

A simple phenotypic method for differentiation between acquired and chromosomal AmpC β -lactamases in *Escherichia coli*

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BACKGROUND. Screening methods for the detection of plasmid-mediated AmpC β -lactamases are technically demanding. The purpose of this study was to assess screening methods for the detection of these enzymes in clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*.

METHODS. Isolates were selected according to a resistance phenotype consistent with production of an AmpC-type β -lactamase. Detection of acquired *ampC* genes was done with a multiplex *ampC*-PCR and sequencing. The phenotypic detection methods evaluated included visual examination of antibiogram plates to identify the presence of scattered colonies located near the edge of the inhibition halo of cefoxitin, cefotaxime, ceftazidime and aztreonam, and a double-disc synergy test using cloxacillin (500 μ g) to inhibit AmpC enzymes.

RESULTS. Seventy-seven isolates were selected from among 6,209 isolates recovered. Acquired *ampC* genes (*bla*_{CMY-2}, *bla*_{DHA-1}, *bla*_{CMY-4} and *bla*_{ACC-1}) were found in 19 (24.7%) of these isolates, including 14 *E. coli*, two *K. pneumoniae* and three *P. mirabilis* isolates. The differential trait for the presence of colonies in the inhibition halo was 100% sensitive and specific. Similar results were obtained for the cloxacillin test, except for the *E. coli* isolates in which specificity was 10.3%.

CONCLUSION. The phenotypic trait described here can be considered useful for suspecting the presence of these enzymes. The cloxacillin test was only useful in isolates lacking a natural AmpC β -lactamase.

Key words: AmpC β -lactamase. CMY. ACC. DHA. Phenotypic detection.

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ANTECEDENTES. El disponer de métodos fenotípicos para la detección de β -lactamasas AmpC plasmídicas sería

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de gran utilidad. El objetivo del presente estudio ha sido el de valorar métodos de cribado para la detección de β -lactamasas AmpC plasmídicas en cepas de *Escherichia coli*, *Klebsiella pneumoniae* y *Proteus mirabilis*.

MÉTODOS. Se seleccionaron las cepas en función del fenotipo de resistencia compatible con la producción de una β -lactamasa de tipo AmpC. La detección de genes *ampC* adquiridos se realizó mediante una técnica de multiplex PCR y secuenciación. Los métodos fenotípicos evaluados fueron el examen visual de las placas de antibiograma para detectar colonias en la proximidad del borde de los halos de inhibición de cefoxitina, cefotaxima, ceftazidima y aztreonam, y una prueba de sinergia con doble disco usando cloxacilina (500 μ g) como inhibidor de enzimas AmpC.

RESULTADOS. Se seleccionaron 77 cepas de las 6.209 aisladas. En 19 (24,7%) cepas que incluyeron 14 *E. coli*, dos *K. pneumoniae* y tres *P. mirabilis* se detectaron genes *ampC* adquiridos (*bla*_{CMY-2}, *bla*_{DHA-1}, *bla*_{CMY-4} y *bla*_{ACC-1}). La característica diferencial de la presencia de colonias en el halo de inhibición mostró una sensibilidad y especificidad del 100%. Se obtuvieron resultados similares con el test de cloxacilina, excepto en cepas de *E. coli* en las que la especificidad fue del 10,3%.

CONCLUSIÓN. La característica fenotípica aquí descrita puede considerarse útil para la sospecha de la presencia de estas enzimas. El test de cloxacilina sólo resulta de utilidad en cepas carentes de una β -lactamasa AmpC cromosómica.

Palabras clave: β -lactamasa AmpC. CMY. ACC. DHA. Detección fenotípica.

Introduction

Plasmid-mediated AmpC β -lactamases constitute an emerging therapeutic problem. Most of these enzymes confer a resistance pattern similar to the overproduction of chromosomal AmpC β -lactamases, which may involve all β -lactam antibiotics except for carbapenems and cepime¹.

Evidence from the nucleotide sequence suggests that genes encoding these enzymes derive from chromosomal *ampC* genes of several *Enterobacteriaceae* which have been integrated into transferable genetic elements facilitating the spread to different microorganisms¹.

Surveillance of plasmid-mediated AmpC β -lactamase-producing organisms is problematic due to the lack of standardized phenotypic methods for its specific detection. Several phenotypic detection methods have been proposed^{2,3}, but none are able to discriminate between acquired and chromosomal AmpC enzymes in organisms that produce endogenous AmpC enzymes.

During a routine surveillance study on β -lactam resistance, we observed that some isolates of *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, with a pattern of resistance compatible with AmpC production, displayed a phenotypical differential trait on antibiogram plates. This consisted in the presence of scattered colonies located near the edge of inhibition around cefoxitin, cefotaxime, ceftazidime and aztreonam discs. The β -lactamases of three randomly selected strains exhibiting this phenotypical feature were characterized and in all cases the plasmidic AmpC β -lactamase CMY-2 was found.

For this reason, a prospective study was performed to investigate whether this phenotype was related to the presence of plasmid-mediated AmpC β -lactamases. The usefulness of a screening test using cloxacillin as inhibitor of AmpC enzymes was also evaluated.

Materials and methods

The isolates included in the study were all clinically relevant isolates of *E. coli*, *K. pneumoniae* and *P. mirabilis* recovered from routine cultures at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) from January 2003 to January 2005.

The susceptibility test was performed by the disc diffusion method according to CLSI (formerly NCCLS) guidelines⁴ with commercial discs (Bio-Rad, Marnes La Coquette, France). The inducible AmpC β -lactamases were detected on primary antibiogram plates by the presence of D-shaped zone of inhibition of aztreonam, ceftazidime or cefepime disc adjacent (2.5-3 cm) to the imipenem or cefoxitin discs. The production of extended-spectrum β -lactamase (ESBL) was assessed by the double-disc synergy method⁵ and by Etest ESBL (AB Biodisk, Solna, Sweden) following the manufacturer's instructions.

Classically, strains expressing AmpC enzymes are resistant to cefoxitin. Nevertheless, as some plasmid-mediated AmpC enzymes (ACC) do not confer resistance to cefoxitin, the selected strains for this study were those showing intermediate resistance or resistance to amoxicillin-clavulanic acid, as well as to cefotaxime or ceftazidime according to CLSI breakpoints, and negative results for ESBL production. The isolates positive for ESBL production that showed intermediate resistance or resistance to cefoxitin were also included.

All selected isolates were tested by a double-disc synergy test based on the utilisation of cloxacillin as inhibitor of AmpC enzymes. Each isolate was inoculated on a Mueller Hinton agar plate, according to CLSI guidelines for disc diffusion susceptibility testing⁴. A disc of cloxacillin (500 μ g) (Neo-Sensitabs, Rosco Diagnostica S/A, Taastrup, Denmark) was placed 2.5 cm (center to center) from ceftazidime (30 μ g) and cefotaxime (30 μ g) discs. After incubation at 37 °C overnight, an enhanced zone of inhibition between any cephalosporin disc and cloxacillin disc was interpreted as evidence of AmpC production.

The presence of acquired *ampC* genes was assessed in all isolates selected according to the above-mentioned phenotype of resistance by a multiplex PCR with six sets of *ampC* family-specific primers as described previously⁶. This method allows to distinguish six groups of AmpC enzymes that include ACC, FOX, MOX (including MOX and CMY-1 related enzymes), DHA, CIT (including LAT, BIL and CMY-2 related enzymes) and EBC (including ACT and MIR enzymes)⁶. Clinical isolates of *Citrobacter freundii*, *Morganella morganii* and *Hafnia alvei* were used as control strains, as well as previously characterised strains carrying CMY-2 or ACC-1^{7,8}. Direct DNA sequencing of PCR products was carried out on both strands by the dideoxy method with fluorescent primers using the Automatic Laser Fluorescent DNA Sequencer (ALF, Pharmacia, Upsala, Sweden). Nucleotide and amino acid sequences were analysed via Internet using the BLAST program (www.ncbi.nlm.nih.gov). Similarly, the full sequences of the *bla*_{CMY}, *bla*_{ACC} and *bla*_{DHA} genes were determined as previously described^{7,9}. The multiplex *ampC*-PCR was considered the reference method to compare the results obtained from the phenotypical detection methods used.

Results and discussion

Among the 6,209 isolates tested, 70 *E. coli*, four *K. pneumoniae* and three *P. mirabilis* met the inclusion criteria and were selected for further analysis.

As shown in table 1, multiplex *ampC*-PCR gave amplification products for 19 (24.7%) isolates, encompassing 14 *E. coli*, two *K. pneumoniae* and three *P. mirabilis* isolates. Sequence analyses showed a 100% identity with *bla*_{CMY-2} (15 isolates), *bla*_{DHA-1} (two isolates; both showed induction on antibiogram plates), *bla*_{CMY-4} (one isolate) and *bla*_{ACC-1} (one isolate). ESBL production was found in 12 (10 *E. coli* and two *K. pneumoniae*) of the 77 isolates selected. None of these isolates yielded a positive multiplex *ampC*-PCR result.

Visual examination of antibiogram plates revealed a differential phenotypical trait manifested by the presence of scattered colonies located near to the edge of inhibition of

TABLE 1. Correlation between genotypes and results obtained from phenotypic detection methods

Organism	No. studied	No. selected	Multiplex <i>ampC</i> PCR results	No. isolates (genes)	Presence of scattered colonies		Cloxacillin double-disk result	
					Positive	Negative	Positive	Negative
<i>E. coli</i>	5,081	70	Positive	14 (11 <i>bla</i> _{CMY-2} , 1 <i>bla</i> _{CMY-4} , 1 <i>bla</i> _{ACC-1} , 1 <i>bla</i> _{DHA-1})	14	0	14	0
			Negative*	56	0	56	52	4
<i>K. pneumoniae</i>	607	4	Positive	2 (1 <i>bla</i> _{CMY-2} , 1 <i>bla</i> _{DHA-1})	2	0	2	0
			Negative*	2	0	2	0	2
<i>P. mirabilis</i>	521	3	Positive	3 (3 <i>bla</i> _{CMY-2})	3	0	3	0
Total No.	6,209	77	Positive	19	19	0	19	0
			Negative	58	0	58	52	6

*Ten *E. coli* and two *K. pneumoniae* carried ESBL.

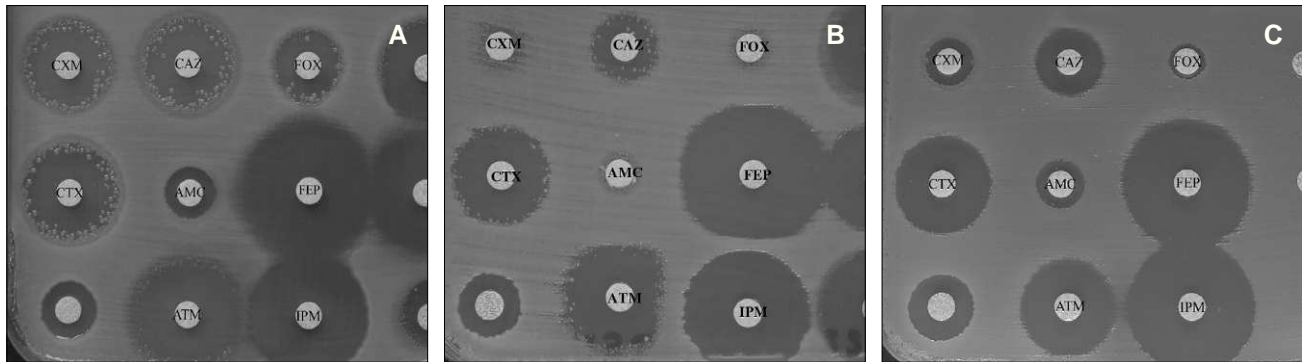


Figure 1. Disk diffusion test. A) *E. coli* isolate carrying *bla*_{CMY-2} as representative of strains carrying CMY-4 or ACC-1. B) *E. coli* isolate carrying DHA-1. C) *E. coli* isolate multiplex *ampC*-PCR negative hyperproducing the chromosomal mediated AmC β -lactamase. CXM: cefuroxime; CAZ: ceftazidime; FOX: cefoxitin; CTX: cefotaxime; AMC: amoxicillin-clavulanic acid; FEP: cefepime; ATM: aztreonam; IPM: imipenem.

cefoxitin, cefotaxime, ceftazidime and aztreonam discs in all 19 isolates carrying plasmid mediated AmpC β -lactamases. The remaining isolates showed a well-delimited edge of inhibition (fig. 1). This phenotypic trait therefore exhibited 100% sensitivity and specificity.

Results from the cloxacillin test correlated with the presence or absence of *ampC* genes in all *K. pneumoniae* and *P. mirabilis*, species without a natural AmpC. Of the 70 *E. coli* tested, this method correctly detected the 14 isolates carrying acquired *ampC* genes, but positive results were obtained also in 52 *E. coli* isolates (including six ESBL producers). No differences were found between the use of cefotaxime or ceftazidime in the cloxacillin double-disc synergy test. Based on these findings, the cloxacillin test exhibited 100% sensitivity and 10.3% specificity in *E. coli* isolates. Although additional testing is required, the positive result of the cloxacillin test in the 52 *E. coli* isolates could be explained by the hyperproduction of chromosomal AmpC β -lactamase.

The isolates negative for the cloxacillin tests (four *E. coli* and two *K. pneumoniae* isolates) were all ESBL producers, in which reduced susceptibility to cefoxitin could probably be due to non-enzymatic resistance mechanisms such as altered permeability.

In this study, CMY-2 was the most prevalent acquired AmpC β -lactamase found and inducible DHA-1, CMY-4, and cefoxitin susceptible ACC-1 were less common. Most plasmid-mediated AmpC β -lactamases identified up to 1998 in the Mediterranean area belonged to the groups CMY-2 to CMY-5 and LAT-1 to LAT-4, but other types, such as FOX-3, FOX-4, ACC-1 and DHA-1, have also recently been reported^{1,8,10,11}. To our knowledge, this is the first time that DHA-1 and CMY-4 have been reported in Spain.

Although the number of isolates carrying plasmid-mediated AmpC betalactamases in our study was small, we have shown that only isolates exhibiting scattered colonies carried acquired *ampC* genes. This distinctive trait should therefore be considered in organisms such as *E. coli* in which the differentiation between plasmidic AmpC and hyperproduction of the chromosomal enzyme is not available based on phenotypic tests. The cloxacillin double-disc synergy test is a simple and reliable method, particularly in isolates lacking a natural AmpC β -lactamase. Further study is required to determine whether this

differential phenotypical trait is also present in isolates carrying AmpC β -lactamases other than CMY-2, CMY-4, DHA-1 and ACC-1. These practical and simple methods for detection of plasmid-mediated AmpC β -lactamases may be useful for enhanced infection control.

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