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Molecular characterisation of acquired and overproduced chromosomal bla_{AmpC} in *Escherichia coli* clinical isolates

Noemí Alonso[a,b,c], Elisenda Miró[a,c], Vanesa Pascual[d], Alba Rivera[a], Maria Simó[e], Maria Consol Garcia[f], Mariona Xercavins[e], Maria Antonia Morera[e], Elena Espejo[f], Mercè Gurguí[a,b,c], Josefa Pérez[e], Mònica Rodríguez-Carballeira[d], Javier Garau[d], Esther Calbo[d,g], Ferran Navarro[a,b,c,*], Beatriz Mirelis[a,b,c], Pere Coll[a,b,c]

[a] *Hospital de la Santa Creu i Sant Pau and Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), Barcelona, Spain*
[b] *Universitat Autònoma de Barcelona, Barcelona, Spain*
[c] *La Red Española de Investigación en Patología Infecciosa (REIPI), Instituto de Salud Carlos III, Madrid, Spain*
[d] *Hospital Universitari MútuaTerrassa, Barcelona, Spain*
[e] *CatLab, Barcelona, Spain*
[f] *Consorci Sanitari de Terrassa, Barcelona, Spain*
[g] *Universitat Internacional de Catalunya, Barcelona, Spain*

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ABSTRACT

Escherichia coli recovered from three hospitals in Barcelona (Spain) were studied to determine the prevalence of isolates with acquired AmpC (ac-AmpC) and/or overproduced chromosomal AmpC (c-AmpC). Mechanisms involved in $bla_{c\text{-}AmpC}$ overexpression, $bla_{ac\text{-}AmpC}$ and the plasmids associated with their distribution as well as the prevalence of plasmid-mediated quinolone resistance (PMQR) in AmpC-producing isolates were also determined. Isolates were selected according to their resistance phenotype. $bla_{ac\text{-}AmpC}$, alterations in the $bla_{c\text{-}AmpC}$ promoter/attenuator, and PMQR genes [*qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib*-cr and *qepA*] were characterised by PCR and sequencing. $bla_{c\text{-}AmpC}$ expression was determined by qRT-PCR. Population structure analysis was performed using PFGE, MLST and phylogenetic group PCR. Plasmids carrying $bla_{ac\text{-}AmpC}$ were characterised by PCR-based replicon typing and S1-PFGE. IncI1 and IncF plasmids were also analysed by plasmid MLST and replicon sequence typing, respectively. Among 21 563 *E. coli* isolates, 240 (1.1%) overproduced AmpC β-lactamases, including 180 (75.0%) harbouring ac-AmpC (132 CMY-2 variants and 48 DHA-1) and 60 (25.0%) c-AmpC enzymes. Three mutation profiles in the $bla_{c\text{-}AmpC}$ promoter/attenuator were associated with a 72.5-, 19.9- and 5.8-fold increased expression, respectively. Moreover, 63.3% of ac-AmpC and 43.3% of c-AmpC isolates belonged to B2, D, E or F phylogenetic groups. PMQR was found in 31% of ac-AmpC isolates [38 *qnrB4*, 8 *aac(6′)-Ib*-cr, 6 *qnrS1* and 3 *qnrB19*] and in 10% of c-AmpC isolates [5 *aac(6′)-Ib*-cr and 1 *qnrS1*]. IncI1-ST12 and IncF were associated with $bla_{CMY\text{-}2}$ and $bla_{DHA\text{-}1}$, respectively. These results suggest that ac-AmpC β-lactamases were the main mechanism of AmpC production. Isolates and plasmids both showed high genetic diversity.

1. Introduction

AmpC β-lactamases are encoded by bla_{AmpC}. These genes are present in the chromosome of some bacterial species but can also be acquired through mobile genetic elements such as plasmids [1].

In *Escherichia coli*, expression of the chromosomal AmpC ($bla_{c\text{-}AmpC}$) is constitutive at a low level owing to the presence of both a weak promoter and an attenuator and it does not confer resistance to β-lactams [2]. However, overproduction of $bla_{c\text{-}AmpC}$ owing to mutations in the promoter/attenuator leads to resistance to penicillins, cephalosporins, cephamycins and/or aztreonam [2,3]. Another mechanism affecting third-generation cephalosporins is the presence of chromosome-borne extended-spectrum AmpC (ESAC) β-lactamases. In this case, the $bla_{c\text{-}AmpC}$ show structural alterations in the R2 loop that can also confer carbapenem resistance [4].

* Corresponding author. Present address: Servei de Microbiologia, Hospital de la Santa Creu i Sant Pau, C/ Sant Quintí 89, 08025 Barcelona, Spain. Tel.: +34 935 537 297; fax: +34 935 537 287.
E-mail address: fnavarror@santpau.cat (F. Navarro).

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Escherichia coli can also acquire other bla_{AmpC} ($bla_{ac\text{-}AmpC}$) (CMY, DHA, ACC, FOX, MOX, ACT, MIR, LAT and CFE), which are found naturally in other Gram-negative bacteria and are able to circulate by plasmid mobilisation. These $bla_{ac\text{-}AmpC}$ undergo an evolution process and hence do not circulate between species with the same genetic features [1]. In general, these $bla_{ac\text{-}AmpC}$ are expressed constitutively, but some, such as $bla_{DHA\text{-}1}$, can be induced by β-lactams, as occurs in other inducible $bla_{c\text{-}AmpC}$ of Enterobacteriaceae (such as *Citrobacter freundii*, *Morganella morganii* and *Enterobacter* spp.) and *Pseudomonas aeruginosa* [1]. In addition, the presence of an ac-AmpC enzyme together with porin alterations can reduce susceptibility to carbapenems [5]. Ac-AmpC β-lactamases have a universal distribution and have been described in strains isolated from clinical, animal, food and water samples [6,7].

Besides knowledge of the genetic elements ($bla_{c\text{-}AmpC}$ or $bla_{ac\text{-}AmpC}$) involved in the expression of AmpC β-lactamases, successful treatment of infections caused by AmpC β-lactamase-producing bacteria depends on the ability to screen these enzymes. The lack of standardised methods for screening AmpC β-lactamases (c-AmpC and ac-AmpC) in Enterobacteriaceae (only the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has published some recommendations [8]) impedes correct detection or an accurate determination of their prevalence in the population. Therefore, distinguishing between ac-AmpC and c-AmpC β-lactamases may be key to the prevention of their diffusion and the effective treatment of bacterial infection. Failures in β-lactam treatment of infections produced by c-AmpC-overproducing or ac-AmpC-producing strains have been reported [9,10].

Moreover, AmpC β-lactamase-producing *E. coli* isolates are frequently co-resistant to other families of antibiotics, such as fluoroquinolones and aminoglycosides, thus posing an even greater challenge for the clinician.

The prevalence of AmpC β-lactamases also depends on the geographic area, the species and the study period, which complicates comparison of data between studies. Although the prevalence of these enzymes around the world is relatively low, many studies show a continuous yearly increase [11,12], particularly in Asia [13]. Recent studies in Spain also report a low but increasing prevalence, notably of ac-AmpC β-lactamases [14–16].

Due to the limited data available on $bla_{c\text{-}AmpC}$ overproduction in *E. coli* in Spain [17–19], the aim of this study was to determine the prevalence of overproduced c-AmpC and ac-AmpC β-lactamase-producing *E. coli* in the Barcelona area. The mechanisms associated with overexpression of $bla_{c\text{-}AmpC}$, $bla_{ac\text{-}AmpC}$ and the plasmids involved in their distribution were evaluated. Because of the correlation between β-lactam resistance and plasmid-mediated quinolone resistance (PMQR), the prevalence of PMQR genes in AmpC-producing *E. coli* isolates was determined. Finally, the population structure of *E. coli* strains carrying the different resistance mechanisms was analysed.

2. Materials and methods

2.1. Study design, bacterial isolates and antimicrobial susceptibility testing

This study was performed at three hospitals and their primary care centres in Barcelona, Spain [Hospital Universitari MútuaTerrassa (HUMT), Hospital de la Santa Creu i Sant Pau (HSCiSP) and Consorci Sanitari de Terrassa (CST)] from June 2010 to November 2011. Altogether these centres serve a population of 1 300 000 people. All isolates were collected consecutively from clinical samples (one per patient) and were selected according to a resistance phenotype compatible with AmpC production as previously described [20]: resistance or reduced susceptibility, according to Clinical and Laboratory Standards Institute (CLSI) breakpoints [21], to amoxicillin/clavulanic acid (<18 mm, >8/4 mg/L) and cefotaxime (<26 mm, >1 mg/L) or ceftazidime (<21 mm, >4 mg/L) or aztreonam (<21 mm, >4 mg/L). Susceptibility studies were carried out using VITEK (bioMérieux, Hazelwood, MO), MicroScan (Dade Behring, West Sacramento, CA) or the disc diffusion method (Rosco Diagnostica A/S, Taastrup, Denmark). All strains were re-tested using the disc diffusion method. All isolates with extended-spectrum β-lactamases (ESBLs) were discarded, except those resistant to cefoxitin (<18 mm, >8 mg/L) or amoxicillin/clavulanic acid. ESBLs were detected by disc diffusion according to CLSI breakpoints [21], PCR and subsequently sequenced. Susceptibility to aminoglycosides (streptomycin, kanamycin, tobramycin, gentamicin, netilmicin and amikacin), sulfonamides, trimethoprim, trimethoprim/sulfamethoxazole, tetracycline, chloramphenicol, fosfomycin, nalidixic acid and ciprofloxacin was also studied in strains that harboured ac-AmpC or overproduced c-AmpC β-lactamases by the disc diffusion technique and was interpreted following CLSI breakpoints [21].

2.2. Molecular characterisation of $bla_{ac\text{-}AmpC}$ and analysis of $bla_{c\text{-}AmpC}$ in overproducer strains

Detection of $bla_{ac\text{-}AmpC}$ was carried out by multiplex PCR as described previously [22]. Screening of mutations in the promoter/attenuator region of $bla_{c\text{-}AmpC}$ was performed by PCR as described previously [3] and was compared with the sequence of *E. coli* K-12 (GenBank accession no. NC_0913.2). ESAC β-lactamases were screened in cefepime-resistant isolates as described previously [23]. All amplicons obtained were purified with a Wizard® SV Gel and the PCR Clean-up System Kit (Promega, Madison, WI) and were sequenced and analysed as previously described [14]. Analysis of $bla_{c\text{-}AmpC}$ expression was performed in triplicate using quantitative real-time PCR (qRT-PCR) [3]. Relative quantification was calculated by the $2^{-\Delta\Delta CT}$ method [2].

The methodologies described in Sections 2.3–2.5 were only performed in isolates confirmed to harbour $bla_{ac\text{-}AmpC}$ or to overproduce $bla_{c\text{-}AmpC}$.

2.3. Molecular typing and determination of phylogenetic groups

The clonal relationship between isolates was determined by pulsed-field gel electrophoresis (PFGE) using the restriction enzyme *Xba*I as described previously [14]. Phylogenetic data were obtained in 28 selected isolates (representative of each resistance mechanism detected) by multilocus sequence typing (MLST) using the Institut Pasteur tools (http://bigsdb.web.pasteur.fr/ecoli/ecoli.html). Phylogenetic groups (A, B1, B2, C, D, E, F and *Escherichia* cryptic clade I) were determined using the Clermont method [24].

2.4. Screening of plasmid-mediated quinolone resistance genes

PMQR genes [*qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib*-cr and *qepA*] were screened by PCR [25,26]. All *E. coli* isolates positive for *aac(6′)-Ib* were digested with *Bts*CI endonuclease or were sequenced to confirm the presence of *aac(6′)-Ib*-cr. The PCR products for *qnr* genes were sequenced and analysed as described previously [14].

2.5. Plasmid typing

Ninety ac-AmpC-producing isolates (50.0%) with different PFGE patterns were selected to analyse their plasmids by PCR-based replicon typing (PBRT) [27]. Plasmid sizes were determined by digestion of whole-genome DNA with S1 endonuclease to linearise the plasmids and PFGE to separate them all [28]. Specific probes for $bla_{ac\text{-}AmpC}$ ($bla_{CMY\text{-}2}$ and $bla_{DHA\text{-}1}$) and replicon groups (IncI1,