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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

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).

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 *ampC*s 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 *ampC* 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.

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-25}), EU515250 (bla_{CMY-27}) and EU515251 (bla_{CMY-40}).

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Transparency Declaration

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