

Plasmid-Encoded AmpC (pAmpC) in *Enterobacteriaceae*: epidemiology of microorganisms and resistance markers

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ABSTRACT

CMY-2 β -lactamase is an important cause of β -lactam resistance in *Enterobacteriaceae* and constitutes the most widespread plasmid-encoded AmpC. Although CMY-2 has been previously recognized in our region, the real prevalence and epidemiology of this resistance marker was uncertain. During August-October 2009, we conducted a multicenter, prospective study to determine pAmpC prevalence and to characterize CMY-2 producing *Escherichia coli* associated plasmids. Plasmid-encoded AmpC prevalence was 0.9 % in enterobacteria in this period, being CMY-2 prevalent and to a lesser extent DHA. Molecular typing of CMY-2- producing *Escherichia coli* isolates showed several lineages. Moreover, replicon typing of *cmy-2*- containing plasmids displayed a broad diversity in Inc/cmy-2 links. Therefore, association of *cmy-2* with specific transposon elements may be responsible for the spread of this resistance marker in *Enterobacteriaceae*.

Key words: plasmid-encoded AmpC, CMY-2 β -lactamase, cephalosporin resistance

RESUMEN

β -lactamasas de tipo AmpC de codificación plasmídica (pAmpC) en *Enterobacteriaceae*: epidemiología de los microorganismos y de los marcadores de resistencia. La β -lactamasa de tipo AmpC de codificación plasmídica CMY-2 es la de mayor diseminación a nivel mundial en *Enterobacteriaceae*. Esta ha sido comunicada esporádicamente en nuestro país. Entre agosto y octubre de 2009 se llevó a cabo un estudio prospectivo y multicéntrico con el objetivo de determinar la prevalencia de AmpC plasmídicas en nuestro medio y de caracterizar a los microorganismos productores y a los plásmidos portadores de estos marcadores de resistencia. La prevalencia de AmpC plasmídicas en enterobacterias en este período fue de 0,9 %. La β -lactamasa CMY-2 fue la enzima prevalente y, en menor medida, la DHA. La tipificación molecular de los aislamientos de *Escherichia coli* productores de CMY-2 mostró la presencia de distintos linajes, y los plásmidos portadores de *cmy-2* pertenecieron a una amplia diversidad de grupos de incompatibilidad. Se determinó la asociación corriente arriba de *cmy-2* con ISEcp1, el cual podría ser responsable de la amplia diseminación de este marcador de resistencia en *Enterobacteriaceae*.

Palabras clave: AmpC plasmídicas, CMY-2, resistencia a cefalosporinas

β -lactamase production constitutes the main β -lactam resistance mechanism in gram-negative bacteria. Resistance to 7- α -methoxy- and oxyiminocephalosporins initially emerged in organisms, such as *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens* and *Pseudomonas aeruginosa*, which overproduced their chromosomal AmpC β -lactamase (11). By the end of the 80's, both cephalexin and oxyimino-cephalosporin resistance emerged among enterobacterial species lacking chromosomal inducible AmpC β -lactamases. Plasmid-encoded *ampC* genes were found to be responsible for this resistant profile (1). Like their counterparts on the chromosome, such

enzymes prefer cephalosporins, displaying low affinity for cefepime, cefpirome and carbapenems, and are not inhibited by commercially available inhibitors (6). Plasmid-encoded AmpC (pAmpC) enzymes have been clustered into nine groups (7), including 90 CMY alleles, 13 variants of ACT and 10 of FOX, 8 variants of DHA and MOX, 5 of MIR and ACC and CFE-1 and LAT-1 (<http://www.lahey.org/Studies/>). Most of these groups are linked to chromosomal genes that represent their possible ancestors (7).

CMY β -lactamases have been reported worldwide in gram-negative bacteria from both nosocomial and community origin, being CMY-2 the most prevalent

(7). This plasmid encoded β -lactamase is related to the chromosomal AmpC gene of *C. freundii*. Plasmids harboring CMY-2 coding genes have been reported in many regions of the world belonging to IncA/C, IncQ and Incl1 replicon type (2, 7). Besides, *cmy-2* genes have been associated upstream with *ISEcp1*, and downstream with *b/c* and *sugE* coding for a lipoprotein and a multidrug resistance protein, respectively (10, 14).

Despite the increasing recognition of CMY β -lactamases worldwide, these enzymes were not reported in Argentina until 2006 in *Shigella flexneri*, and later in *Citrobacter koseri*, *Klebsiella pneumoniae*, *Escherichia coli* and *Proteus mirabilis* (Cejas D. et al. 2008. Presented at the XIII Jornadas Argentinas de Microbiología, Rosario, Argentina; Radice M. et al. 2007. Presented at the 47 ICAAC, Chicago, USA) (8, 13). Plasmid-encoded AmpC β -lactamases have been sporadically reported, although little was known about the real incidence and epidemiology of this resistance marker.

We conducted a prospective multicenter study in order to determine the prevalence of pAmpC, to identify different enzymes and to characterize the association of their coding genes to mobile elements.

All *E. coli* and all non-inducible chromosomal AmpC producing enterobacteria recovered from seven different hospitals between August-October 2009 were included. Those isolates that displayed resistance to cefoxitin (FOX) and/or inhibition zones for cefotaxime (CTX) ≤ 27 mm and/or ceftazidime (CAZ) ≤ 22 mm were further analyzed.

Susceptibility was determined by diffusion and dilution tests according to Clinical and Laboratory Standards Institute (CLSI) guidelines (4). Phenotypic detection of AmpC β -lactamases was performed by the disk diffusion synergy test using (300 μ g) phenylboronic acid (APB) disks (15). Molecular confirmation was conducted by multiplex-PCR amplification of pAmpC coding genes (12) on plasmid DNA extracted as described by Kado et al. (9). *ampC* genes were identified using the following primers: *cmy* (CMY-F: ATG ATG AAA AAA TCG TTA TGC T and CMY-R: TTA TTG CAG CTT TTC AAG AAT GCG) and *dha* (DHA-F: TCT GTC TGG TGA ATC TGA CGA and DHA-R CTC ATC CTC CAT AAA ACA GCC) and amplicon sequencing. *cmy-2* containing plasmids were transformed into *E. coli* DH5 α and transformants were selected on Luria Bertani plates supplemented with (10 μ g/ml) ceftazidime. Replicon typing of *cmy* containing plasmids was performed as described by

Carattoli et al. (3) on the transformant cells. The genetic context of *cmy-2* was determined by PCR mapping and sequencing, using different primer combinations that are shown in Figure 1. Molecular typing of *E. coli* isolates was carried out by PCR amplification of enterobacterial repetitive intergenic consensus sequences (ERIC-PCR) and a dendrogram was built with the Treecon program, using UPGMA algorithm and applying the DICE correlation coefficient.

A total of 2202 enterobacteria were isolated within this period. Among them, 82.9 % corresponded to *E. coli*, 7.9 % to *K. pneumoniae* and 4.4 % to *P. mirabilis*. Resistance to FOX was 1.2 %, 2.28 % and 1.03 % in *E. coli*, *K. pneumoniae* and *P. mirabilis* respectively. Four point one percent of *E. coli* and 24.6 % of *K. pneumoniae* isolates displayed inhibition zones ≤ 27 mm for CTX and/or ≤ 22 mm for CAZ. The synergy test using APB was positive for 21 isolates, suggesting the presence of AmpC β -lactamases. Multiplex PCR for AmpC coding genes rendered positive results on plasmid extracted DNA from 19 isolates. These pAmpC-producing isolates were mainly recovered from urinary tract infections. They were resistant to ampicillin, amoxicillin/clavulanic acid, cephalotin and cefoxitin, and susceptible to cefepime, imipenem and meropenem. According to CLSI 2009 breakpoints, many of these isolates were categorized as intermediate even susceptible to CTX and/or CAZ (Table 1). If the oxyimino-cephalosporin susceptibility is interpreted according to current CLSI 2011 breakpoints (5), all isolates should be categorized as resistant to both CAZ and CTX by the disk diffusion test. Three isolates should be categorized as intermediate for CAZ and only 1 for CTX by dilution tests according to current breakpoints (5). Susceptibility to ciprofloxacin, gentamicin, amikacin and trimethoprime/sulfametoxazole was variable.

Using *cmy* primers, a 1100 bp amplicon was obtained on 17 *E. coli* DNA samples, while conducting amplification of *dha* genes, a 1100 bp amplicon was obtained for *P. mirabilis* and *K. pneumoniae* plasmids. Amplicon sequences corresponded to *cmy-2* and *dha-1* respectively. Replicon typing of *cmy-2* encoding plasmids recognized different Inc groups: BO, K, I1, Y, F (Table 1). *cmy-2* was related upstream with specific transposable element *ISEcp1* and downstream with *b/c* and *sugE* in good agreement with previously reported flanking regions (Figure 1) (10, 14). Molecular typing of CMY-2- producing *E. coli* isolates indicated the presence of several lineages

Table 1. Epidemiological data of AmpC-producing isolates, susceptibility profile and genetic characterization of the resistance marker

Hospital	Isolate	Isolate origin	Samples	Antimicrobial susceptibility						<i>pampC</i>	Inc group	
				(diffusion test: mm/ interpretation ⁽⁴⁾)		(dilution test: µg/ml/ interpretation ⁽⁴⁾)		FOX	CAZ	CTX	FEP	
H1	<i>E. coli</i> 4820	Nosocomial	skin	6 R/ >128 R	8 R/ 64 R	26 S/ ≤ 2 S	cmy-2	BO				
	<i>E. coli</i> 4598	Nosocomial	abdominal fluid	6 R/ 64 R	10 R/ 32 R	17 I/ 16 I	28 S/ ≤ 2 S	cmy-2	BO/K			
H2	<i>E. coli</i> 47688	Community	urine	10 R/ 32 R	12 R/ 32 R	17 I/ 16 I	28 S/ ≤ 2 S	cmy-2	Y			
	<i>E. coli</i> 47914	Community	urine	10 R/32 R	12 R/ 16 I	17 I/ 8 S	28 S/ ≤ 2 S	cmy-2	I1			
<i>P. mirabilis</i> 9216-3078	Community	urine	14 R/ 16 I	14 R/ 8 S	16 I/ 4 S	26 S/ ≤ 2 S	dha-1	ND				
	<i>E. coli</i> 9233-3045	Community	urine	10 R/ 64 R	14 R/ 16 I	18 I/ 8 S	33 S/ ≤ 2 S	cmy-2	K			
H3	<i>E. coli</i> 9234-26	Nosocomial	abdominal fluid	9 R/ 64 R	11 R/ 32 R	18 I/ 16 I	30 S/ ≤ 2 S	cmy-2	F/K/BO			
	<i>E. coli</i> 9316-3009	Community	urine	6 R/ 128 R	12 R/ 16 I	19 I/ 8 S	25 S/ ≤ 2 S	cmy-2	F/I1/K			
	<i>E. coli</i> 9238-3013	Community	urine	8 R/ 128 R	13 R/ 16 I	15 I/ 16 I	24 S/ ≤ 2 S	cmy-2	F/BO/K			
	<i>E. coli</i> 9272-3113	Community	urine	7 R/ 64 R	11 R/ 16 I	15 I/ 8 S	24 S/ ≤ 2 S	cmy-2	F			
	<i>E. coli</i> 9288-3031	Community	urine	6 R/ > 128 R	16 I/ 32 R	18 I/ 32 I	22 S/ ≤ 2 S	cmy-2	I1			
	<i>E. coli</i> 3945440-1	Nosocomial	abdominal fluid	6 R/ > 128 R	10 R/ 32 R	13 R/ 32 I	25 S/ ≤ 2 S	cmy-2	Y			
H4	<i>E. coli</i> 3937856	Nosocomial	abdominal fluid	6 R/ > 128 R	6 R/ 128 R	13 R/ 64 R	24 S/ ≤ 2 S	cmy-2	BO/K			
	<i>K. pneumoniae</i> 3905864-1	Community	urine	6 R/ 64 R	6 R/ >128 R	15 I/ 4 S	25 S/ ≤ 2 S	dha-1	ND			
H5	<i>E. coli</i> M178/09	Nosocomial	perianal abscess	11 R/ 32 R	14 R/ 16 I	18 I/ 8 S	26 S/ ≤ 2 S	cmy-2	BO/K			
	<i>E. coli</i> 3335	Nosocomial	blood	8 R/ 64 R	13 R/ 32 R	18 I/ 8 S	30 S/ ≤ 2 S	cmy-2	BO/K			
H6	<i>E. coli</i> 5031	Nosocomial	urine	6 R/ 32 R	14 R/ 16 I	15 I/ 32 I	23 S/ ≤ 2 S	cmy-2	K/BO			
	<i>E. coli</i> ELA069956	Community	urine	12 R/ 64 R	16 I/ 8 S	17 I/ 8 S	30 S/ ≤ 2 S	cmy-2	ND			
H7	<i>E. coli</i> CLB020365	Community	urine	12 R/ 32 R	15 I/ 8 S	17 I/ 8 S	29 S/ ≤ 2 S	cmy-2	N			

(4) reference's number

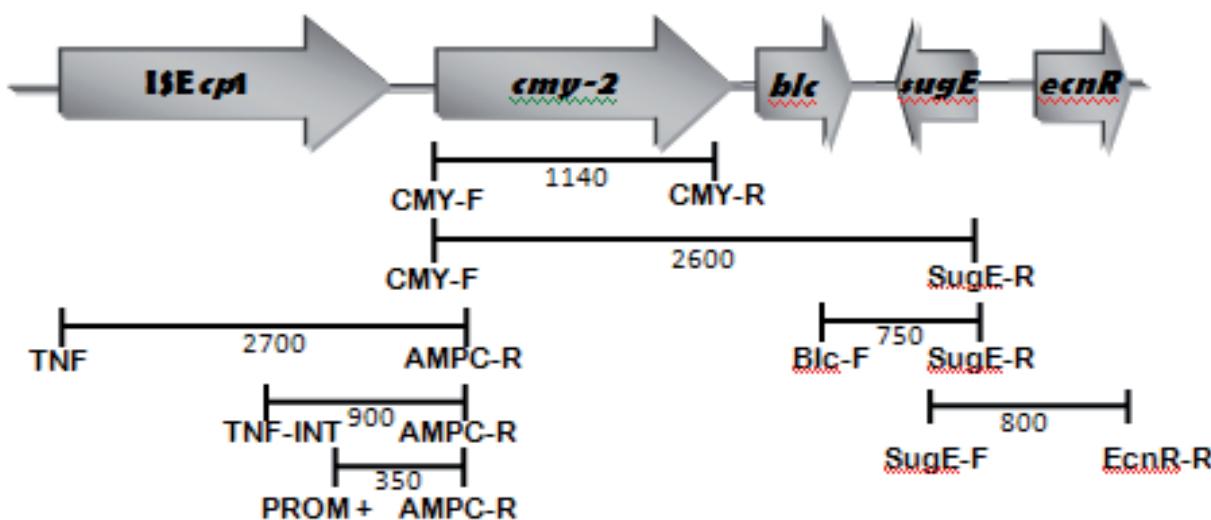


Figure 1. Genetic context of *cmy-2*

ISEcp1: Insertion sequence *Ecp1*; *blc*: outer membrane lipoprotein coding gene, lipocalin; *sugE*: gene encoding for small multidrug resistance protein; *ecnR*: coding gene for a transcriptional regulatory protein, entericidinR.

Lines below indicate the amplified fragments, their sizes (bp) and primers used: SugE-R: GCC TGA TAT GTC CTG GAT CGT; SugE-F: AGC ATG GCG ATA CTG ACG AT; Blc-F: CAT TCC TGG TTG TCG CGT GT; EcnR-R: GGA TTG AGA GGG CAC GAT; ECNR- 3'F: TGT TTA TGC ACT CCC TCC CG; TNF: ACC TAG ATT CTA CGT CAG TACT; TNF-INT: ATT CTA CAC TCA CCT CAC AAC G; PROM +: TGC TCT GTG GAT AAC TTG C; AMPC-R: CCC TGG TAG ATA ACG GCA

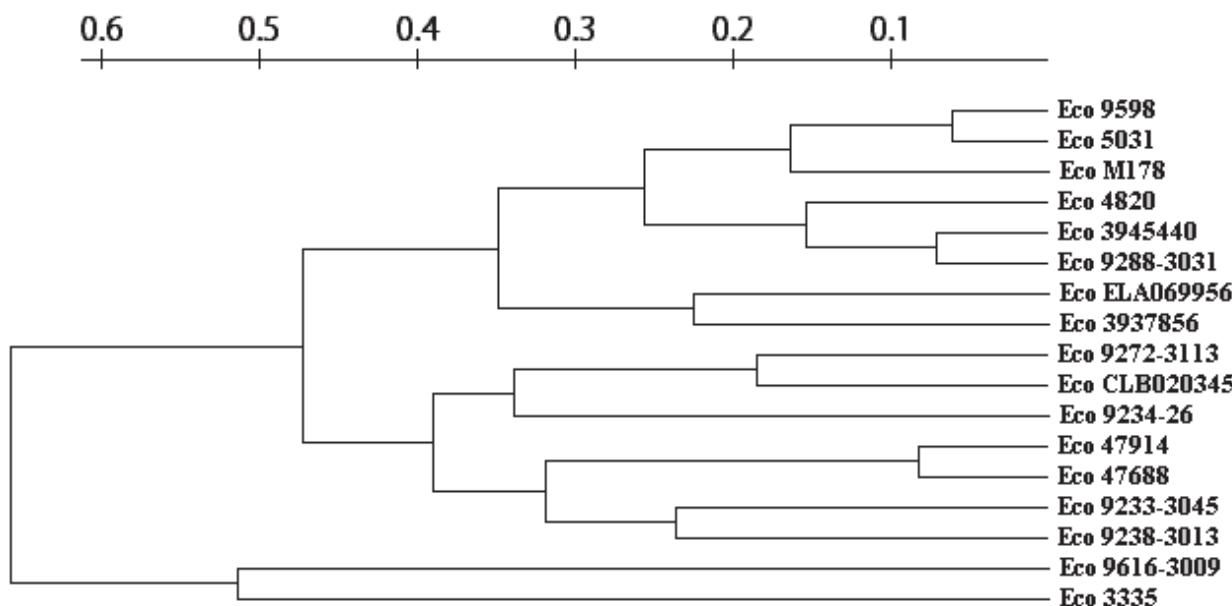


Figure 2. Genetic relationship of *E. coli* AmpC-producing isolates

(Figure 2).

Prevalence of pAmpC among enterobacterial isolates recovered within this period was 0.9 %, with CMY-2 being prevalent (17/19) and to a lesser extent DHA (2/19). CMY-2 was responsible for the 23 % third generation cephalosporin resistance observed in *E. coli*. Current CLSI interpretative criteria showed to be accurate in detecting all pAmpC producers. The APB-based screening method displayed 100 % sensitivity and 99 % specificity. Two *E. coli* that hyperproduced their chromosomal AmpC rendered positive phenotypic screening but negative genotypic detection for pAmpC coding genes. Although IncA/C, IncQ and IncI1 have been associated to *cmy-2* in many regions of the world (2), IncK, IncF, IncY and IncBO replicons in *cmy-2* containing plasmids were also detected in this study. The analyzed *cmy-2* context agrees completely with the conserved region reported for Type I, II and III environments described in *Salmonella enterica* and *E. coli*, in which *cmy-2* genes are associated with the insertion sequence IS*Ecp1* that not only mobilizes the downstream-located genes but also provides a strong promoter sequence for high level β-lactamase expression.

Considering that CMY-2-producing *E. coli* isolates included in this study corresponded to several lineages and that the resistant marker displayed a wide diversity of Inc/cmy-2 associations, the spread of *cmy-2* in *Enterobacteriaceae* may be associated to specific transposable elements responsible for its mobilization.

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