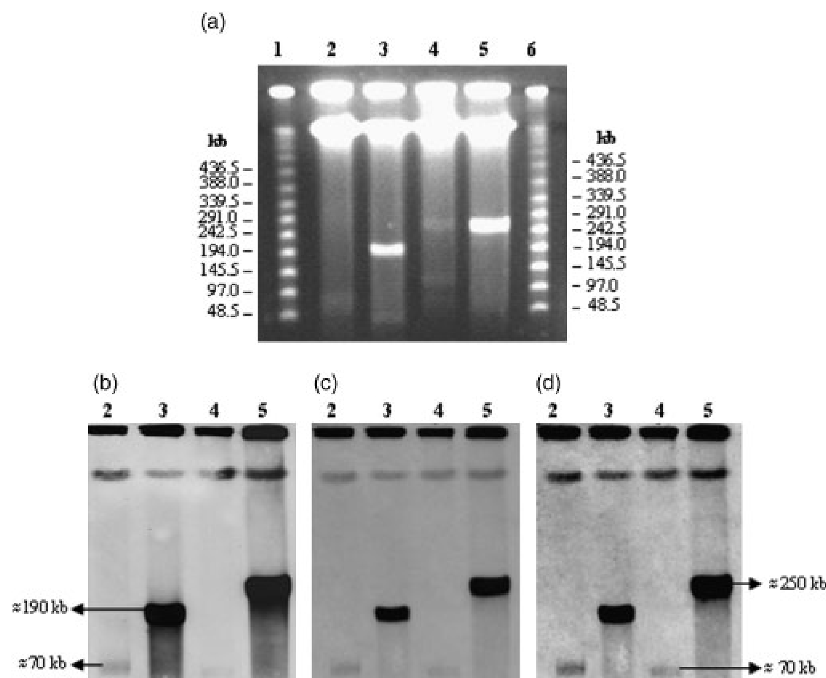

Fig. 1. S1-PFGE hybridized with $bla_{\text{DHA-1}}$, *qnr*B and IncL/M probes. Lanes 1 and 6: λ ladder, PFG marker; lane 2: N4112 (*Serratia marcescens* donor); lane 3: N4112 Tc (*Escherichia coli* tranconjugant); lane 4: N4114 (*E. coli* donor); lane 5: N4114 Tc (*E. coli* transconjugant); (a) S1-PFGE; (b) Southern hybridization with the $bla_{\text{DHA-1}}$ probe; (c) Southern hybridization with the IncL/M probe; (d) Southern hybridization with the *qnr*B probe.

that IncL/M is the most frequently found incompatibility group among the *Enterobacteriaceae* carrying $bla_{\text{DHA-1}}$ genes studied in our setting (96.6%; 28 from 29 isolates) (data not published). Curiously, *qnr*B4 genes have frequently been linked to the broad-host-range IncL/M plasmids (Carattoli, 2009). The presence of both resistance genes on the same plasmid and the reported increase in PMQR could perhaps account for the increasing number of isolates harbouring $bla_{\text{DHA-1}}$ genes (Park *et al.*, 2007; Tamang *et al.*, 2008; Strahilevitz *et al.*, 2009). The possibility that $bla_{\text{DHA-1}}$ genes may be mobilized by a vector with a greater capacity to spread could perhaps explain the recently widespread distribution of $bla_{\text{DHA-1}}$ genes.

Southern hybridization analysis revealed the colocalization of $bla_{\text{DHA-1}}$ and *qnr*B resistance genes on the same conjugative plasmid (Fig. 1). In *S. marcescens* and *E. coli* donor strains, $bla_{\text{DHA-1}}$ and *qnr*B genes hybridized to an approximately 70 kb-sized plasmid. Plasmids coharbouring these resistances in their transconjugants were larger than in wild strains; that in the *S. marcescens* transconjugant was around 190 kb, while that in the *E. coli* transconjugant was around 250 kb. All plasmids belonged to the IncL/M group (Fig. 1). These discrepancies in the size between donors and their respective transconjugants could be explained by cointegrates formed during the conjugation process (García *et al.*, 2005; Tamang *et al.*, 2008). Care should therefore be taken in molecular epidemiology studies when plasmid size is only estimated in transconjugants because it could be overestimated.

To sum up, this is the first report of an isolate of *S. marcescens* harbouring a pACBL. The observation of scattered colonies near the edge of the inhibition zones was the only phenotypic method that led us to suspect the presence of a pACBL in a chromosomal AmpC producer. Our results suggest an *in vivo* horizontal transfer of a plasmid coharbouring $bla_{\text{DHA-1}}$ and *qnr*B resistance genes between *S. marcescens* and *E. coli* isolates.

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References

Alvarado A, Garcillán-Barcia M & de la Cruz F (2008) A PCR-based method for Classification of conjugative plasmids based on relaxase sequences. The International Society for Plasmid

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Published by Blackwell Publishing Ltd. All rights reserved

Biology and other Mobile Genetic Elements (ISPB), 30 August–5 September 2008, Gdansk, Poland, p. 134.

Barton BM, Harding GP & Zuccarelli AJ (1995) A general method for detecting and sizing large plasmids. *Anal Biochem* **226**: 235–240.

Carattoli A (2009) Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob Agents Ch* **53**: 2227–2238.

Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL & Threlfall EJ (2005) Identification of plasmids by PCR-based replicon typing. *J Microbiol Meth* **63**: 219–228.

García A, Navarro F, Miró E, Mirelis B, Campoy S & Coll P (2005) Characterization of the highly variable region surrounding the $bla_{CTX-M-9}$ gene in non-related *Escherichia coli* from Barcelona. *J Antimicrob Chemoth* **56**: 819–826.

Mata C, Miró E, Rivera A, Mirelis B, Coll P & Navarro F (2010) Prevalence of acquired AmpC beta-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *ampC* genes at a Spanish hospital from 1999 to 2007. *Clin Microbiol Infec* **16**: 472–476.

Mirelis B, Rivera A, Miró E, Mesa R, Navarro F & Coll P (2006) A simple phenotypic method for differentiation between acquired and chromosomal AmpC beta-lactamases in *Escherichia coli*. *Enferm Infec Micr Cl* **24**: 370–372.

Naumiuk L, Baraniak A, Gniadkowski M *et al*. (2004) Molecular epidemiology of *Serratia marcescens* in two hospitals in Danzig, Poland, over a 5-year period. *J Clin Microbiol* **42**: 3108–3116.

Park YJ, Yu JK, Lee SG, Oh EJ & Woo GJ (2007) Prevalence and diversity of *qnr* alleles in AmpC-producing *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Serratia marcescens*: a multicentre study from Korea. *J Antimicrob Chemoth* **60**: 868–871.

Pérez-Pérez F & Hanson N (2002) Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* **40**: 2153–2162.

Pitout JDD (2008) Multiresistant *Enterobacteriaceae*: new threat of an old problem. *Expert Rev Anti Infect Ther* **6**: 657–669.

Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA & Hooper DC (2006) qnr prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. *Antimicrob Agents Ch* **50**: 2872–2874.

Strahilevitz J, Jacoby GA, Hooper DC & Robicsek A (2009) Plasmid-mediated Quinolone resistance: a multifaceted threat. *Clin Microbiol Rev* **22**: 664–689.

Tamang MD, Seol SY, Oh JY *et al*. (2008) Plasmid-mediated Quinolone resistance determinants *qnr*A, *qnr*B, and *qnr*S among clinical isolates of *Enterobacteriaceae* in a Korean hospital. *Antimicrob Agents Ch* **52**: 4159–4162.

Walther-Rasmussen J & Hoiby N (2007) Class a carbapenemases. *J Antimicrob Chemoth* **60**: 470–482.

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ANNEX III

8.3. Prevalence of acquired AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes at a Spanish hospital from 1999 to 2007.

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4. Swidsinski A, Mendling W, Loening-Baucke V *et al.* Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 2005; 106: 1013–1023.
5. Gillis JC, Wiseman LR. Secnidazole. A review of its antimicrobial activity, pharmacokinetic properties and therapeutic use in the management of protozoal infections and bacterial vaginosis. *Drugs* 1996; 51: 621–638.
6. Dubreuil L, Calvet L, Behra J. Le secnidazole: activite antibactérienne sur les anaérobies stricts. *Antibiotiques* 2005; 7: 239–246.
7. De Backer E, Verhelst R, Verstraelen H *et al.* Antibiotic susceptibility of *Atopobium vaginae*. *BMC Infect Dis* 2006; 6: 51.
8. Ferris MJ, Masztal A, Aldridge KE, Fortenberry JD, Fidel PL Jr, Martin DH. Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infect Dis* 2004; 4: 5.
9. Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathol Microbiol Scand B Microbiol Immunol* 1971; 217 (suppl 217): S1–S90
10. Davis B, Glover DD, Larsen B. Analysis of metronidazole penetration into vaginal fluid by reversed-phase high-performance liquid chromatography. *Am J Obstet Gynecol* 1984; 149: 802–803.
11. Mattila J, Mannisto PT, Mantyla R, Nykanen S, Lamminsivu U. Comparative pharmacokinetics of metronidazole and tinidazole as influenced by administration route. *Antimicrob Agents Chemother* 1983; 23: 721–725.
12. Lamp KC, Freeman CD, Klutman NE, Lacy MK. Pharmacokinetics and pharmacodynamics of the nitroimidazole antimicrobials. *Clin Pharmacokinet* 1999; 36: 353–373.
13. Theron MM, Janse Van Rensburg MN, Chalkley LJ. Nitroimidazole resistance genes (nimb) in anaerobic gram-positive cocci (previously *Peptostreptococcus* spp.). *J Antimicrob Chemother* 2004; 54: 240–242.
14. Fethers KA, Fairley CK, Hocking JS, Gurrin LC, Bradshaw CS. Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clin Infect Dis* 2008; 47: 1426–1435.

Prevalence of acquired AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes at a Spanish hospital from 1999 to 2007

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Abstract

In 2007, a significant increase in acquired *ampC* genes in *Enterobacteriaceae* from 0.06% in 1999 to 1.3% was observed. *Proteus mirabilis* showed the highest prevalence (0.95%) and CMY-2 was the most prevalent AmpC enzyme (66.7%). Other enzymes such as CMY-4, DHA-1, ACC-1, and three new enzymes called CMY-25, CMY-27 and CMY-40 were detected. Seven out of the 117 isolates (6%) also produced an extended-spectrum β-lactamase. As acquired AmpC enzymes are likely to become a serious public health issue worldwide, close surveillance is necessary to curb their spread.

Keywords: AmpC β-lactamases, antimicrobial resistance mechanism, epidemiology of resistance

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Acquired AmpCs appeared in the late 1980s and have been detected mainly in isolates of *Klebsiella* spp., *Escherichia coli*, *Proteus mirabilis* and *Salmonella* spp. although they have also been identified in other species including natural AmpC producers [1]. These enzymes confer resistance to most β-lactams – including cephamycins – with the exception of cefepime and carbapenems [2].

Most acquired *ampC*s derive from chromosomal *amp*C genes of the family *Enterobacteriaceae* (*Citrobacter freundii*, *Enterobacter* spp., *Morganella morganii* and *Hafnia alvei*) whereas the origin of others remains unknown. Isolates harbouring acquired *ampC*s are usually multi-resistant [3–6], limiting the therapeutic options even further. In this context, we aimed to determine the prevalence of acquired AmpCs in *Enterobacteriaceae* isolates lacking inducible chromosomal *amp*C genes at a Spanish hospital from January 1999 to December 2007.

Isolates were obtained from routine cultures at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). When there were multiple isolates from a patient within a 30-day period, only one was considered for analysis. Isolates were identified using standard methods [7]. The disc diffusion susceptibility test was performed according to Clinical Laboratory Standards Institute (CLSI) guidelines [8], using commercially available discs (Bio-Rad, Marnes La Coquette, France). The production of Extended-spectrum beta-lactamase (ESBL) was studied using the double-disc synergy test

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TABLE 1. Nucleotide changes (shown in bold) with the corresponding amino acid substitutions in the newly acquired AmpCs and antibiotic susceptibility

	Amino acid position									Susceptibility to β-lactam antibiotics (mg/L)[c]	Non-β-lactam antibiotics[d]
	125	146	153	180	214	221	252	273	338		
CMY-2	CGC (Arg)	AGG (Arg)	CAT (His)	GCG (Ala)	AAC (Ala)	TGG (Trp)	CGC (Arg)	GCG (Ala)	TCC (Ser)	–	–
CMY-25[a]	–	AG**T** (Ser)	–	–	–	–	–	–	–	FOX (>256); CXM (>256); CTX (>256); CRO (>256); CAZ (>256); FEP (8); AZT (64*); AMC (>256); TZP (64*); IMP (1*); ERT (2*)	STR
CMY-27[e]	–	–	–	–	–	TG**T** (Cys)	–	–	–	FOX (>256); CXM (>256); CTX (>256); CRO (>256); CAZ (>256); FEP (4); AZT (32); AMC (>256); TZP (6); IMP (0.75); ERT (0.50)	NAL; CIP; TMP; TET
CMY-40[b]	AG**T** (Ser)	A**C**G (Thr)	C**GC** (Arg)	**A**CG (Thr)	A**G**C (Ser)	–	C**A**C (His)	G**A**G (Glu)	T**A**C (Tyr)	FOX (>256); CXM (>256); CTX (>256); CRO (>256); CAZ (>256); FEP (4); AZT (48*); AMC (192*); TZP (96); IMP (1.5*); ERT (0.50)	NAL; CIP; TMP; STR;

[a,b]Additionally, three[a] and 28[b] silent mutations were detected.
[c]Susceptibility to β-lactam antibiotics was performed by Etest (AB; Biodisk). The antibiotics tested were: FOX, cefoxitin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; AZT, aztreonam; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; IMP, imipenem; ERT, ertapenem. *Scattered colonies were observed within the inhibition halo.
[d]Susceptibility to non-β-lactam antibiotics was tested by disc diffusion. Antibiotics tested were: SSS, sulphonamides; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; STR, streptomycin; KAN, kanamycin; TOB, tobramycin; AMK, amikacin; GEN, gentamicin; NET, netilmicin; NEO neomycin; NAL, nalidixic acid; CIP, ciprofloxacin.
[e]Antibiotic susceptibility was performed for both CMY-27 producers. Susceptibility results were identical except for trimethoprim (only one was resistant).

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and confirmed when necessary by Etest ESBL (AB Biodisk, Solna, Sweden) [3,9].

As some acquired AmpCs do not confer resistance to cefoxitin, the strains selected for this study were those showing intermediate resistance or resistance to amoxicillin-clavulanic acid, cefotaxime or ceftazidime according to CLSI breakpoints [8], and negative results for ESBL production. Isolates which screened positive for ESBL production and showed intermediate susceptibility or resistance to amoxicillin-clavulanic or cefoxitin were also included [10]. Acquired AmpCs were characterized using the previously described multiplex PCR [2]. Amplicons were purified and sequenced as described previously [9]. PCR-positive isolates were tested using a double-disc synergy test based on the utilization of cloxacillin (500 μg) as inhibitor of AmpC enzymes (except *Escherichia coli* strains). All PCR-positive isolates were also tested for the presence of scattered colonies in the inhibition halo of cefoxitin, cefotaxime, ceftazidime and aztreonam [10].

Among the 27 119 isolates of *Enterobacteriaceae* lacking inducible chromosomal AmpC β-lactamases, 437 isolates were studied as putative acquired AmpC producers. We obtained amplicons in 117: 75 *E. coli*, 20 *P. mirabilis*, 16 *K. pneumoniae*, four *K. oxytoca* and two *S. enterica*. The remaining 320 isolates were ESBL producers, hyperproducers of chromosomal AmpC enzymes (*E. coli*), or hyperproducers of class A enzymes (*Klebsiella* spp.). Moreover, other non-enzymatic resistance mechanisms such as altered permeability may also have been present in these isolates. A few of the 117 isolates included in this study have been described previously [7,9,10].

The 117 isolates were recovered from urine (66.7%), fluids and tissue (14.5%), blood (10.3%), respiratory tract (3.4%) or other samples (5.1%). Most samples were from ambulatory patients (64.1%).

The overall prevalence of *Enterobacteriaceae* carrying acquired *ampCs* was 0.43%, rising from 0.06% (1999) to 1.3% (2007). This significant increase (p <0.001; contingency table-chi-square test was used for evaluation; SPSS V15 software; SPSS Inc., Chicago, IL, USA), which occurred mainly in the last 3 years, could have been as a result of the emergence of *Enterobacteriaceae*-producing DHA (16 out of 40 in 2007) and the increase of CMY-2-producing *P. mirabilis*. The highest prevalence was found in *P. mirabilis* (0.95%), as in a recent survey in Polish hospitals where acquired AmpCs were observed

TABLE 2. Prevalence and distribution of acquired AmpCs among *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes

	1999	2000	2001	2002	2003	2004	2005	2006	2007	1999–2007
E. coli (*n*)	2283	2068	1820	2109	2440	2285	2385	2315	2224	*n* = 19929
CMY-2	1	6	1	1	3	8	4	14[a]	15	53 (70.7%)
CMY-4							1		1	2 (2.7%)
CMY-27								2[a]		2 (2.7%)
CMY-40							1			1 (1.3%)
DHA-1							4[b]	3	8[c]	15 (20%)
ACC-1						1			1	2 (2.7%)
Total (%)	1 (0.04)	6 (0.29)	1 (0.05)	1(0.05)	3(0.12)	9(0.39)	10 (0.42)	19 (0.82)	25 (1.12)	75 (0.38)
K. pneumoniae (*n*)	214	222	181	181	288	295	273	339	394	*n* = 2387
CMY-2			1	1[d]		1	1			4 (25%)
CMY-25								1		1 (6.3)
DHA-1							2	2	6[e]	10 (62.5)
ACC-1							1			1 (6.3)
Total (%)		1 (0.44)	1 (0.55)			1 (0.34)	4 (1.46)	3 (0.84)	6 (1.52)	16 (0.67)
P. mirabilis (*n*)	280	140	201	178	267	249	248	262	270	*n* = 2095
CMY-2		1				1[f]	4[f,g]	6	7[f]	19 (95%)
DHA-1								1		1 (5%)
Total (%)		1 (0.71)				1 (0.80)	4 (1.21)	7 (2.67)	7 (2.60)	20 (0.95)
K. oxytoca (*n*)	0	0	45	88	65	70	98	76	87	*n* = 509
DHA-1								2[h]	2[h]	4 (100%)
Total (%)								2 (2.63)	2 (2.30)	4 (0.79)
S. enterica (*n*)	352	148	290	208	182	231	141	125	94	*n* = 1771
CMY-2	1							1		2 (100%)
Total (%)	1 (0.28)							1 (0.80)		2 (0.11)
Others[i]										*n* = 428
Overall prevalence (%)	0.06	0.31	0.08	0.04	0.09	0.38	0.53	1.01	1.3	0.43

[a]Two of these isolates showed identical ERIC and PFGE patterns and spread among patients was established.
[b]Two of these isolates showed identical ERIC and PFGE patterns but no epidemiological relationship was established between patients.
[c]Two isolates also harboured a CTX-M-14.
[d]This isolate also harboured a CTX-M-1.
[e]Three isolates also harboured a CTX-M-15. Two of these isolates showed identical PFGE patterns. Patient spread was established.
[f]PFGE results showed a cluster of five *P. mirabilis* (four identical PFGE and one probably related pattern; all carrying CMY-2). One of these strains was isolated in 2004, another in 2005 and the remaining three in 2007. No epidemiological relationship was established between patients.
[g]One of these isolates also harboured a CTX-M-2 (first report in Catalonia).
[h]PFGE results showed a cluster of two *K. oxytoca* (one strain isolated in 2006 and the other in 2007). Both were isolated from the same patient over an interval of 8 months.
[i]The species included here are: *C. koseri* (211 isolates), *Shigella* spp. (101 isolates), *P. vulgaris* (108 isolates) and *P. penneri* (eight isolates). No acquired AmpCs were found.

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exclusively in *P. mirabilis* (20.5%) [11]. Other studies found no acquired-AmpC-producing *P. mirabilis* [5] or found it at a lower rate (0.75%) [12]. It is of note that in our setting, *P. mirabilis* is acquiring different types of β-lactamases, including AmpCs [9].

CMY-2 has a worldwide distribution. In our study, it was the predominant enzyme (66.7%), followed by DHA-1 (25.6%). DHA-1 was mainly associated with *Klebsiella* spp. and was the only acquired AmpC detected in *K. oxytoca*. Less commonly found enzymes were ACC-1, CMY-4, CMY-25, CMY-27 and CMY-40. The last three are reported here for first time (their amino acid substitutions and the corresponding susceptibility test results are shown in Table 1). Seven (6%) out of the 117 acquired-AmpC-producing isolates also produced an ESBL (Table 2). The prevalence and type of acquired AmpCs differs depending upon the geographical area, the species studied and the period of study [4,11–14], possibly as a result of the selection criteria used. For this reason, it is difficult to compare the prevalence of acquired AmpCs between studies.

Resistance of the AmpC-producers to non-β-lactam antibiotics was high. Isolates showed resistance to nalidixic acid (74.4%), ciprofloxacin (51.3%), tetracycline (67.5%), chloramphenicol (43.6%), sulphonamides (61.5%), trimethoprim (43.6%) and aminoglycosides such as streptomycin (52.1%), kanamycin (43.6%), gentamicin (36.7%) and tobramycin (34.2%).

The cloxacillin test was positive for all analysed isolates. Using the disc diffusion method, 86.3% of isolates showed an inhibition halo to third-generation cephalosporins (13–33 mm) and aztreonam (13–43 mm). Most of these (91.1%) showed scattered colonies near the edge of the inhibition zones. Both these phenotypic tests are useful to detect the presence of acquired AmpCs in *Enterobacteriaceae* lacking inducible chromosomal AmpC. Nevertheless, as previously reported [15], the cloxacillin test does not allow differentiation between chromosomal and acquired AmpC enzymes. PCR is the still the most reliable test in these cases.

The clonal diffusion of these enzymes was analysed by clinical and molecular epidemiology. Enterobacterial repetitive intergenic consensus (ERIC)-PCR was used as a first approach for *E. coli* strains [2,9]. Those that showed ERIC patterns with >80% homology were then analysed by pulsed-field gel electrophoresis (PFGE) [3]. PFGE was also used to study the clonal relationship of the remaining isolates. Results are shown in Table 2.

Acquired-AmpC-producing organisms are likely to remain undetected in many clinical laboratories as there is a lack of standardized phenotypic methods [10,16]. In a multi-centre Spanish study, only 53.2% of 57 laboratories were able to detect *E. coli* and *K. pneumoniae* producing acquired AmpC [17].

There are very few reports from Europe regarding the epidemiology of acquired-AmpCs over a period of several years [4], and to date, this is the first from Spain. Our findings support the view that the prevalence and diversity of acquired *ampC* genes is increasing. Knowledge about the prevalence and diffusion of this emergent resistance may be helpful to establish preventive measures that will curb their spread.

Nucleotide Sequence Accession Number

The new β-lactamase gene sequences were submitted to the GenBank under accession numbers EU515249 ($bla_{CMY\text{-}25}$), EU515250 ($bla_{CMY\text{-}27}$) and EU515251 ($bla_{CMY\text{-}40}$).

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Transparency Declaration

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References

1. Philippon A, Arlet G, Jacoby A. Plasmid-determined AmpC-Type β-lactamases. *Antimicrob Agents Chemother* 2002; 46: 1–11.
2. Pérez-Pérez FJH, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002; 40: 2153–2162.
3. Blanc V, Mesa R, Saco M *et al.* ESBL- and plasmidic class C β-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Vet Microbiol* 2006; 118: 299–304.
4. Hopkins KL, Batchelor MJ, Liebana E, Deheer-Graham AP, Threlfal EJ. Characterisation of CTX-M and *Amp*C genes in human isolates of *Escherichia coli* identified between 1995 and 2003 in England and Wales. *Int J Antimicrob Agents* 2006; 28: 180–192.
5. Song W, Kim JS, Kim HS *et al.* Increasing trend in the prevalence of plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking chromosomal *amp*C gene at a Korean university hospital from 2002 to 2004. *Diagn Microbiol Infect Dis* 2006; 55: 219–224.
6. Pitout JD, Gregson DB, Church DL, Laupland KB. Population-based laboratory surveillance for AmpC β-lactamase-producing *Escherichia coli*, Calgary. *Emerg Infect Dis* 2007; 13: 443–448.
7. Navarro F, Pérez-Trallero E, Marimón JM, Aliaga R, Gomariz M, Mirelis B. CMY-2-producing *Salmonella enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca, Proteus mirabilis* and *Escherichia coli* strains isolated in

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Spain (October 1999–December 2000). *J Antimicrob Chemother* 2001; 48: 383–389.

8. Clinical and Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing: Seventeenth Informational (suppl) M100-S17*.PA, USA: CLSI, Wayne, 2007.
9. Aragón LM, Mirelis B, Miró E *et al.* Increase in β-lactam-resistant *Proteus mirabilis* strains due to CTX-M and CMY-type as well as new VEB- and inhibitor-resistant TEM-type β-lactamases. *J Antimicrob Chemother* 2008; 61: 1029–1032.
10. Mirelis B, Rivera A, Miró E, Mesa RJ, Navarro F, Coll P. A simple phenotypic method for differentiation between acquired and chromosomal AmpC β-lactamases in *Escherichia coli*. *Enferm Infecc Microbiol Clin* 2006; 24: 370–372.
11. Empel J, Baraniak A, Literacka E *et al.* and the Beta-PL Study Group. Molecular survey of β-lactamases conferring resistance to newer β-lactams in *Enterobacteriaceae* isolates from Polish hospitals. *Antimicrob Agents Chemother* 2008; 52: 2449–2454.
12. Adler H, Fenner L, Walter P *et al.* Plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes: prevalence at a Swiss university hospital and occurrence of the different molecular types in Switzerland. *J Antimicrob Chemother* 2008; 61: 457–458.
13. Álvarez M, Tran JH, Chow N, Jacoby GA. Epidemiology of conjugative plasmid-mediated AmpC β-lactamase in the United States. *Antimicrob Agents Chemother* 2004; 42: 533–537.
14. Woodford N, Reddy S, Fagan EJ *et al.* Wide geographic spread of diverse acquired AmpC β-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. *J Antimicrob Chemother* 2007; 59: 102–105.
15. Yen Tan T, Yong LS, He J, Hsien T, Yang L. Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *Antimicrob Agents Chemother* 2009; 53: 146–149.
16. Hanson ND. AmpC β-lactamases: what do we need to know for the future? *J Antimicrob Chemother* 2003; 52: 2–4.
17. Conejo MC, Mata C, Navarro F, Pascual Aand the GEMARA collaborative group. Detection and reporting betalactam resistance phenotypes in *Escherichia coli* and *Klebsiella pneumoniae*: a multicentre proficiency study in Spain. *Diagn Microbiol Infect Dis* 2008; 62: 317–325.

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ANNEX IV

8.4. Plasmid typing and genetic context of AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes: findings from a Spanish hospital 1999-2007.

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Plasmid typing and genetic context of AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal *ampC* genes: findings from a Spanish hospital 1999–2007

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Objectives: To gain insights into *ampC* transmission between bacterial strains.

Methods: We examined the genetic context of 117 acquired *ampC* genes from 27119 Enterobacteriaceae collected between 1999 and 2007. Plasmid analysis was carried out by PCR-based replicon or relaxase typing, S1-PFGE and Southern hybridization. I-CeuI/PFGE was used for isolates not characterized by plasmid analysis. PCR reactions were used to map the genetic organization of the *ampC* genes.

Results: Among the isolates studied, 81.2% of *ampC* genes were located on plasmids of known Inc/MOB groups, 7.7% were chromosomally located and 11.1% were not determined. A/C, I1 and K were the most commonly found replicons in plasmids carrying bla_{CMY-2}, while L/M replicons were associated with bla_{DHA-1}. bla_{ACC-1} was linked to I1 and MOB_{F11} plasmids; bla_{CMY-27} was associated with IncF and MOB_{P12} plasmids; the plasmid carrying bla_{CMY-25} could not be typed, and bla_{CMY-40} was chromosomally located. All 87 isolates carrying bla_{CMY-2}, bla_{CMY-4}, bla_{CMY-25}, bla_{CMY-27}, bla_{CMY-40} or bla_{ACC-1} displayed the transposon-like structures IS*Ecp1*/ΔIS*Ecp1*-bla_{CMY}-*blc*-*sugE* or ΔIS*Ecp1*-bla_{ACC-1}-*gdha*. The most prevalent structure in bla_{DHA-1} (93.3% of cases) was identical to that described in the *Klebsiella pneumoniae* pTN60013 plasmid. Remarkably, in three isolates containing chromosomal bla_{CMY-2}, this gene was mobilized by conjugation.

Conclusions: Although plasmids are the main cause of the rapid dissemination of *ampC* genes among bacteria, we need to be aware that other mobile genetic elements such as integrative and conjugative elements (ICEs) can be involved in the mobilization of these genes.

Keywords: incompatibility groups, relaxases, mobile genetic elements, resistance gene dissemination

Introduction

AmpC β-lactamases confer resistance to most β-lactams except cefepime and carbapenems.[1] Although most of the AmpC enzymes are intrinsic and chromosomally encoded, some are also found on plasmids. This is due to the action of mobile genetic elements (MGEs) in several species that can capture these chromosomal genes and transfer them into mobilizable and/or conjugative plasmids, and subsequently disseminate them into many bacterial species that naturally lack these genes.[1–5]

As plasmids are the principal vehicles for the dissemination of a great variety of resistance genes, their study and understanding is critical for reversing the increasing trend in antibiotic resistance rates worldwide. PCR-based replicon typing (PBRT)[6] is currently the method of choice for plasmid characterization in clinically relevant bacteria. However, this method has several limitations in identifying divergent or novel replicons.[7,8] A new method based on the characterization of plasmid-encoded relaxases and covering a wider diversity of transmissible plasmids from γ-proteobacteria has recently been proposed.[8]

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There is little information regarding the association of plasmid-mediated *ampC* genes with specific plasmid families. The few reports to date are mainly based on biased collections (e.g. only one *ampC* gene reported, or a single host studied)[7] and mainly focused on bla_{CMY-2}, while reports on other *ampC* genes such as bla_{DHA-1} and bla_{ACC-1} are either scarce or non-existent. The aim of this study was to determine the plasmid families and the genetic loci involved in the dissemination of acquired *ampC* genes into 117 Enterobacteriaceae lacking chromosomal AmpC enzymes. These isolates were recovered at Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) over a 9-year period (1999–2007). The predominantly acquired AmpC β-lactamase was found to be CMY-2 (n=78), followed by DHA-1 (n=30). Less commonly found enzymes were ACC-1 (n=3) and CMY-2 variants [CMY-4 (n=2), CMY-27 (n=2), CMY-25 (n=1) and CMY-40 (n=1)].[9]

Methods

Bacterial isolates

A total of 27119 isolates of Enterobacteriaceae lacking inducible chromosomal AmpC enzymes were investigated between 1999 and 2007. The criteria for selecting putative acquired AmpC producers have been previously described.[9] Among these isolates, 117 carried acquired *ampC* genes, including 75 *Escherichia coli*, 20 *Proteus mirabilis*, 16 *Klebsiella pneumoniae*, 4 *Klebsiella oxytoca* and 2 *Salmonella enterica*. Clonal relationships were established for one cluster of five *P. mirabilis* isolates, three clusters of two identical *E. coli* isolates and one cluster of two identical *K. pneumoniae* isolates.[9]

Conjugation experiments

Conjugation assays were performed using a broth mating method at 37°C. A modified *E. coli* HB101 strain, UA6190 (aminoglycosides and rifampicin resistant), was used as the recipient strain. This recipient also expresses a green fluorescent protein (GFP) marker, integrated in the chromosome by the suicide mini-transposon delivery plasmid pAG408 together with two aminoglycosides resistance genes as described previously.[10] Transconjugants were selected on Luria–Bertani (LB) agar supplemented with ceftazidime (10 mg/L) and rifampicin (100 mg/L), and were exposed to UV illumination to check for GFP fluorescence. The frequency of transfer was expressed as the ratio of transconjugants to total recipient cells.

Susceptibility testing of transconjugants

Disc diffusion susceptibility tests were performed on transconjugant strains, according to CLSI guidelines,[11] using commercially available Neo-Sensitabs discs (Rosco Diagnostica S/A).

PBRT

PBRT[6] was used to identify the major incompatibility (Inc) groups of plasmids present in transconjugants, or in parental strains when conjugation did not occur. When *ampC* genes were not associated with any of the Inc groups obtained by PBRT, ColE, IncU and IncR plasmids were tested.[12] Template DNA was prepared by extraction of total DNA using the GenElute™ Bacterial Genomic DNA kit (Sigma).

Characterization of the relaxase MOB family

A PCR-based MOB amplification method[8,13] was performed to identify the relaxase MOB family of plasmids carrying the *ampC* genes that could not be characterized by PBRT. Briefly, this new plasmid characterization methodology classifies plasmids according to their *mob* region; this encodes the plasmid relaxase, which is found in all conjugative and mobilizable plasmids. Conjugative plasmids can be classified into six MOB families (MOB_F, MOB_H, MOB_Q, MOB_C, MOB_P and MOB_V) based on the amino acid sequence of their relaxases. Subgroups of five relaxase MOB families prevalent in the γ-proteobacteria (MOB_P, MOB_F, MOB_C, MOB_H and MOB_Q) were studied. When the relaxase was the only determinant obtained from plasmids mobilizing *ampC* genes, amplicons obtained from the MOB PCR were purified and sequenced. Primers and PCR conditions used to amplify these relaxases are listed in Table S1 (available as Supplementary data at *JAC* Online).

Plasmid profiles and Southern blot analysis

When possible, plasmid analysis was carried out in the transconjugant or donor strains by DNA linearization with S1 nuclease followed by PFGE as previously described.[14] Plasmid sizes were estimated using Fingerprinting II Informatix™ software (Bio-Rad). DNA was transferred from S1-PFGE gels onto nylon membranes by Southern blotting. Purified DNA products obtained from the PCR of *ampC* genes, bla_{ESBL} genes, Inc group or MOB relaxase amplicons were used as probes for hybridization of the S1-PFGE blots. These probes were labelled with the Amersham ECL Direct Nucleic Acid Labelling and Detection Systems, according to the manufacturer's instructions (GE Healthcare).

I-CeuI analysis

The chromosomal locations of *ampC* and bla_{ESBL} genes were investigated by digestion of the entire DNA with I-CeuI, followed by PFGE.[15] This technique was applied to isolates that could not be characterized by the methods mentioned above.

Detection of the flanking regions of acquired ampC genes

An overlapping PCR strategy was used to map the regions surrounding these genes. The regions surrounding *ampC* genes most frequently described in the literature were explored.[1–5,16–19] In the case of bla_{CMY-2}, bla_{CMY-4}, bla_{CMY-25}, bla_{CMY-27}, bla_{CMY-40}, the presence of IS*Ecp1*, *blc* and *sugE* genes was studied. As truncated versions of IS*Ecp1* (at either the 3′ or 5′ end) have been described, several primers were used to explore this region. For bla_{DHA-1}, searched genes were IS*CR1* (*orf513*), IS*26*, *orf2* (conserved region of unknown function in *Morganella* species), *ampR*, *qacE*Δ*1* and *sul1*; for bla_{ACC-1}, searched genes were IS*26*, IS*Ecp1* and *gdha*. Primers and PCR conditions are listed in Table S1.

Results

Conjugation experiments

All 117 isolates from the collection were studied. Among these, conjugative transfer of the *ampC* gene was detected for 97 isolates (82.9%). Conjugation frequencies ranged from 10^{-2} to 10^{-8}. Conjugation experiments involving CMY-4, CMY-25 and CMY-40 donors did not produce any transconjugants, despite testing under several different conjugation conditions (data not shown).

Susceptibility testing

Most donor strains showed high levels of resistance to all non-β-lactam antimicrobial agents tested (Table 1). However, the

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Table 1. Non-β-lactam antibiotic susceptibility testing

		Non-β-lactam antibiotics (% of resistant isolates)[a]						
AmpC enzymes	D or Tc (*n*)	NAL	CIP	SSS	TMP	SXT	TET	CHL
CMY-2[b]	D (78)	76.9	47.4	73.1	43.6	39.7	80.7	59.0
	Tc (66)	0.0	0.0	50.0	3.0	3.0	47.0	42.4
DHA-1[c]	D (30)	76.7	60.0	46.6	46.7	36.7	43.3	16.7
	Tc (26)	61.5	0.0	15.4	15.4	7.7	11.5	0.0
ACC-1	D (3)	2	2	2	1	0	2	0
	Tc (3)	0	0	2	0	0	0	0
CMY-27	D (2)	2	2	0	1	0	2	0
	Tc (2)	0	0	0	0	0	0	0
CMY-4[d]	D (2)	2	2	2	2	2	2	1
CMY-25[d]	D (1)	0	0	0	0	0	0	0
CMY-40[d]	D (1)	1	1	0	1	0	0	0

NAL, nalidixic acid; CIP, ciprofloxacin; SSS, sulphonamides; TMP, trimethoprim; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; D, clinical strain; Tc, transconjugant.
[a]When the number of strains is less than 10, the resistant isolates are expressed as *n* rather then %.
[b]Two of these isolates also harboured an ESBL; the plasmid mobilizing *ampC* was conjugative in one case.
[c]Five of these isolates also harboured an ESBL; plasmids mobilizing these genes were conjugative in all cases.
[d]Transconjugants were not obtained in isolates carrying CMY-4, CMY-25 or CMY-40.

percentage of transconjugant strains showing co-resistance to non-β-lactam antibiotics was lower. CMY-2 and DHA-1 enzymes displayed higher rates of resistance to non-β-lactam antibiotics, while isolates carrying ACC-1, CMY-25 and CMY-27 were susceptible to most antibiotics tested. Resistance to sulphonamides, tetracycline and chloramphenicol were mainly present in isolates carrying $bla_{\text{CMY-2}}$, while 61.5% of $bla_{\text{DHA-1}}$-carrying transconjugants displayed reduced susceptibility to nalidixic acid (Table 1).

Plasmid characterization

The first approaches to characterize plasmids carrying *ampC* genes were PBRT and Southern hybridization using the amplicons obtained by PBRT as probes. Eleven of the 18 replicons tested, including I1, L/M, N, FIA, FIB, FIC, A/C, K, B/O and F, were involved in the dissemination of *ampC* genes. The most representative, alone or with other replicons, were A/C (n=30), L/M (n=25), I1 (n=25), K (n=10) and the F group (n=10) (Table 2). The *ampC* genes were also found in plasmids with two or more replicons in 15 isolates (13%). The multi-replicon combinations identified were I1+F (n=3), I1+K (n=2), A/C+FIB+F (n=2), L/M+FIA (n=2), L/M+FIC (n=2), L/M+N (n=1), A/C+I1 (n=1) and ColE+MOB_{P11} (n=1) (Table 2). Characterization of plasmids carrying *ampC* gene was possible in 91 isolates in this first step (78%).

As a second approach, the remaining 26 non-typeable isolates were tested using PCR for ColE, IncU and IncR replicons and for the relaxase MOB family. None of the isolates was positive for IncU or IncR plasmids. Hybridization with the ColE replicons and the MOB relaxase amplicons found in these isolates allowed us to characterize another four plasmids carrying *ampC* genes: two MOB_{F11} plasmids, one MOB_{P12} plasmid and one co-integrate of a ColE-like plasmid and a MOB_{P11} plasmid (Table 2). MOB_{F11} sequencing revealed that one case was compatible with the relaxase of the R46 (IncN-like) plasmid, while a novel MOB_{F11} relaxase was found for the other case (submitted to GenBank under accession number F421285). The translated sequence showed 79% amino acid identity (44/56) to TraI of IncN plasmid R46, and 51% (31/61) to TraI of pCT14. Sequencing of the MOB_{P11} and MOB_{P12} amplicons revealed relaxases identical to TraI of the IncP-1α plasmid RP4, and to NikB of the Incα plasmid R64, respectively.

The 95 characterized strains showed that *ampC* genes were generally located on large plasmids of various sizes. The predominant plasmids present in the 78 strains carrying CMY-2, or CMY-2 plus an extended-spectrum β-lactamase (ESBL), belonged to Inc groups A/C (33%), I1 (23%) and K (10%). $bla_{\text{CMY-2}}$ was also found in multi-replicon plasmids (14%) (Table 2). On the other hand, all but one of the plasmids associated with DHA-1 (or DHA-1 plus ESBL) belonged to the L/M group. In most cases, L/M was the only replicon present (67%), or it was present in multi-replicon plasmids (17%). Genes encoding ACC-1, CMY-4 and CMY-27 were carried by IncI1, IncF, MOB_{F11} (an IncN-like and a novel relaxase) or MOB_{P12} (IncI1-like relaxase) plasmids.

No differences were observed in plasmid content among clonally related isolates, except for the cluster of two *E. coli* containing $bla_{\text{CMY-27}}$. The *ampC* gene was located on an IncF plasmid in one case, while in the other case it was located on a MOB_{P12} plasmid.

The remaining 22 isolates were non-typeable by the methods used: (i) in nine isolates, no plasmids were observed in the S1-PFGE gel, or the plasmids present did not hybridize with the *ampC* gene; (ii) in three isolates, plasmids carrying *ampC* genes were not solved by PBRT or by relaxase-typing methods; (iii) in six isolates, multiple hybridization bands with *ampC* and other replicon probes (four $bla_{\text{CMY-2}}$ and two $bla_{\text{DHA-1}}$) were found [when total DNA from several independent transconjugant colonies was analysed by S1-PFGE and hybridization with *ampC*

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Table 2. Plasmid families associated with acquired AmpC β-lactamases in Enterobacteriaceae

Enzyme (*n*)	Replicons/relaxases[a]	Isolates (*n*)	Strains (*n*)	Sizes (kb)	Total (%)
CMY-2/CMY-2+ESBL (78)	A/C	*E. coli* (15), *K. pneumoniae* (2), *P. mirabilis* (8), *S. enterica* (1)	26	~95 to 365	33
	I1	*E. coli* (16), *P. mirabilis* (1), *S. enterica* (1)	18	~80 to 350	23
	K	*E. coli* (7), *P. mirabilis* (1)	8	~80 to 115	10
	I1+K	*E. coli* (2)	2	~90 and 95	3
	I1+F	*E. coli* (3)	3	~85 to 135	4
	A/C+FIB+F	*E. coli* (2)	2	~160 and 340	3
	A/C+FIA+FIB	*E. coli* (1)	1	~300	1
	A/C+I1	*E. coli* (1)	1	~415	1
	F	*E. coli* (1)	1	~190	1
	ColE+MOB_{P11}	*P. mirabilis* (1)	1	≈100	1
	chromosomal location	*P. mirabilis*[b] (8)	8	—	10
	non-typeable	*E. coli*[c] (3), *K. pneumoniae*[d] (2)	5	~110 to 425	6
	degraded	*E. coli* (2)	2	—	3
DHA-1/DHA-1+ESBL (30)	L/M	*E. coli*[e] (8), *K. pneumoniae*[f] (9), *K. oxytoca* (3)	20	~70 to 295	67
	L/M+FIA	*E. coli* (2)	2	~155 and 170	7
	L/M+FIC	*K. pneumoniae*[g] (1), *K. oxytoca* (1)	2	~110 and 150	7
	N	*E. coli* (1)	1	~50	3
	L/M+N	*P. mirabilis* (1)	1	~105	3
	non-typeable	*E. coli*[h] (2)	2	~75 to 310	7
	degraded	*E. coli* (2)	2	—	7
ACC-1 (3)	I1	*E. coli* (1)	1	~95	33
	MOB_{F11}	*E. coli* (1), *K. pneumoniae* (1)	2	~65 and 75	67
CMY-4 (2)	F	*E. coli* (1)	1	~120	50
	non-typeable	*E. coli* (1)	1	~40	50
CMY-27 (2)	F	*E. coli* (1)	1	~75	50
	MOB_{P12}	*E. coli* (1)	1	~250	50
CMY-25 (1)	non-typeable	*K. pneumoniae*[i] (1)	1	~380	100
CMY-40 (1)	chromosomal location	*E. coli* (1)	1	—	100

[a]F includes all the IncF plasmids amplified by F-simplex PCR using the PBRT method; MOB_{P11} belongs to the MOB_P relaxase family (includes IncP-1 plasmids); MOB_{P12} belongs to the MOB_P relaxase family (includes IncI, K and B/O); MOB_{F11} belongs to the MOB_F relaxase family (includes IncN and IncW).
[b]One *P. mirabilis* also harboured $bla_{CTX\text{-}M\text{-}2}$ (also located on the chromosome). These isolates are not clonally related. An SXT/R391-like element was involved in the mobilization of $bla_{CMY\text{-}2}$ genes in seven out of the eight *P. mirabilis*.
[c]The plasmid carrying $bla_{CMY\text{-}2}$ in one *E. coli* could not by typed either by PBRT or by relaxase typing. This isolate also carried copies of $bla_{CMY\text{-}2}$ on the chromosome. The remaining two *E. coli* showed multiple hybridization bands with the *ampC* gene and the replicon probes involved, and copies on the chromosome.
[d]One *K. pneumoniae* also harboured $bla_{CXT\text{-}M\text{-}1}$ on the plasmid containing $bla_{CMY\text{-}2}$. Both showed multiple hybridization bands with the *ampC* gene and the replicon probes involved, and copies on the chromosome.
[e]Two *E. coli* also harboured $bla_{CTX\text{-}M\text{-}14}$ genes on different plasmids belonging to the IncI group (~90 kb).
[f]One *K. pneumoniae* also harboured $bla_{CXT\text{-}M\text{-}15}$ genes on the same IncL/M plasmid (~170 kb).
[g]One *K. pneumoniae* also harboured $bla_{CXT\text{-}M\text{-}15}$ genes on the same cointegrate IncL/M+FIC (~155 kb).
[h]Both isolates showed multiple hybridization bands with $bla_{DHA\text{-}1}$ and L/M probes. They also had copies on the chromosome.
[i]The plasmid carrying $bla_{CMY\text{-}25}$ could not by typed either by PBRT or by relaxase typing. This isolate also carried copies of $bla_{CMY\text{-}25}$ on the chromosome.

probes, we observed several patterns: one or two hybridization bands of different sizes depending on the specific transconjugant (data not shown)]; and (iv) four isolates were degraded during S1-PFGE and therefore could not be characterized (Table 2).

I-CeuI/PFGE analysis was applied to all the unsolved isolates except those that were degraded during S1-PFGE. A chromosomal location was confirmed in the nine isolates (eight *P. mirabilis* with CMY-2, and one *E. coli* with CMY-40) without *ampC* plasmid hybridization signals in the S1-PFGE gel. The eight *P. mirabilis* strains were not clonally related. Four of these isolates were positive for MOB_{H12} amplicons, compatible with the presence of an SXT/R391-like element; for three of these *P. mirabilis* isolates containing a chromosomal copy of $bla_{CMY\text{-}2}$, the gene had been mobilized by conjugation.

Two of the three non-characterized plasmids carrying *ampC* genes, and all isolates showing multiple hybridization bands

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with the replicon and the *ampC* probes, also had copies of the *ampC* gene located on the chromosome.

In three of the seven isolates with *ampC* and bla_{ESBL} genes, both genes were located on the same plasmid; in two isolates, bla_{ESBL} genes (one $bla_{CTX\text{-}M\text{-}2}$ and one $bla_{CTX\text{-}M\text{-}15}$) were located on the chromosome; in the remaining two, *ampC* and bla_{ESBL} genes were located on different plasmids.

Detection of the flanking regions of acquired ampC genes

Genetic organization of $bla_{CMY\text{-}2}$ *and its derivatives*

IS*Ecp1* and the genes *blc* (outer membrane lipoprotein) and *sugE* (drug efflux channel) were found in all the screened isolates (Figure 1). IS*Ecp1* is responsible for the transfer of the $bla_{CMY\text{-}2\text{-}like}$-*blc*-*sugE* region from the chromosome of *Citrobacter freundii* to a plasmid.[4] However, truncation of IS*Ecp1* was observed in 54.8% (46/84) of the isolates. IS*Ecp1* was truncated at the 5′ end in 28.6% (24/84) of these, as amplicons were not obtained when using the a–b or a–d primer combinations, whilst c–d primers produced amplification products (Table S1 and Figure 1). In the remaining 26.2% (22/84) of the isolates, IS*Ecp1* was truncated at the 3′ end; in these cases, a–d primers successfully amplified a product of 1584 bp instead of the 2164 bp expected.

Genetic organization of $bla_{DHA\text{-}1}$

We found a structure composed of seven consecutive open reading frames (ORFs) in 28 out of 30 $bla_{DHA\text{-}1}$-carrying isolates (93%). The genes were *orf-2* (98% identical to *orf-1* from *Morganella morganii* chromosome, accession no. AF055067),[5] $bla_{DHA\text{-}1}$, *ampR*, *qacE*Δ*1*, *sul1*, *orf-5* and the IS*26* element. One of the remaining two isolates had the IS*26* element downstream of $bla_{DHA\text{-}1}$; this was not found upstream or downstream of $bla_{DHA\text{-}1}$ in the other isolate (Figure 1). Screening for IS*CR1* was negative in all but one of the isolates. In this case, IS*CR1* could not be linked with the $bla_{DHA\text{-}1}$ gene, although Expand Long Template PCR System (Roche Applied Science) was also performed to efficiently amplify the large genomic DNA fragments (∼20 kb).

Genetic organization of $bla_{ACC\text{-}1}$

In all three isolates carrying $bla_{ACC\text{-}1}$, an IS*Ecp1* element truncated at the 5′ end was found upstream of this gene, while *gdha* (a gene derived from the chromosome of *Hafnia alvei*) was found downstream (Figure 1). IS*26* was not present in any of the isolates.

Discussion

In this work we characterized the genetic context of the largest collection to date of plasmid-mediated AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal AmpC enzymes,[9] describing the widest variety of *ampC* genes in a single study. The prevalence of AmpC enzymes is increasing,[1,9,20] mainly due to the transfer of these genes into mobilizable and conjugative plasmids.

Our results showed a close relationship between each *ampC* gene and the plasmid involved. Plasmids belonging to the A/C and I1 Inc groups are the most frequently reported $bla_{CMY\text{-}2}$ carriers.[3,4,7,20–23] In the present study, A/C and I1 Inc groups were also the most prevalent plasmids carrying $bla_{CMY\text{-}2}$, followed by IncK plasmids. Although the $bla_{DHA\text{-}1}$ gene has mainly been associated with IncFII plasmids in previous reports,[7,21,22] our collection exhibited a clear association with IncL/M replicons. Several recent studies have also described the association of $bla_{DHA\text{-}1}$ with IncL/M plasmids.[24,25] This new trend could be explained by a direct link between $bla_{DHA\text{-}1}$ and *qnrB* to the IncL/M plasmids identified in the present collection.[26] This association could explain why the 61.5% of transconjugants carrying $bla_{DHA\text{-}1}$ displayed reduced susceptibility to nalidixic acid.[26]

The three $bla_{ACC\text{-}1}$ genes were carried by I1 ($n=1$) and MOB_{F11} ($n=2$) plasmids. One of the MOB_{F11} relaxases had a sequence not previously reported. Data on plasmids carrying $bla_{ACC\text{-}1}$ are scarce. To our knowledge, the plasmid location of $bla_{ACC\text{-}1}$ has only been reported once, and the plasmid could not be typed by PBRT.[23] Thus, this is the first time that plasmids carrying $bla_{ACC\text{-}1}$ have been typed.

The fact that PBRT also failed to detect two out of three $bla_{ACC\text{-}1}$-carrying plasmids of our collection and that one of the MOB_{F11} relaxases found has a novel sequence suggests that the $bla_{ACC\text{-}1}$ gene could be carried by new backbones undetected by PBRT.

Plasmid associations of the recently-described $bla_{CMY\text{-}25}$, $bla_{CMY\text{-}27}$ were expected to be similar to those obtained for $bla_{CMY\text{-}2}$, as is the case for other $bla_{CMY\text{-}2}$ variants.[7] However, only one $bla_{CMY\text{-}27}$ was carried by an IncIα plasmid, as identified by the relaxase-typing method. One $bla_{CMY\text{-}4}$ and one $bla_{CMY\text{-}27}$ were carried by IncF plasmids, which are less commonly found associated with $bla_{CMY\text{-}2}$ and usually appear in multi-replicon plasmids; the plasmid carrying $bla_{CMY\text{-}25}$ found in a *K. pneumoniae* isolate could not be solved either by PBRT or by relaxase typing.

Among the remaining 22 isolates that were not solved by plasmid analysis, nine had the *ampC* gene chromosomally located, and six showed multiple hybridization bands with *ampC* and replicon probes. It is also remarkable that these six isolates also had copies of *ampC* located on the chromosome. A picture of broad gene dissemination should be kept in mind, since different hybridization bands were obtained from different transconjugants arising from the same donor. This suggests either that different co-integrations from plasmid harbouring *ampC* genes are formed during conjugation, or that a highly active MGE is involved in the *ampC* genes – moving between different replicons in the donor strain. Further studies are needed to address this issue.

Moreover, in seven out of eight *P. mirabilis* where $bla_{CMY\text{-}2}$ was located on the chromosome, *amp*C genes were mobilized by SXT/R391-like integrative and conjugative elements (ICEs).[27]

Furthermore, we analysed the regions surrounding the *ampC* genes. The genetic organization of $bla_{CMY\text{-}2}$ and its variants was highly conserved. All the isolates carried the transposon-like element IS*Ecp1* (IS*Ecp1*/ΔIS*Ecp1*-bla_{CMY}-*blc*-*sugE*), as documented in previous reports.[3,4,16] A well-conserved structure was also found in all isolates carrying $bla_{ACC\text{-}1}$ genes (ΔIS*Ecp1*-$bla_{ACC\text{-}1}$-*gdha*). However, although IS*26* is commonly related to the transmission of ACC-1 enzymes (and normally appears within IS*Ecp1*),[18,19] none of our isolates harboured this insertion sequence. The genetic organization of $bla_{DHA\text{-}1}$ was more variable. Mobilization of DHA-1 enzymes has been associated with

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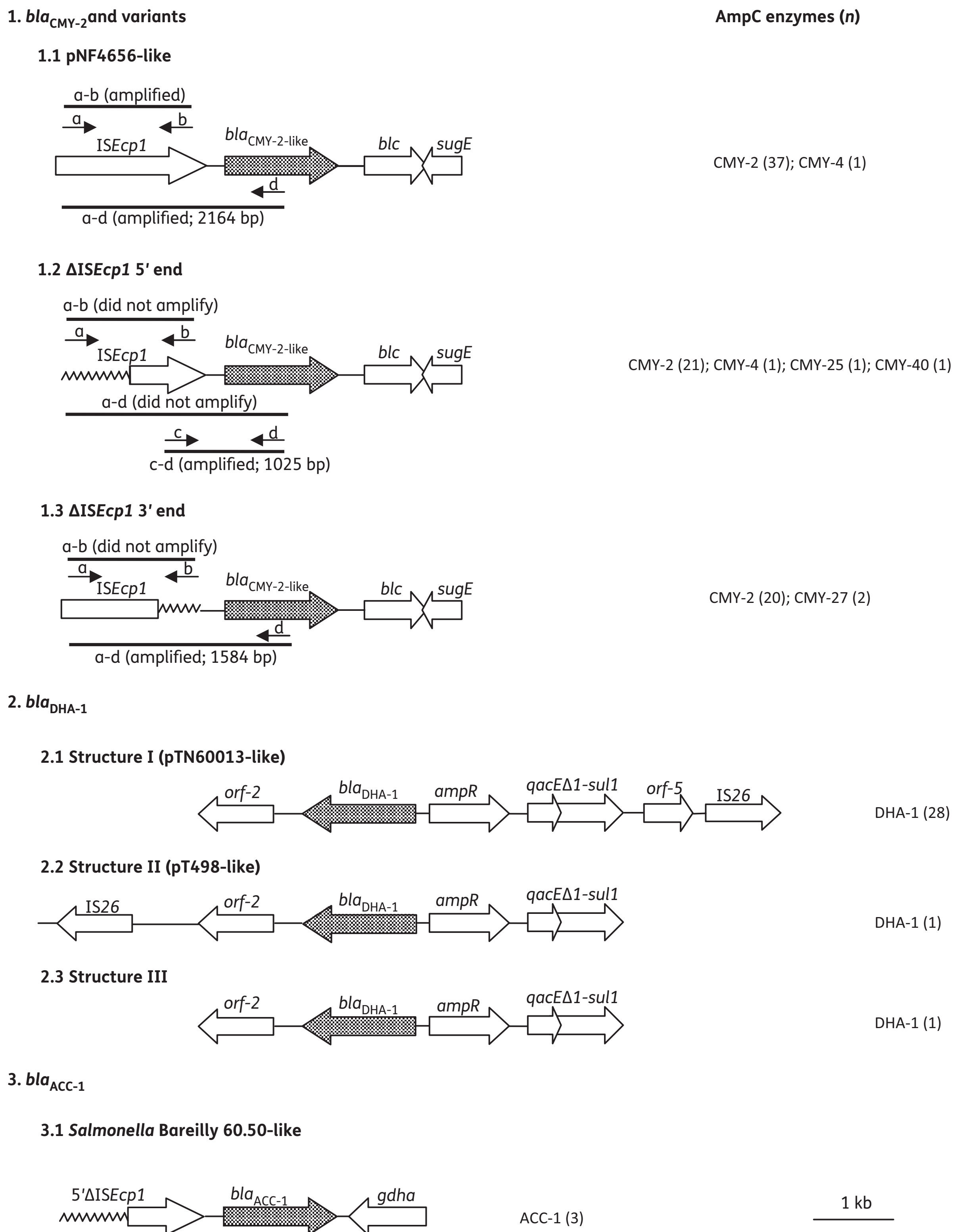

Figure 1. Genetic organization of the *ampC* genes. *ampC* genes are represented by filled arrows, while the surrounding genes are represented by white arrows. Truncated genes are represented by disrupted arrows. (1.1) 45.2% of the isolates showed an identical structure to pNF4656 (AY581207). (1.2) 28.6% of the isolates showed IS*Ecp1* truncated at the 5′ end. (1.3) 26.2% of the isolates showed IS*Ecp1* truncated at the 3′ end. (2.1) 93.3% of the isolates showed an identical structure to pTN60013 (AJ971345). (2.2) One isolate showed an identical structure to pT498 (AY705809). (2.3) In one isolate, IS*26* was not present either upstream or downstream of bla_{DHA-1}. (3.1) All three isolates showed an identical structure to *S. enterica* serovar Bareilly 60.50 (AY856832).

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IS*26* or class 1 integron-bearing IS*CR1* elements.[3,5,17] We found three similar structures (Figure 1), but IS*CR1* was not involved in any of them. Structures I and II were identical to those described in the *K. pneumoniae* plasmids pTN60013 (AJ971345) and pT498 (AY705809), respectively. The most prevalent was structure I (93.3% of cases). IS*26* and *qacE*Δ*1* and *sul1* genes – the last two belong to the 3′ conserved sequence of class 1 integrons – were present in both structures. In the single isolate displaying structure III, only *qacE*Δ*1* and *sul1* genes were found.

Although plasmids have been shown to be the main vehicles for the fast dissemination of *ampC* genes among clinically relevant bacteria, other MGEs may also play an important role in the marked increase in the prevalence of these enzymes. Further studies focusing not only on plasmids but also on other MGEs such as ICEs are needed to appreciate the complex processes involved in the dissemination of antibiotic resistance genes worldwide.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Jacoby GA. AmpC β-lactamases. *Clin Microbiol Rev* 2009; **22**: 161–82.

2 Su LH, Chu C, Cloeckaert A *et al.* An epidemic of plasmids? Dissemination of extended-spectrum cephalosporinases among *Salmonella* and other *Enterobacteriaceae*. *FEMS Immunol Med Microbiol* 2008; **52**: 155–68.

3 Naseer U, Haldorsen B, Simonsen GS *et al.* Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. *Clin Microbiol Infect* 2009; **16**: 171–8.

4 Verdet C, Gautier V, Chachaty E *et al.* Genetic context of plasmid-carried $bla_{CMY-2-like}$ genes in *Enterobacteriaceae*. *Antimicrob Agents Chemother* 2009; **53**: 4002–6.

5 Wei ZQ, Chen YG, Yu YS *et al.* Nosocomial spread of multi-resistant *Klebsiella pneumoniae* containing a plasmid encoding multiple β-lactamases. *J Med Microbiol* 2005; **54**: 885–8.

6 Carattoli A, Bertini A, Villa L *et al.* Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**: 219–28.

7 Carattoli A. Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob Agents Chemother* 2009; **53**: 2227–38.

8 Garcillán-Barcia MP, Francia MV, de la Cruz F. The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol Rev* 2009; **33**: 657–87.

9 Mata C, Miró E, Rivera A *et al.* Prevalence of acquired AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes at a Spanish hospital from 1999 to 2007. *Clin Microbiol Infect* 2010; **16**: 472–6.

10 Suarez A, Güttler A, Strätz M *et al.* Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. *Gene* 1997; **196**: 69–74.

11 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement M100-S17*. CLSI, Wayne, PA, USA, 2007.

12 García-Fernández A, Fortini D, Veldman K *et al.* Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*. *J Antimicrob Chemother* 2009; **63**: 274–81.

13 Alvarado A, Garcillán-Barcia M, de la Cruz F. A PCR-based method for classification of conjugative plasmids based on relaxase sequences. In: *Abstracts of the 2008 International Plasmid Biology Conference, Gdansk, Poland, 2008*. p.134. International Society for Plasmid Biology (ISPB), Pittsburgh, PA, USA.

14 Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. *Anal Biochem* 1995; **226**: 235–40.

15 Liu SL, Hessel A, Sanderson KE. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. *Proc Natl Acad Sci USA* 1993; **15**: 6874–8.

16 Kang MS, Besser TE, Call DR. Variability in the region downstream of the bla_{CMY-2} β-lactamase gene in *Escherichia coli* and *Salmonella enterica* plasmids. *Antimicrob Agents Chemother* 2006; **50**: 1590–3.

17 Verdet C, Benzerara Y, Gautier V *et al.* Emergence of DHA-1-producing *Klebsiella* spp. in the Parisian region: genetic organization of the *ampC* and *ampR* genes originating from *Morganella morganii*. *Antimicrob Agents Chemother* 2006; **50**: 607–17.

18 Partridge SR. Genetic environment of IS*Ecp1* and bla_{ACC-1}. *Antimicrob Agents Chemother* 2007; **51**: 2658–9.

19 Doloy A, Verdet C, Gautier V *et al.* Genetic environment of acquired bla_{ACC-1} β-lactamase gene in *Enterobacteriaceae* isolates. *Antimicrob Agents Chemother* 2006; **50**: 4177–81.

20 Mataseje L, Neumann N, Crago B *et al.* Characterization of cefoxitin-resistant *Escherichia coli* isolated from recreational beaches and private drinking water in Canada between 2004 and 2006. *Antimicrob Agents Chemother* 2009; **53**: 3126–30.

21 Hopkins KL, Batchelor MJ, Liebana E *et al.* Characterisation of CTX-M and *AmpC* genes in human isolates of *Escherichia coli* identified between 1995 and 2003 in England and Wales. *Int J Antimicrob Agents* 2006; **28**: 180–92.

22 González-Sanz R, Herrera-León S, de la Fuente M *et al.* Emergence of extended-spectrum β-lactamases and AmpC-type β-lactamases in

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human *Salmonella* isolated in Spain from 2001 to 2005. *J Antimicrob Chemother* 2009; **64**: 1181–6.

23 Dierikx C, van Essen-Zandbergen A, Veldman K *et al.* Increased detection of extended spectrum β-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet Microbiol* 2010; **145**: 273–8.

24 Mata C, Miró E, Mirelis B *et al.* *In vivo* transmission of a plasmid coharbouring $bla_{\text{DHA-1}}$ and *qnr*B genes between *Escherichia coli* and *Serratia marcescens*. *FEMS Microbiol Lett* 2010; **308**: 24–8.

25 Diestra K, Miró E, Marti C *et al.* Multiclonal epidemic of *Klebsiella pneumoniae* isolates producing DHA-1 in a Spanish hospital. *Clin Microbiol Infect* 2011; **17**: 1032–6.

26 Mata C, Miró E, Toleman M *et al.* Association of $bla_{\text{DHA-1}}$ and *qnr*B genes carried by broad-host-range plasmids among isolates of *Enterobacteriaceae* at a Spanish hospital. *Clin Microbiol Infect* 2011: doi:10.1111/j.469-0691.2011.03539.x.

27 Mata C, Navarro F, Miró E *et al.* Prevalence of SXT/R391-like integrative and conjugative elements carrying $bla_{\text{CMY-2}}$ in *Proteus mirabilis*. *J Antimicrob Chemother* 2010; **66**: 2266–70.

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ANNEX V

8.5. Prevalence of SXT/R391-like integrating conjugative elements carrying *bla*$_{CMY-2}$ in *Proteus mirabilis*

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Prevalence of SXT/R391-like integrative and conjugative elements carrying *bla*$_{CMY-2}$ in *Proteus mirabilis*

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Objectives: To characterize the vectors involved in the dissemination of *bla*$_{CMY-2}$ genes in clinical isolates of *Proteus mirabilis* collected between 1999 and 2007.

Methods: Plasmid analysis of 19 *P. mirabilis* carrying *ampC* genes was performed by PCR-based replicon typing, S1-PFGE and Southern hybridization with *ampC* and replicon probes. Isolates that could not be characterized were examined for the presence of SXT/R391-like elements. To demonstrate the involvement of these elements in the dissemination of *bla*$_{CMY-2}$, we performed a PCR amplification of the integrase (*int*) and toxin/antitoxin (*TA*) genes from SXT/R391-like integrative conjugative elements (ICEs). Later on, I-Ceu-I PFGE gels and hybridization with *bla*$_{CMY-2}$, *int* and *prfC* probes were performed. The genetic organization of *bla*$_{CMY-2}$ was also studied.

Results: *ampC* genes were located on large conjugative plasmids in 11 of the 19 (58%) *P. mirabilis* studied. However, in eight of these isolates a plasmid was not involved in the mobilization of *ampC* genes. I-Ceu-I PFGE and hybridization analyses revealed that *bla*$_{CMY-2}$ were chromosomally located in these eight *P. mirabilis* isolates. The genetic organization of *bla*$_{CMY-2}$ and hybridization analyses revealed that *bla*$_{CMY-2}$ was carried by an ICE almost identical to ICE*Pmi*Jpan1 in seven out of these eight isolates.

Conclusions: The prevalence of ICEs carrying *bla*$_{CMY-2}$ was surprisingly high [37% (7 out of 19)]. This is the first study giving prevalence data on ICEs carrying *bla*$_{CMY-2}$ genes. These results suggest the need to study these mobile genetic elements in the context of dissemination of acquired AmpC β-lactamases and also of other β-lactamases, such as extended-spectrum β-lactamases and carbapenemases.

Keywords: mobile genetic elements, AmpC β-lactamases, Enterobacteriaceae

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Introduction

Proteus mirabilis causes 90% of *Proteus* infections in humans and is responsible for urinary tract, wound and bloodstream infections. These isolates naturally lack *bla* genes on their chromosome, and are therefore generally susceptible to all β-lactam antibiotic agents. However, they have demonstrated a great ability to acquire resistance genes, such as inhibitor-resistant TEM β-lactamases (IRTs), extended-spectrum β-lactamases (ESBLs) and acquired *ampC* β-lactamases.[1–3]

Acquired AmpCs confer resistance to most β-lactams, including third-generation cephalosporins and cephamycins, with the exception of cefepime and carbapenems, and they are poorly inhibited by β-lactamase inhibitors.[4] CMY-2 is the most widely distributed acquired AmpC worldwide.[3,4] Plasmids are thought to be the main mechanism involved in the acquisition of these genes in *P. mirabilis*, via mobile genetic elements such as IS*Ecp1*, IS*5*, IS*10* and IS*1294*.[5,6] Nevertheless, there are a few reports in which the acquired *ampC* genes in *P. mirabilis* are located on the chromosome. During the preparation of this manuscript, a paper was published that described the mobilization of *bla*$_{CMY-2}$ via an integrative and conjugative element (ICE) in one *P. mirabilis* isolated in Japan.[6]

ICEs are self-transferable mobile genetic elements that are able to move from the chromosome of a donor to the chromosome of a recipient bacterium through a process of excision, conjugation and integration.[7] ICEs are composed of a highly conserved set of genes that mediate their core functions, and variable regions. The latter are subject to high recombination rates, allowing the ICEs to capture foreign genes such as antibiotic or heavy metal resistance genes.[6–8]

SXT and R391 ICEs were first discovered in isolates of *Vibrio cholerae* and *Providencia rettgeri*, respectively, but they have since been found in many other bacteria.[6,7] More than 30 elements belonging to the SXT/R391-like family have now been described, all possessing a highly conserved integrase gene.

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The encoded integrase mediates the site-specific integration of the ICE into the 5′ end of *prfC*, a conserved gene encoding the peptide chain release factor 3.[7]

The aim of this study was to characterize the vectors involved in the dissemination of bla_{CMY-2} genes in clinical isolates of *P. mirabilis* collected between 1999 and 2007.

Materials and methods

Bacterial isolates

A total of 2095 *P. mirabilis* isolates were examined for the presence of acquired AmpCs at a Spanish hospital between January 1999 and December 2007. The criteria used for selecting suspected AmpC producers has been described previously.[3] Out of these, 19 isolates harboured acquired *ampC* genes (18 bla_{CMY-2} and one bla_{CMY-2} plus $bla_{CTX-M-2}$).[3] Clonal relationships were established for five isolates (four identical PFGE and one probably related pattern). However, these isolates were not epidemiologically related.[3] Isolates were recovered from urine (45%), fluids and tissue (35%), and blood and respiratory sputum samples (10%). Eighty-five percent of patients carrying these isolates were between 70 and 96 years old.

Conjugation assays and susceptibility testing

Conjugation assays were performed using a broth mating method at 37°C.[9] A modified *Escherichia coli* HB101 (UAB190) was used as the recipient strain [rifampicin and aminoglycoside resistant and green fluorescent protein (GFP) producing]. Transconjugants were selected on Luria Bertani (LB) agar supplemented with ceftazidime (10 mg/L) and rifampicin (100 mg/L). Disc diffusion susceptibility tests were performed on parental and transconjugant strains according to CLSI guidelines,[10] using commercially available discs (Neo-Sensitabs, Rosco Diagnostica).

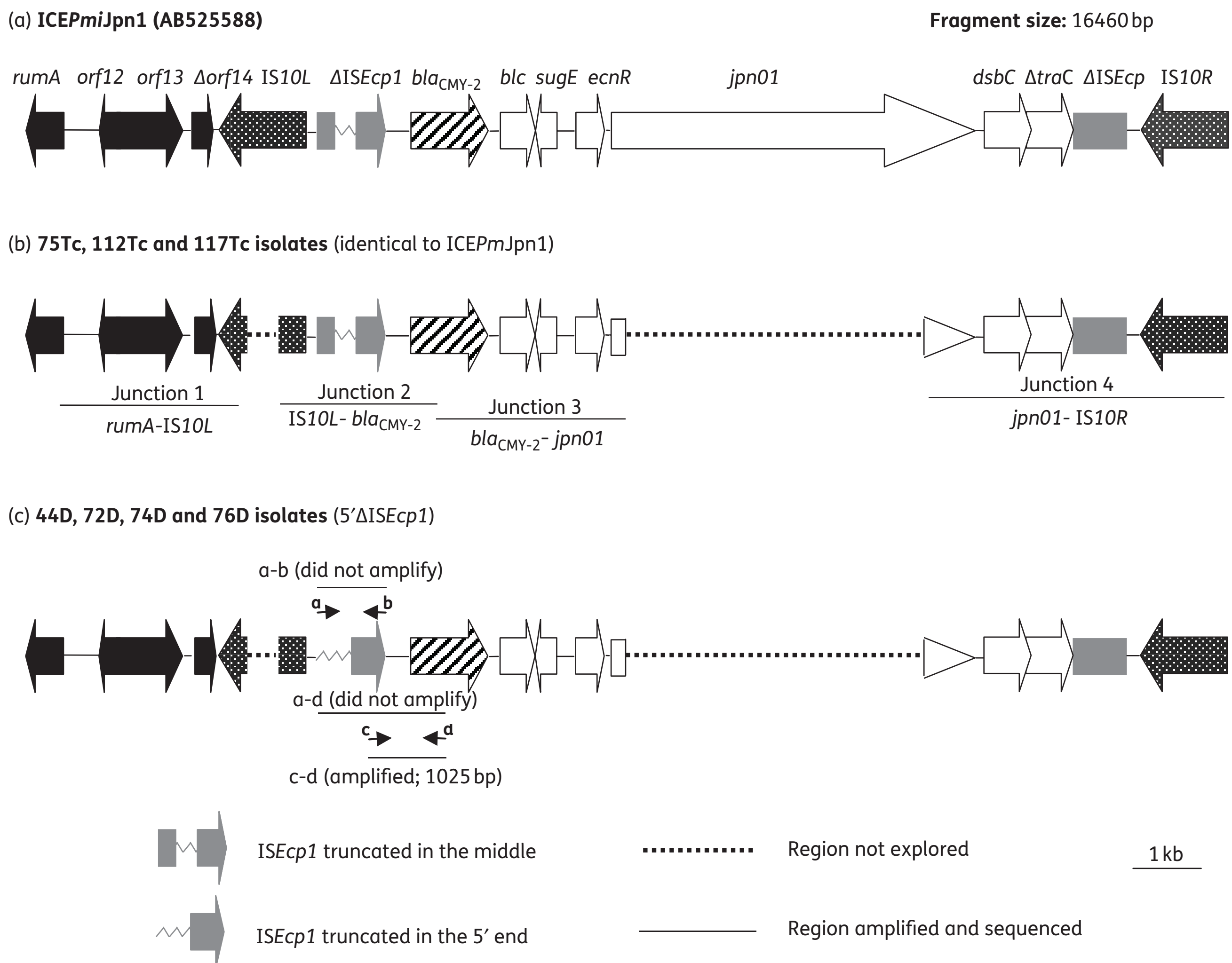

Figure 1. Genetic organization of bla_{CMY-2} in the seven *P. mirabilis* with SXT/R391 ICEs. Black arrows represent the conserved genes of the ICE. Dark grey stippled arrows represent the Tn*10* transposon. Light grey arrows represent the truncated IS*Ecp1*. Hatched arrows represent the bla_{CMY-2} gene. White arrows represent other genes present in this region. Thin black arrows represent different primer combinations used to explore this region. 'D' indicates donor isolates that did not produce transconjugants, 'Tc' indicates transconjugants. (a) 16460 bp region of the ICE*Pmi*Jpn1 used as a model to map the surrounding regions of the bla_{CMY-2} gene in the isolates of the study. (b) Schematic representation of the regions amplified and sequenced. Isolates 75Tc, 112Tc and 117Tc are identical to ICE*Pmi*Jpn1. (c) Isolates displaying an identical organization to ICE*Pmi*Jpn1, except for the IS*Ecp1* element that appears truncated in the 5′ end. Primer information is listed in Table S1.

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Plasmid analysis

Plasmid analysis was carried out by PCR-based replicon typing (PBRT),[11,12] DNA linearization with S1 nuclease followed by PFGE and Southern hybridization using *ampC* and replicon probes, as described previously.[9]

SXT/R391-like ICE analysis

Chromosomal DNA analysis in donor, transconjugant and recipient strains was performed by digesting the entire DNA with the I-Ceu-I enzyme, followed by PFGE.[13] PCR analysis was performed to determine the presence of two conserved SXT/R391 genes in all *P. mirabilis* isolates carrying the *ampC* gene on the chromosome. This included amplification of the integrase (*int*) and the toxin–antitoxin (*TA*) system and was carried out using the SXTINT F and SXTINT R primers for *int* and the SXTTA F and SXTTA R primer for *TA* (Table S1, available as Supplementary data at *JAC* Online). The presence of the circular intermediate form of the ICE was explored using the LE4 and RE4 primers (Table S1).[6]

Purified DNA products obtained from the PCR of *ampC*, *int* and *prfC* genes were used as probes to hybridize with the I-Ceu-I PFGE gels. To demonstrate that *ampC* genes were carried by the SXT/R391-like ICEs, an overlapping PCR amplification was performed using the model ICE*Pmi*Jpan1 (AB525688).[6] A section of 16460 bp was analysed by amplification of four regions, called junctions 1–4, covering most of the region from *rumA*, a conserved gene from the ICE, to the right-hand copy of IS*10* at the end of the *Tn*10 transposon (Figure 1). Sequencing of the purified PCR products of these four regions using transconjugant templates was performed. The primers used for this analysis are listed in Table S1.

Results and discussion

AmpC β-lactamases have been increasingly described in *P. mirabilis*.[3] In this study we characterized the vectors involved in the carriage of bla_{CMY-2} by 19 *P. mirabilis* isolates at a Spanish hospital between 1999 and 2007. Plasmid analysis by PBRT, S1-PFGE and Southern hybridization revealed that *ampC* genes were located on large conjugative plasmids in 11 of the 19 (58%) *P. mirabilis* studied, most of them belonging to the IncA/C group (8 out of 11; 73%). The remaining three isolates carried the *ampC* genes on plasmids belonging to the IncI1/Iγ, IncK and ColE groups (Table 1). However, in 8 of the 19 isolates a plasmid was not involved in the mobilization of the *ampC* genes (Table 1). I-Ceu-1 PFGE and hybridization analyses revealed that bla_{CMY-2} were chromosomally located in these isolates (Figure S1, available as Supplementary data at *JAC* Online). Furthermore, these isolates were not clonally related. Acquired

Table 1. Non-β-lactam antibiotic resistance profiles in donor and transconjugant *P. mirabilis* strains, location of *ampC* genes and plasmid size

Strains[a]	Resistance enzymes	Transconjugants[b]	Year of isolation	Non-β-lactam antibiotic resistance: clinical strain	Non-β-lactam antibiotic resistance: transconjugant	Replicon typing/resistance enzymes/plasmid size (kb)/ICEs
10	CMY-2	yes	2000	NAL, SSS, TMP, SXT, TET, CHL	SSS, TET, CHL	A/C^{CMY-2} (∼168)
27	CMY-2	no	2004	NAL, TMP, SXT, TET, CHL	—	—
28	CMY-2	yes	2004	NAL, SSS, TET	SSS, TET	A/C^{CMY-2} (∼165)
43	CMY-2	yes	2005	NAL, CIP, SSS, TMP, SXT, TET, CHL	SSS, TET, CHL	A/C^{CMY-2} (∼162)
44[c]	CMY-2, CTX-M-2	no	2005	NAL, CIP, SSS, TMP, SXT, TET, CHL	—	ICE^{CMY-2}
45	CMY-2	yes	2005	NAL, CIP, TMP, TET	none	K^{CMY-2} (∼82)
71	CMY-2	yes	2006	TET	none	ColE^{CMY-2} (∼97)
72[c]	CMY-2	no	2006	NAL, SSS, TET	—	ICE^{CMY-2}
73	CMY-2	yes	2006	NAL, CIP, SSS, TET, CHL	SSS, TET, CHL	A/C^{CMY-2} (∼159)
74[c]	CMY-2	no	2006	TMP, TET	—	ICE^{CMY-2}
75[c]	CMY-2	yes	2006	TET	none	ICE^{CMY-2}
76[c]	CMY-2	no	2006	NAL, SSS, TMP, SXT, TET, CHL	—	ICE^{CMY-2}
111	CMY-2	yes	2007	NAL, CIP, SSS, TET, CHL	SSS, TET, CHL	A/C^{CMY-2} (∼155)
112[c]	CMY-2	yes	2007	NAL, TET	none	ICE^{CMY-2}
113	CMY-2	yes	2007	NAL, CIP, SSS, TET, CHL	SSS, TET, CHL	A/C^{CMY-2} (∼170)
114	CMY-2	yes	2007	NAL, TMP, TET	none	I1/Iγ^{CMY-2} (∼83)
115	CMY-2	no	2007	NAL, CIP, SSS, TMP, SXT, TET, CHL	—	A/C^{CMY-2} (∼97)
116	CMY-2	yes	2007	NAL, CIP, SSS, TET, CHL	SSS, TET, CHL	A/C^{CMY-2} (∼150)
117[c]	CMY-2	yes	2007	NAL, CIP, TET	none	ICE^{CMY-2}

NAL, nalidixic acid; CIP, ciprofloxacin; SSS, sulphonamides; TMP, trimethoprim; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol.
Other antibiotics used for susceptibility testing were imipenem and chloramphenicol. As the recipient used for conjugation was resistant to several aminoglycoside agents, this antimicrobial group was not included in the study.
NAL*, the transconjugant strain resistant to NAL, displayed reduced susceptibility (14–18 mm).
[a]Names assigned to the strains.
[b]Refers to plasmids carrying *ampC* genes conjugated into the modified *E. coli* HB101 (UA6190) recipient strain.
[c]bla_{CMY-2} genes were carried by an SXT/R391-like ICE in these isolates.

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ampC genes have been described previously in the chromosome of *P. mirabilis*. Several IS*Ecp1*-mediated transpositions have been found to be involved in the mobilization of these *ampC* genes into the chromosome of a multidrug-resistant *P. mirabilis* clone in Europe.[14]

Remarkably, in three isolates from the study (75, 112 and 117) containing chromosomal bla_{CMY-2}, the gene was mobilized by conjugation. Moreover, I-Ceu-I PFGE gels displayed different patterns for the three transconjugants obtained (75Tc, 112Tc and 117Tc) and the recipient *E. coli* HB101. An insertion on the chromosome was observed in all three transconjugants; this involved the ~660 kb band from the recipient cell disappearing and a larger band of various sizes appearing in the transconjugants (Figure S1). All these data suggested that a putative ICE could be involved in the dissemination of bla_{CMY-2} in these isolates. These differences in size could be explained by the formation of different tandem arrays of the ICEs.[8]

PCR amplification of the *int* and *TA* genes from the SXT/R391 ICE family was positive in seven of the eight *P. mirabilis* with *ampC* genes located on the chromosome. Results obtained using RE4 and LE4 primers yielded a 546 bp product, suggesting the presence of a circular intermediate form of an ICE in all seven isolates positive for *int* and *TA*.

The integrase of the SXT/R391-like ICEs enables site-specific integration into the 5′ end of the chromosomal *prfC* gene.[7,8] Hybridization with the *prfC* probe demonstrated that this conserved gene was located in the ~660 kb band from the recipient cell (Figure S1). No hybridization was observed in the *P. mirabilis* donor isolates, presumably because of sequence differences between the *P. mirabilis prfC* and the *E. coli prfC* probe (Figure S1).

Hybridization analyses with labelled bla_{CMY-2} and SXT/R391 *int* family probes in the donor and transconjugant strains indicated that bla_{CMY-2} genes and the SXT/R391 element were located in the same portion of the genome in all but one of the isolates tested (Figure S1). It is remarkable that the 27D *P. mirabilis* isolate without the SXT/R391-like element showed at least four copies of bla_{CMY-2} on its chromosome (Figure S1). In this isolate neither a plasmid nor an SXT/R391-like ICE was involved in the dissemination of this gene.

The local genetic organization of the bla_{CMY-2} gene confirmed that bla_{CMY-2} was located inside an ICE, through an IS*10*-mediated transposition, in the seven *P. mirabilis* analysed (Figure 1). All the isolates analysed displayed an identical structure to that described in the *P. mirabilis* carrying ICE*Pmi*Jpan1, with the exception of isolates 44D, 72D, 74D and 76D, which showed an IS*Ecp1* truncated at the 5′ end instead of in the middle of the element. In these isolates, amplicons were not obtained with the primer combinations a–b and a–d, whilst combination of primers c and d amplified successfully (Figure 1).

The fact that ICEs carrying bla_{CMY-2} have been found in two widely distant parts of the world, Japan and Spain, probably indicates that ICEs play an important role in the dissemination of these resistance genes.

Although acquisition of *ampC* genes in Enterobacteriaceae has been mainly associated with plasmids, it is remarkable that the prevalence of ICEs carrying bla_{CMY-2} in our setting was surprisingly high. SXT/R391 ICEs related to the mobilization of bla_{CMY-2} were recovered in 37% (7 out of 19) of the *P. mirabilis* carrying these genes between 1999 and 2007. Furthermore, the seven *P. mirabilis* were collected during the last 3 years (Table 1), suggesting an increasing trend in mobilization of bla_{CMY-2} by this vector. This is the first study worldwide giving prevalence data on ICEs carrying bla_{CMY-2} genes. These results also suggest the necessity for surveillance of mobile genetic elements such as ICEs in the context of the dissemination of acquired AmpC β-lactamases and perhaps also for other resistance mechanisms, such as ESBLs and carbapenemases.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Aragón L, Mirelis B, Miró E *et al*. Increase in β-lactam-resistant *Proteus mirabilis* strains due to CTX-M- and CMY-type as well as new VEB- and inhibitor-resistant TEM-type β-lactamases. *J Antimicrob Chemother* 2008; **61**: 1029–32.

2 Endimiani A, Luzzaro F, Brigante G *et al*. *Proteus mirabilis* bloodstream infections: risk factors and treatment outcome related to the expression of extended-spectrum β-lactamases. *Antimicrob Agents Chemother* 2005; **49**: 2598–605.

3 Mata C, Miró E, Rivera A *et al*. Prevalence of acquired AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal *ampC* genes at a Spanish hospital from 1999 to 2007. *Clin Microbiol Infect* 2010; **16**: 472–6.

4 Jacoby G. AmpC β-lactamases. *Clin Microbiol Rev* 2009; **22**: 161–82.

5 Verdet C, Gautier V, Chachaty E *et al*. Genetic context of plasmid-carried bla_{CMY-2}-like genes in Enterobacteriaceae. *Antimicrob Agents Chemother* 2009; **53**: 4002–6.

6 Harada S, Ishii Y, Saga T *et al*. Chromosomally encoded bla_{CMY-2} located on a novel SXT/R391-related integrating conjugative element in a *Proteus mirabilis* clinical isolate. *Antimicrob Agents Chemother* 2010; **54**: 3545–50.

7 Wozniak RA, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 2010; **8**: 552–63.

8 Burrus V, Waldor MK. Formation of SXT tandem arrays and SXT-R391 hybrids. *J Bacteriol* 2004; **186**: 2636–45.

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9 Mata C, Miró E, Mirelis B *et al*. *In vivo* transmission of a plasmid coharbouring bla_{DHA-1} and *qnr*B genes between *Escherichia coli* and *Serratia marcescens*. *FEMS Microbiol Lett* 2010; **308**: 24–8.

10 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth Informational Supplement M100-S19*. CLSI, Wayne, PA, USA, 2009.

11 García-Fernández A, Fortini D, Veldman K *et al*. Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*. *J Antimicrob Chemother* 2009; **63**: 274–81.

12 Carattoli A, Bertini A, Villa L *et al*. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**: 219–28.

13 Liu S, Hessel A, Sanderson K. Genomic mapping with I-*Ceu* I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. *Proc Natl Acad Sci USA* 1993; **15**: 6874–8.

14 D'Andrea MM, Literacka E, Zioga A *et al*. Evolution and spread of a multidrug-resistant *Proteus mirabilis* clone with chromosomal AmpC-type cephalosporinases in Europe. *Antimicrob Agents Chemother* 2011; **55**: 2735–42.

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ANNEX VI

8.6. Association of *bla*$_{DHA-1}$ and *qnr*B genes carried by broad-host-range plasmids among isolates of *Enterobacteriaceae* at a Spanish hospital.

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Association of bla_{DHA-1} and *qnr*B genes carried by broad-host-range plasmids among isolates of *Enterobacteriaceae* at a Spanish hospital

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Abstract

A collection of 30 DHA-1-*Enterobacteriaceae* producers was examined for the presence of *qnr* genes. PCR-based replicon typing, plasmid profile and Southern hybridisation analyses revealed that all isolates co-harboured bla_{DHA-1} and *qnr*B genes on the same plasmid. All but one of these plasmids belonged to the L/M group. Genetic organization analyses of a randomly selected isolate revealed the co-localization of both genes on an IS*26*-composite transposon. As plasmids carrying both genes seem to have a high prevalence and a worldwide distribution, care should be taken when quinolones are used to treat infections caused by DHA-1 producers.

Keywords: IncN, IS*26*-composite transposon, L/M incompatibility group, plasmid-mediated AmpC β-lactamases, plasmid-mediated quinolone resistance

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DHA-1, an inducible acquired AmpC enzyme conferring resistance to most β-lactamams, and QnrB, a plasmid-mediated enzyme conferring low resistance to quinolones, have been widely associated with clinical isolates of the family *Enterobacteriaceae* [1,2]. Although these enzymes were first described in 1992 and 2006, respectively [3,4], it was not until some years later that their presence noticeably increased, and they became the most prevalent enzymes of their groups in some countries [1,5,6]. As a strong correlation has been reported between these two resistance mechanisms [6], the aim of this study was to analyse the presence of *qnr* genes on a large characterized collection of DHA-1-*Enterobacteriaceae* producers to determine whether they were localized on the same or different plasmids. As in some cases bla_{DHA-1} and *qnr*B genes have been linked together on a complex sul1-type integron [7], we also analysed their genetic organization on the plasmid.

A total of 30 *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes encoding bla_{DHA-1} or bla_{DHA-1} plus $bla_{CTX-M-14,-15}$ were collected from a Spanish hospital (Hospital de la Santa Creu i Sant Pau, Barcelona) from 2005 to 2007 [8]. Bacterial isolates were mainly recovered from urine (76.7%) and most of them (83.3%) were from patients aged 70 to 95 years old. Occurrence of these resistance determinants increased over this period from six cases in 2005 to 16 cases in 2007 [8]. No clonal relationship was established between these isolates except for one cluster of two *E. coli*, one cluster of two *K. pneumoniae* co-producing DHA-1 plus CTX-M-15 and one cluster of two *K. oxytoca* [8].

Using the broth mating method [9], transfer of bla_{DHA-1} genes via conjugation was possible in 86.7% (26/30) of the tested strains, with a conjugation frequency ranging from 10^{-5} to 10^{-8} transconjugants per recipient. Transconjugants were selected on LB agar supplemented with ceftazidime (10 mg/L) and rifampin (100 mg/L). Among the five isolates co-harbouring bla_{DHA-1} plus $bla_{CTX-M14,-15}$ genes, both resistant determinants were transferred in three of them (101-Tc, 103-Tc and 104-Tc) (Table 1).

Antibiotic agents used in the disc diffusion susceptibility test in donor and transconjugant strains are listed in Table 1. All donor and transconjugant strains showed high-level resistance to most β-lactam antibiotic agents, as expected by an *amp*C-producer. Antibiotics displaying higher activity against AmpC β-lactamases were imipenem, cefepime and aztreonam, with percentages of susceptible isolates of 100%, 92.3% and 84.6%, respectively (data not shown). These isolates also showed reduced susceptibility to other antibiotic families (Table 1). Non-β-lactam antibiotics transferred via conjugation were nalidixic acid (61.5%), sulphonamides (30.8%), trimethoprim (15.4%), tetracycline (11.5%) and trimethoprim-sulphamethoxazole (7.7%) (Table 1).

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TABLE 1. Non-β-lactam resistance profiles in donor and transconjugant strains, location of *bla* and *qnr*B genes and plasmid size

Strains-D/Tc[a]	Species	Isolation data[b]	Non-β-lactam resistance profile[c]		Replicon typing/resistant enzymes/plasmid size (kb)[d]
			Donors	Transconjugants	
35-Tc	*E. coli*	01/05	SSS,SXT, TET, NAL, CIP	SSS	L/M-FIA$^{DHA-1,QnrB}$ (≈171)
36-Tc	*E. coli*	06/05	SSS, TET	NAL	L/M$^{DHA-1,QnrB}$ (≈209)
37-Tc	*E. coli*	07/05	SSS, TMP, SXT, TET, NAL, CIP	SSS, NAL	L/M$^{DHA-1,QnrB}$ (≈100 and 312)[e]
38-Tc	*E. coli*	12/05	SSS, TMP, SXT, NAL, CIP	–	L/M$^{DHA-1,QnrB}$ (≈79)
62-Tc	*E. coli*	06/06	SSS, TMP, SXT, TET, CHL, NAL, CIP	SSS, NAL	L/M$^{DHA-1,QnrB}$ (≈74 and 164)[e]
63-Tc	*E. coli*	07/06	NAL, CIP	NAL	L/M$^{DHA-1,QnrB}$ (≈293)
64-D	*E. coli*	12/06	–	–	F, FIB (lysed)
95-Tc	*E. coli*	01/07	NAL	NAL	L/M$^{DHA-1,QnrB}$ (≈236)
96-Tc	*E. coli*	06/07	SSS, TMP, SXT, NAL, CIP	NAL	L/M$^{DHA-1,QnrB}$ (≈96)
97-Tc	*E. coli*	07/07	SSS, TET, CHL, NAL, CIP	NAL	L/M$^{DHA-1,QnrB}$ (≈173)
98-Tc	*E. coli*	07/07	TET, NAL, CIP	TET, NAL	N$^{DHA-1,QnrB}$ (≈52)
99-Tc	*E. coli*	08/07	SSS, TMP, SXT, TET, NAL, CIP	NAL	L/M-FIA$^{DHA-1,QnrB}$ (≈155)
100-D	*E. coli*	07/07	SSS, TMP, SXT, TET, NAL, CIP	–	L/M, F, FIB (lysed)
101-Tc	*E. coli*	09/07	NAL	NAL	L/M$^{DHA-1,QnrB}$ (≈156), I1/I$\gamma^{CTX-M-14}$ (≈88)
102-Tc	*E. coli*	12/07	SSS, TMP, SXT, TET, NAL, CIP	TMP, TET, NAL	L/M$^{DHA-1,QnrB}$ (≈93), F-FIB (≈148)
40-D	*K. pneumoniae*	01/05	–	–	L/M$^{DHA-1,QnrB}$ (≈79)
41-Tc	*K. pneumoniae*	06/05	NAL	NAL	L/M$^{DHA-1,QnrB}$ (≈202)
66-D	*K. pneumoniae*	08/06	TMP, CHL, NAL	–	L/M$^{DHA-1,QnrB}$ (≈72)
67-Tc	*K. pneumoniae*	10/06	–	–	L/M$^{DHA-1,QnrB}$ (≈72)
103-Tc	*K. pneumoniae*	02/07	TMP, NAL, CIP	TMP, SXT	L/M$^{DHA-1,QnrB,CTX-M-15}$ (≈168)
104-Tc	*K. pneumoniae*	03/07	TMP, NAL, CIP	TMP	L/M-FIC$^{DHA-1,QnrB,CTX-M-15}$ (≈153)
105-Tc	*K. pneumoniae*	06/07	SSS, TMP, SXT, TET, CHL, NAL, CIP	–	L/M$^{DHA-1,QnrB}$ (≈72)
106-Tc	*K. pneumoniae*	06/07	NAL	NAL	L/M$^{DHA-1,QnrB}$ (≈72)
107-Tc	*K. pneumoniae*	08/07	NAL, CIP	–	L/M$^{DHA-1,QnrB}$ (≈72)
108-Tc	*K. pneumoniae*	10/07	SSS, TMP, SXT, TET, CHL, NAL, CIP	–	L/M$^{DHA-1,QnrB}$ (≈70)
68-Tc	*K. oxytoca*	11/06	–	–	L/M$^{DHA-1,QnrB}$ (≈72)
69-Tc	*K. oxytoca*	12/06	SSS, TMP, SXT, TET	–	L/M$^{DHA-1,QnrB}$ (≈72)
109-Tc	*K. oxytoca*	06/07	SSS, TMP, SXT, TET, NAL, CIP	NAL	L/M-FIC$^{DHA-1,QnrB}$ (≈153)
110-Tc	*K. oxytoca*	08/07	NAL	NAL	L/M$^{DHA-1,QnrB}$ (≈112)
77-Tc	*P. mirabilis*	10/06	SSS, TMP, SXT, TET, NAL, CIP	SSS, TMP, SXT, TET, NAL	L/M-N$^{DHA-1,QnrB}$ (≈103)

Ampicillin, piperacillin, cephalotin, cefuroxime, cefotaxime, ceftazidime, cefepime, aztreonam, cefoxitin, amoxicillin-clavulanic acid, piperacillin-tazobactam and imipenem were also used for susceptibility testing. As the recipient used for conjugation was resistant to aminoglycoside antibiotics, this antibiotic family was not included in the study.
NAL, nalidixic acid; CIP, ciprofloxacin; SSS, sulphonamides; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol.
[a]Names assigned to the strains. D, donor strains due to the inability to obtain transconjugants; Tc, transconjugant strains.
[b]Isolation date of donor strains (month/year).
[c]The disc diffusion susceptibility test was performed according to Clinical Laboratory Standards Institute (CLSI) guidelines. All transconjugant strains resistant to NAL displayed reduced susceptibility (14–18 mm; intermediate as clinical category).
[d]Plasmid analyses results given are from transconjugant strains with the exception of 40-D, 64-D, 66-D and 100-D due to the inability to obtain transconjugants in these isolates.
[e]*bla*$_{DHA-1}$, L/M replicon and *qnr*B probes hybridized in both plasmids present in this isolate.

Screening of *qnr* genes in all 30 isolates was carried out by PCR amplification [10]. *qnrB* genes were present in all isolates tested. These results were in agreement with other reports that found a close association between *qnr*B, especially *qnr*B4, and *bla*$_{DHA-1}$ determinants in isolates of the family *Enterobacteriaceae* [6,11–13].

PCR-based replicon typing, plasmid profile and Southern hybridization analyses were performed in all 30 DHA-1 producers (Table 1) as previously described [9]. Two of these isolates were lysed during the S1-PFGE and therefore could not be analysed. To determine whether both resistance genes were localized on the same or different plasmids, hybridization with *bla*$_{DHA-1}$ and *qnr*B probes was performed. Results revealed the co-localization of *bla*$_{DHA-1}$ and *qnr*B resistance genes on the same conjugative plasmid in all 28 isolates tested (Table 1). All but one of the plasmids associated with *qnr*B, *bla*$_{DHA-1}$ or *bla*$_{DHA-1}$ plus *bla*$_{ESBL}$ belonged to the broad-host-range L/M plasmids (Table 1), while *bla*$_{DHA-1}$ has traditionally been found on narrow-host-range IncFII plasmids [14]. The single isolate not linked to the IncL/M group, carried both resistance genes on an IncN plasmid. This is the first time that both *bla*$_{DHA-1}$ and *qnr*B genes have been described on an IncN plasmid [14]. The new localization of *bla*$_{DHA-1}$ and *qnr*B4 genes in broad-host-range L/M plasmids has been postulated as a possible cause of the lately widespread distribution of *bla*$_{DHA-1}$ genes [9]. Our results reinforce this idea, together with the fact that a strong association between *qnr*B and *bla*$_{DHA-1}$ has been evidenced worldwide [2,6,7,15] and that recent reports have found *bla*$_{DHA-1}$ and *qnr*B genes also in L/M plasmids [16].

The genetic organization of these 30 isolates carrying *bla*$_{DHA-1}$ has been previously analysed by overlapping PCR amplification, exploring the most frequent regions surrounding *amp*C genes described in the literature (data not shown). As 93.3% (28/30) of the isolates displayed the same genetic organization, an IS*26* element and a region from the *Morganella morganii* genome found upstream and downstream of *bla*$_{DHA-1}$, respectively, one of them was randomly selected for further analyses. Plasmid extraction of this *E. coli* isolate

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was performed. Isolated plasmids were sequenced by Eurofins MWG Operon using GS FLX Titanium series chemistry technology. Partial analysis of the sequenced plasmid, called pEC37Tc, showed that bla_{DHA-1} and *qnr*B4 were localized together on an IS*26*-composite transposon, most likely involved in their mobilization (Fig. 1). Other non-resistance genes found in this structure were the 3′CS extreme of a class 1 integron and the stress-inducible psp (phage shock protein) operon (Fig. 1). Under a wide variety of environmental signals, the psp operon is highly transcribed to conserve membrane integrity, efficient translocation and maintenance of the proton motive force [17]. This structure, carrying beneficial traits to the bacteria such as antibiotic resistance genes and a wide response to environmental factors, may contribute to the maintenance of the plasmid in the bacteria. Linkage of bla_{DHA-1} and *qnr*B4 genes on similar structures has so far been described in isolates of *Klebsiella* spp, mainly *K. pneumoniae* [7,18,19]. To our knowledge, this is the first time that this genetic organization is described in an *E. coli* strain.

The presence of *qnr* genes has been associated with an increase in the quinolone MIC values and treatment failures when quinolones are used to treat quinolone-susceptible enterobacteria [15]. As plasmids carrying both resistance determinants seem to have a high prevalence and a worldwide distribution, care should be taken when quinolones are used to treat infections caused by DHA-1-*Enterobacteriaceae* producers.

Nucleotide Sequence Accession Number

The GenBank accession number of the sequence presented here is HQ700359 (pEC37Tc).

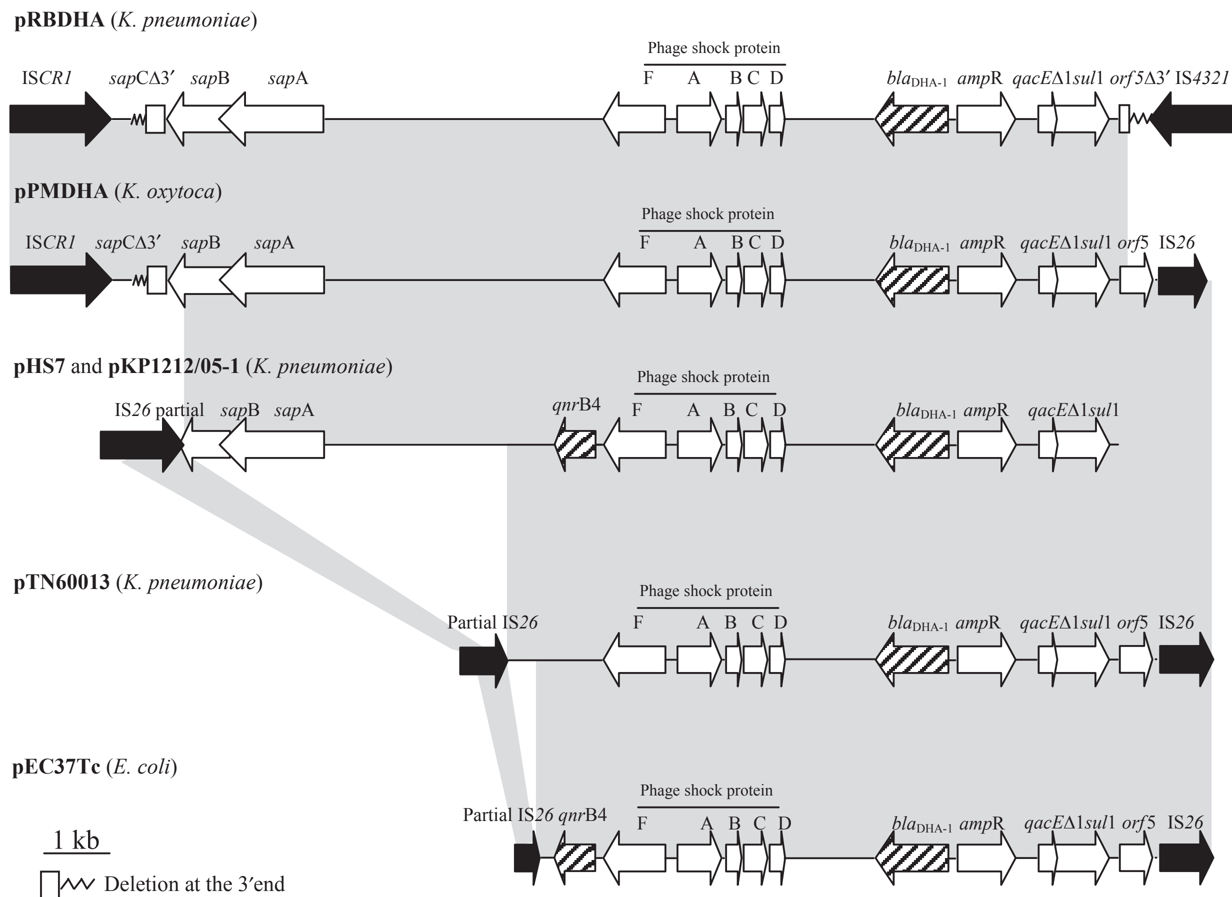

FIG. 1 Genetic organization of pEC37Tc in comparison with the common regions of other plasmids carrying bla_{DHA-1} and *qnr*B genes. GenBank accession numbers of pRBDHA, pMPDHA, pHS7, pKP1212/05-1, pTN60013 and pEC37Tc are AJ971343, AJ971344, EF683583, FJ943500, AJ971345 and HQ700359, respectively. Genes are represented by arrows. The resistant genes *qnr*B4 and bla_{DHA-1} are represented by filled arrows. Insertion sequences are represented by black arrows. Shaded regions are regions of 100% identity. Although *qnr*B4 genes are present in all the plasmids shown, the arrow for this gene is not drawn in pRBDHA, pMPDHA and pTN60013 because it is not annotated in the submitted GenBank sequence.

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Transparency Declaration

The authors declare that they have no conflict of interest.

References

1. Jacoby G. Ampc β-lactamases. *Clin Microbiol Rev* 2009; 22: 161–182.
2. Cattoir V, Nordmann P. Plasmid-mediated quinolone resistance in gram-negative bacterial species: an update. *Curr Med Chem* 2009; 16: 1028–1046.
3. Gaillot O, Clement C, Simonet M, Philippon A. Novel transferable β-lactam resistance with cephalosporinase characteristics in *Salmonella enteritidis*. *J Antimicrob Chemother* 1997; 39: 85–87.
4. Jacoby GA, Walsh KE, Mills DM *et al. Qnr*B, another plasmid-mediated gene for quinolone resistance. *Antimicrob Agents Chemother* 2006; 50: 1178–1182.
5. Song W, Kim J, Kim H *et al.* Increasing trend in the prevalence of plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking chromosomal *amp*C gene at a Korean university hospital from 2002 to 2004. *Diagn Microbiol Infect Dis* 2006; 55: 219–224.
6. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev* 2009; 22: 664–689.
7. Verdet C, Benzerara Y, Gautier V, Adam O, Ould-Hocine Z, Arlet G. Emergence of DHA-1-producing *Klebsiella* spp. in the parisian region: genetic organization of the *ampC* and *ampR* genes originating from *Morganella morganii*. *Antimicrob Agents Chemother* 2006; 50: 607–617.
8. Mata C, Miró E, Rivera A, Mirelis B, Coll P, Navarro F. Prevalence of acquired AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *amp*C genes at a Spanish hospital from 1999 to 2007. *Clin Microbiol Infect* 2010; 16: 472–476.
9. Mata C, Miró E, Mirelis B *et al. In vivo* transmission of a plasmid co-harbouring *bla*$_{\text{DHA-1}}$ and *qnr*B genes between *Escherichia coli* and *Serratia marcescens*. *FEMS Microbiol Lett* 2010; 308: 24–28.
10. Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, Hooper DC. *Qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. *Antimicrob Agents Chemother* 2006; 50: 2872–2874.
11. Park YJ, Yu JK, Lee SG, Oh EJ, Woo GJ. Prevalence and diversity of *qnr* alleles in *amp*C-producing *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Serratia marcescens*: a multicentre study from Korea. *J Antimicrob Chemother* 2007; 60: 868–871.
12. Tamang MD, Seol SY, Oh JY *et al.* Plasmid-mediated quinolone resistance determinants *qnr*A, *qnr*B, and *qnr*S among clinical isolates of *Enterobacteriaceae* in a Korean hospital. *Antimicrob Agents Chemother* 2008; 52: 4159–4162.
13. Pai H, Seo MR, Choi TY. Association of *qnr*B determinants and production of extended-spectrum β-lactamases or plasmid-mediated AmpC β-lactamases in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2007; 51: 366–368.
14. Carattoli A. Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob Agents Chemother* 2009; 53: 2227–2238.
15. Martinez-Martinez L, Cano ME, Rodriguez-Martinez JM, Calvo J, Pascual A. Plasmid-mediated quinolone resistance. *Expert Rev Anti-Infect Ther* 2008; 6: 685–711.
16. Diestra K, Miró E, Marti C *et al.* Multiclonal epidemic of *Klebsiella pneumoniae* isolates producing DHA-1 in a Spanish hospital. *Clin Microbiol Infect* doi: 101111/j1469-0691201003319x. 2011.
17. Huvet M, Toni T, Sheng X *et al.* The evolution of the phage shock protein (psp) response system: interplay between protein function, genomic organization and system function. *Mol Biol Evol* 2011; 28: 1141–1155.
18. Hu FP, Xu XG, Zhu DM, Wang MG. Coexistence of *qnr*B4 and *qnr*S1 in a clinical strain of *Klebsiella pneumoniae*. *Acta Pharmacol Sin* 2008; 29: 320–324.
19. Jiang Y, Yu D, Wei Z, Shen P, Zhou Z, Yu Y. Complete nucleotide sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKP048, carrying *bla*$_{\text{KPC-2}}$, *bla*$_{\text{DHA-1}}$, *qnr*B4, and *arm*A. *Antimicrob Agents Chemother* 2010; 54: 3967–3969.

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