Spanish Multicenter Study of the Epidemiology and Mechanisms of Amoxicillin-Clavulanate Resistance in Escherichia coli

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Epidemiology and resistance mechanisms to amoxicillin-clavulanate in *Escherichia coli*: A Spanish multicenter study

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Abstract

We conducted a prospective multicenter study in Spain to characterize the mechanisms of resistance to amoxicillin/clavulanate (AMC) in *Escherichia coli*. Up to 44 AMC-resistant *E. coli* isolates (CMI ≥32/16 μg/mL) were collected at each of the seven participant hospitals. Resistance mechanisms were characterized by PCR and sequencing. Molecular epidemiology was studied by pulsed field gel electrophoresis (PFGE) and by multilocus sequence typing. Overall AMC resistance was 9.3%.

The resistance mechanisms detected in the 257 AMC-resistant isolates were: OXA-1 production (26.1%), hyperproduction of penicinillinase (22.6%), production of plasmidic AmpC (19.5%), hyperproduction of chromosomic AmpC (18.3%), and production of inhibitor-resistant TEM (IRT) (17.5%). The IRTs identified were TEM-40 (33.3%), TEM-30 (28.9%), TEM-33 (11.1%), TEM-32 (4.4%), TEM-34 (4.4%), TEM-35 (2.2%), TEM-54 (2.2%), TEM-76 (2.2%), TEM-79 (2.2%), and the new TEM-185 (8.8%). By PFGE, a high degree of genetic diversity was observed although two well-defined clusters were detected in the OXA-1-producing isolates: the C1 cluster consisting of 19 phylogroup A/ST88 isolates, and the C2 cluster, 19 phylogroup B2/ST131 isolates (16 of them producing CTX-M-15); each of the clusters was detected in six different hospitals. In total, 21.8% of the isolates were serotype O25b/phylogroup B2. AMC resistance in *E. coli* is widespread in Spain at the hospital and community levels. A high prevalence of OXA-1 was found. Although resistant isolates were genetically diverse, clonality was linked to OXA-1-producing isolates of the STs 88 and 131. Dissemination of IRTs was frequent, and the epidemic O25b/B2/ST131 clone carried many different mechanisms of AMC resistance.

Introduction

Escherichia coli is an important etiologic agent for both nosocomial- and community-acquired infections in humans (10, 14, 23). Amoxicillin-clavulanate (AMC) is one of the most widely used antibiotics in many countries (3, 12, 16). In Spain, a 34.7% increase in community use of AMC was recorded from 2000 to 2006 (21). Recently, blood isolates of *E. coli* non-susceptible to AMC increased from 9.3% (2003) to 25.9% (2010) in Spain, according to the European Antimicrobial Resistance Surveillance Network (EARS-Net) (9, 21).

Enzymatic mechanisms of *E. coli* resistance to AMC include: hyperproduction of plasmid-mediated class A β-lactamases such as TEM-1 and SHV-1 (19,32), plasmid-mediated AmpC-type β-lactamase (p-AmpC) (22), chromosomal AmpC β-lactamase (c-AmpC) (22), production of inhibitor-resistant TEM (IRT) β-lactamases (18,28), plasmid-mediated β-lactamase OXA-1 (33), and complex mutant TEM (CMT) enzymes than combine IRT- and extended-spectrum β-lactamase (ESBL)-type substitutions (27).

In spite of the significant increase in AMC use in the last years, there is little recent information available about the prevalence of AMC resistance mechanisms in *E. coli*; most previous studies analyzed strains isolated more than 10 years ago from single hospitals in the United States (13, 29), France (17), and Spain (20, 25).

Accordingly, the aims of this prospective Spanish national multicenter study were to investigate the epidemiology and mechanisms of AMC resistance in clinical isolates of *E. coli* causing both community- and nosocomial infections.

Material and methods

Study design and bacterial isolates

A prospective multicenter study was designed to obtain *E. coli* isolates resistant to AMC (MIC ≥32/16 μg/mL and/or disk inhibition zone ≤13 mm according to Clinical and Laboratory Standards Institute [CLSI] [8]) from clinical samples collected between January 2010 and May 2010. Seven university hospitals of six Spanish Autonomous Communities and members of the Spanish Network for the Research in Infectious Diseases (REIPI) participated. Investigators at these hospitals were asked to collect up to 22 consecutive community- and 22 nosocomial-acquired, non-duplicated isolates of *E. coli* resistant to AMC.

Nosocomial-acquired isolates were defined as those acquired at least 48 hours after hospital admission. Putatively community-acquired isolates were those isolated in the community or within 48 hours of hospital admission.

Susceptibility testing

The disk diffusion and/or microdilution susceptibility tests were performed using different automated systems in each participating laboratory. All isolates were submitted to the antibiotic laboratory of the Centro Nacional de Microbiología (Majadahonda, Madrid), where additional confirmatory antibiotic susceptibility testing was performed with the agar dilution method according to the CLSI guidelines (7). Control strains used were *E. coli* ATCC 25922 and *E. coli* ATCC 35218. The production of extended spectrum β-lactamases (ESBLs) was studied by the double-disc synergy test and/or Etest ESBL using cefotaxime and ceftazidime as substrates (AB Biodisk, Solna, Sweden).

Molecular characterization of mechanisms of resistance to AMC

109 The *bla*$_{TEM}$ gene and its promoter region were assessed by PCR and sequencing
110 (11). Sequences were compared to those of the *bla*$_{TEM-1}$ gene and its promoter region in
111 public databases (GenBank accession no. AB194682) (30). In those isolates in which
112 the promoter region could not be amplified with these primers, the possibility of linkage
113 of *bla*$_{TEM}$ alleles to IS26-like elements, as suggested previously (1), was assessed with
114 primers IS26-F (5′-GCG GTA AAT CGT GGA GTG AT-3′) and TEMi-R (5′-TCT TTT
115 ACT TTC ACC AGC GTT-3′).

116 Genes coding for p-AmpC (CIT, DHA, ACC, EBC, MOX, and FOX) and
117 *bla*$_{OXA-1}$ were characterized by PCR amplification with specific primers and sequencing
118 (2, 24, 26) in all isolates. *E. coli* isolates with a negative result for p-AmpC β-
119 lactamases but that displayed a resistance phenotype consistent with AmpC production
120 on the basis of their resistance to AMC and cefoxitin, and inhibition with phenyl
121 boronic acid and cloxacillin (cefotetan/cefotetan-cloxacillin E-test [AB Biomerieux,
122 Solna, Sweden]), were categorized as c-AmpC.

123 Isolates only carrying the *bla*$_{TEM-1}$ or *bla*$_{SHV-1}$ genes with resistance to ampicillin,
124 AMC, and cefazolin but susceptibility to the remaining β-lactam antibiotic families
125 were considered penicillinase hyperproducers. In the case of SHV-1, an increase of
126 ceftazidime MIC was also considered compatible with a hyperproduction of this
127 enzyme (19).

128 Genes coding ESBL enzymes (CTX-M, SHV-type, and TEM-type) were studied
129 by PCR and sequencing in all AMC-resistant *E. coli* isolates with an phenotype
130 consistent with ESBL-production on the basis of their resistance to the extended
131 spectrum cephalosporins whose activity was recovered in presence of clavulanate (24).

Phylogenetic groups and O25b type detection

The phylogenetic groups of AMC-resistant *E. coli* isolates were determined by a multiplex PCR assay described by Clermont et al. (4). To search for an ST131/B2/O25b *E. coli* clone, the O25b type detection was performed with an allele-specific PCR (5).

Molecular epidemiology

The genetic relationship between the AMC-resistant *E. coli* isolates was determined by pulsed-field gel electrophoresis (PFGE) after total chromosomal DNA digestion with *Xba*I (24).

A selected sample of 43 AMC-resistant *E. coli* isolates, representing the two major PFGE clusters (19 isolates each) and 5 IRTs-producing isolates belonging to serotype O25b phylogroup B2 isolates, was studied further by multilocus sequence typing (MLST), according to the University College Cork (Éire) scheme for *E. coli* (http://mlst.ucc.ie/mlst/dbs/Ecoli; data last accessed July 20, 2011).

Statistical analyses

Differences in the prevalence of mechanisms of resistance and phylogroups between different groups were assessed by Fisher's exact test. Association was determined by calculation of the odds ratio (OR) with 95% confidence intervals (CI). The null hypothesis was rejected for values of $P < 0.05$. Statistical analyses were performed using GraphPad Prism version 3.02 software (GraphPad Software, Inc., San Diego, CA, USA).

Results and Discussion

157 ***Bacterial isolates and mechanisms of AMC resistance***

158 A total of 257 clinical AMC-resistant *E. coli* isolates were collected, 110 of them
159 (43%) produced nosocomial-acquired infections, and 147 (57%) putatively community-
160 acquired infections; 170 (65.9%) were from urine, 30 (12%) from wound, 19 (7.4%)
161 from blood, and 38 (14.7%) from other clinical samples.

162 During the study period, the participant hospitals had a prevalence of AMC
163 resistance in *E. coli* (MIC ≥ 32/16 μg/ml) of 9.3% (range: 3.3%–13.5%).

164 The mechanisms of resistance detected were: production of OXA-1 (67 isolates,
165 26.1%), hyperproduction of penicinillinase (58, 22.6%; 53 TEM-1 and 5 SHV-1),
166 production of p-AmpC (50, 19.5%), hyperproduction of c-AmpC (47, 18.3%), and
167 production of IRTs (45, 17.5%). In one AMC-resistant isolate with the ESBL
168 phenotype, $bla_{\text{TEM-12}}$ was detected linked to an IS26 element upstream of bla_{TEM}. In two
169 isolates, no enzymatic mechanism of resistance to AMC was identified. Two different
170 mechanisms of AMC resistance were present in 13 isolates: OXA-1+p-AmpC (7
171 isolates, 2.7%) and OXA-1+c-AmpC (6 isolates, 2.3%).

172 The IRTs identified in this study were TEM-40 (15, 33.3%), TEM-30 (13,
173 28.9%), TEM-33 (5, 11.1%), TEM-32 (2, 4.4%), TEM-34 (2, 4.4%), TEM-35 (1,
174 2.2%), TEM-54 (1, 2.2%), TEM-76 (1, 2.2%), TEM-79 (1, 2.2%), and the new TEM-
175 185 (4, 8.8%), first described in this study.

176 Of the 50 p-AmpC β-lactamases detected in our study, 37 (74%) were CMY-2;
177 11 (22%), DHA-1; 1 (2%), CMY-30; and 1 (2%), CMY-42 (2%).

178 There were some relevant geographical differences in the prevalence of the
179 AMC resistance mechanisms. For instance, p-AmpC mechanism was more prevalent in
180 the isolates from the Sant Pau Hospital, Catalonia (45.7%, $P = 0.0001$) in comparison
181 with isolates from the other hospitals; c-AmpC was also more prevalent in isolates from

182 the Vall d'Hebron Hospital, Catalonia (37.5%, P = 0.022); OXA-1 was more prevalent
183 in isolates from the Complejo Hospitalario A Coruña, Galicia (46.5%, P = 0.0019); and
184 IRT was more prevalent in isolates from the Gregorio Marañón Hospital, Madrid, (30%,
185 P=0.038).

186 Previous studies detected the hyperproduction of penicillinases (13, 25, 29),
187 mainly TEM-1, and AmpC production (17, 20) as the most common mechanisms of
188 resistance to AMC in *E. coli*. A 2011 study showed that of 50 ampicillin/sulbactam
189 resistant *E. coli* isolates from the United States, 96% produced $bla_{\text{TEM-1}}$, 8%, $bla_{\text{CMY-2}}$,
190 and 8%, $bla_{\text{OXA-1}}$ (32).

191 Some information about p-AmpC production in AMC-resistant *E. coli* has been
192 published (17, 20, 25, 29); one study from the United States reported that of 69 *E. coli*
193 isolates resistant to AMC, 5.8% produced CIT (13).

194 Production of OXA-1 in *E. coli* has been sporadically described previously (13,
195 17, 20, 25). The highest reported OXA-1 prevalence (15.3%) was observed in isolates
196 from the United States collected between 1990 and 1994 (29); by the end of last
197 century, this figure was 7.6% (25) and 2.6% (20) in two Spanish hospitals.

198 The prevalence of IRTs found in this study is among the highest reported in
199 AMC-resistant *E. coli* (13, 20, 25, 29). However, of 255 *E. coli* isolates resistant to
200 AMC studied in France in 1996-1998, 37.7% produced IRTs (17). Recently, in a single
201 Spanish hospital (18) 11.8% of AMC-resistant *E. coli* produced IRTs.

202 Overexpression of $bla_{\text{TEM-1}}$ has been associated with promoters PaPb, P4 and
203 other (11, 15, 31); but in this study this association was not clearly observed since
204 50.9% of our TEM-1 producers had the most commonly found P3 promoter (Table 1).
205 However, our findings are consistent with a previous report of a 54.5% prevalence of
206 P3/TEM-1 among ampicillin/sulbactam resistant *E. coli* isolates (32). In our study, the

most prevalent strong promoter was PaPb, as previously described (32) (Table 1). Among our IRTs, 62.2% had promoters other than P3, most of which were PaPb (Table 1). Implications of the promoter variations in the AMC resistance requires further elucidation. The occurrence of IS26 located at different positions upstream of the bla_{TEM} gene has been recently described (1), but the influence of this insertion in the expression of the bla_{TEM} is unclear so far.

A total of 37 isolates (14.4%) produced ESBLs: 28 (75.7%) CTX-M-15, 7 (18.9%) CTX-M-14, 1 (2.7%) SHV-12, and 1 (2.7%) TEM-12. All but one of them had an additional AMC resistance mechanism, mainly OXA-1 (67.6%), p-AmpC (13.5%), c-AmpC (13.51%), and IRTs (5.4%). Complex mutant TEM β-lactamases were not detected.

In relation to the AMC resistance mechanisms, no significant difference were found between community and nosocomial isolates, except for $bla_{\text{TEM-1}}$ promoters other than P3 which were more frequent in nosocomial AMC-resistant *E. coli* ($P < 0.05$).

Table 2 shows the distribution of $MIC_{50,}$ MIC_{90}, MIC ranges and the percentages of isolates with MIC > 32/16 µg/mL according to the molecular mechanisms of resistance to AMC. OXA-1 producing isolates had AMC MICs lower than isolates with other AMC resistance mechanisms ($P < 0.03$).

Resistance to non-β-lactam antibiotics in relation to the mechanism AMC-resistance is detailed in Table 3. OXA-1-producing isolates were more resistant to ciprofloxacin, cotrimoxazole, and aminoglycosides than IRT, AmpC, or TEM-1 producers ($P < 0.001$).

Phylogenetic groups and O25b type detection

Of the 257 AMC-resistant *E. coli* isolates, 76 (29.6%) belonged to phylogenetic group A, 32 (12.4%) to group B1, 104 (40.5%) to group B2, and 45 (17.5%) to group D.

Phylogroup A was more frequent in OXA-1 producers (36, 53.7%; $P < 0.0003$); phylogroup B2, in IRT producers (24, 53.3%; $P = 0.06$), p-AmpC producers (21, 42%; $P = 0.87$), and TEM-1 producers carrying promoters other than P3 (16, 61.6%; $P < 0.03$); and phylogroup D, in P3/TEM-1 producers (13, 48.1%; $P < 0.0001$).

Of the 104 phylogroup B2 isolates, 56 (21.8% of all isolates) were serotype O25b, being detected in all seven participant hospitals. Of the O25b/B2 isolates, 22 (39.3%) were OXA-1 producers, 15 (26.8%) were TEM-1 producers with different promoters, 11 (19.6%) were p-AmpC producers, 8 (14.3%) were c-AmpC producers, and 5 (8.9%) were IRT producers. Five of these isolates produced both OXA-1 and AmpC β-lactamases.

Molecular epidemiology

A high degree of genetic diversity was observed according to PFGE, as 235 different PFGE patterns were obtained from the 257 AMC-resistant *E. coli* isolates analyzed. However, two well-defined clusters were detected: cluster C1 (genetic linkage > 90%) consisting of 19 OXA-1-producing isolates of phylogroup A, detected in six of the seven participant hospitals; and cluster C2 (genetic linkage > 80%) consisting of 19 AMC-resistant isolates of phylogoup B2 and serotype O25b, detected in six of the seven participant hospitals. Of these 19 C2 isolates, 12 produced OXA-1 and CTX-M-15; 3 produced OXA-1 only; 3 produced CTX-M-15 and c-AmpC; and one produced CTX-M-15 and DHA. Figure 1 shows the PFGE profiles of the 67 OXA-1-producing isolates.

By MLST, the PFGE C1 cluster was identified as ST88, and the PFGE C2 cluster as ST131.

Dissemination of OXA-1 β-lactamase in AMC-resistant *E. coli* isolates in Spain is due in part to the clonal spread of the epidemic ST131 clone producing CTX-M-15 and OXA-1, and to the spread of the ST88 phylogroup A clone only producing OXA-1, an association first described here. ST88 has been previously described in association with c-AmpC production in a French hospital (6). On the Website of the University College of Cork (Ireland), 23 ST88 *E. coli* isolates are registered and were recovered from infections of humans and domestic animals (http://mlst.ucc.ie/mlst/dbs/Ecoli, data last accessed July 20, 2011)

In 24 O25b/B2 isolates, including 19 C2 isolates and five IRTs, MLST was performed and showed that all of them were ST131. We found that O25b/B2/ST131 *E. coli* isolates not only carried CTX-M-15 and OXA-1 enzymes as previously reported, but also TEM-30, TEM-34, TEM-40, and TEM-54 IRT enzymes. To the best of our knowledge, this is the first description of IRTs-producing isolates belonging to the ST131 international clone.

TEM-185 characterization

The new IRT TEM-185 has a double amino acid substitution at positions 69 (M→L) and 165 (W→L) in comparison with TEM-1 (GenBank accession number 1446016); these positions are frequently modified in IRT enzymes. TEM-185 is similar to TEM-39 except that the latter has an additional substitution at position 276 (N→D) (http://www.lahey.org/studies/temtable.asp, data last accessed August 2, 2011).

TEM-185 was detected in four *E. coli* belonging to phylogroup A; they were isolated in two geographically distant Spanish hospitals with two isolates each; two of

279 them were closely related (genetic similarity >85% and same P4 promoter), the two
280 additional isolates were genetically unrelated and had two different promoters, P4 and
281 Pdel. These findings suggest that several clones can spread TEM-185.
282
283 **Concluding remarks**
284 Our findings suggest a complex epidemiological background in which *E. coli*
285 acquire AMC resistance by several mechanisms, including clonal (ST131, ST88) and
286 non-clonal spread, dissemination of mobile genetic elements carrying different *bla*
287 genes, and eventual mutations in individual isolates as a response to selective
288 antimicrobial pressure.
289
290
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Table 1. The frequency of promoter regions and insertions and deletions identified in TEM-producing *Escherichia coli* isolates.

TEM type (n)	Promoter class				IS26 insertion*	IS911 insertion#	C31→T39deletion
	P3	PaPb	P4	Pdel			
TEM-1 (53)	27	18	2	-	4	1	1
TEM-40 (15)	6	6	3	-	-	-	-
TEM-30 (13)	5	6	1	-	1	-	-
TEM-33 (5)	2	3	-	-	-	-	-
TEM-185 (4)	-	-	3	1	-	-	-
TEM-32 (2)	2	-	-	-	-	-	-
TEM- 34 (2)	2	-	-	-	-	-	-
TEM-35 (1)	-	1	-	-	-	-	-
TEM-76 (1)	-	1	-	-	-	-	
TEM-79 (1)	-	1	-	-	-	-	-

All data represent the number of affected isolates, n.

*The IS26 element was detected upstream of bla_{TEM}, inserted at promoter positions 150 (n=3) or 46 (n=2) according to the Sutcliffe numbering system (16).

#The IS911 element was detected upstream of bla_{TEM-1}, inserted at promoter position 46.

Table 2. Distribution of $MIC_{50,}$ MIC_{90}, MIC range, and percentage of amoxicillin/clavulanate resistant *Escherichia coli* isolates with MIC>32/16 µg/ml in relation to the molecular mechanisms of resistance.

Resistance mechanism (n)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)	Isolates with MIC > 32/16 µg/ml
OXA-1 (54)	32/16	64/32	16/8-128/64	40.3%
IRT (45)	64/32	128/64	16/8->128/64	93.3%
p-AmpC (43)	64/32	128/64	32/16->128/64	92%
c-AmpC (41)	64/32	128/64	32/16-128/64	83%
P3/TEM-1 (27)	64/32	64/32	16/8->128/64	66.7%
Pdf3/TEM-1* (26)	64/32	>128/64	16/8->128/64	84.6%
OXA-1+AmpC (13)	64/32	64/32	32/16-64/32	53.8%

*Isolates with bla_{TEM-1} and with promoters other than P3.

Table 3. Resistance to non-β-lactam antibiotics in relation to the mechanism of resistance to amoxicillin/clavulanate in *Escherichia coli*.

Resistance mechanism or source (n)	Ciprofloxacin, n (%)	Gentamicin, n (%)	Tobramycin, n (%)	Amikacin, n (%)	Cotrimoxazole, n (%)
TEM-1 (53)	27 (51.8)	7 (13.2)	10 (20.4)	0	30 (56.6)
IRTs (45)	17 (37.8)	4 (8.9)	4 (8.9)	0	24 (53.3)
OXA-1 (67)	57 (85.1)	34 (50.7)	55 (82.1)	12 (17.9)	55 (82.1)
AmpC (97)	51 (52.6)	17 (17.5)	23 (23.7)	1 (1)	44 (45.4)
Total (257)	149 (58)	61 (23.7)	86 (33.5)	12 (4.7)	149 (58)

Figure 1. Dendrogram illustrating the percentage linkage of pulsed-field-gel-electrophoresis profiles of 67 OXA-1-producing *Escherichia coli* isolates resistant to amoxicillin-clavulanate.