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CLINICAL MICROBIOLOGY - SHORT COMMUNICATION

Conjugative plasmidic AmpC detected in *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* human clinical isolates from Portugal

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Abstract

AmpC is a type of β-lactamase enzyme produced by bacteria; these enzymes are classified in Class C and Group 1, and these confer resistance to cephamycin. Enterobacterales producing AmpC are reported worldwide and have great clinical importance due to therapeutic restriction and epidemiological importance once the easy dissemination by plasmidic genes to other bacteria is a real threat. These genes are naturally found in some enterobacteria as *Enterobacter cloacae*, *Morganella morganii*, and *Citrobacter freundii*, but other species have demonstrated similar resistance phenotype of AmpC production. Genes carried in plasmids have been described in these species conferring resistance to cefoxitin and causing therapeutic failure in some bacterial infections. This work detected and described five clinical strains of *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* that presented plasmid ampC (pAmpC) isolated from the north of Portugal collected in 2009. AmpC production was confirmed by inhibition of the enzyme by cloxacillin and boronic acid in agar diffusion tests. Also, PCR (polymerase chain reaction) was performed for the detection of gene universal to AmpC, bla_{ampC}, and others to AmpC group: bla_{ACC}, bla_{CIT}, bla_{CMY}, bla_{DHA}, and bla_{EBC}. The conjugation in liquid medium for 24 h was realized to determine if gene is localized in chromosome or plasmid. The isolates and their conjugants showed phenotypic characteristics and bla_{CMY} and bla_{CIT} were detected by PCR corroborating the AmpC characteristics observed in these bacteria. Confirmation of transfer of plasmid containing genes encoding AmpC is of high epidemiological relevance to the hospital studied and demonstrated the importance of AmpC surveillance and studies in hospital and community environments in order to choose the appropriate therapy for bacterial infections.

Keywords β-Lactamase · Boronic acid · Cloxacillin · Conjugative plasmid · Nosocomial infection

Introduction

Escherichia coli, *Klebsiella pneumoniae*, and *Proteus mirabilis* are the main Gram-negative pathogens responsible for nosocomial infections, a serious public health problem. Some risk factors in the development of these infections are burns, presence of endotracheal tubes, nasal tubes, blood catheters, and hospitalization for more than 2 weeks [1, 2]. In addition to the high rates of infection in hospitals, the growing resistance of these nosocomial pathogens to antimicrobials has an economic burden in the treatment of these infections. These pathogens have been reported resistant to important drugs to treat infectious diseases as cefotaxime, amoxicillin, nitrofurantoin, and trimethoprim plus sulfamethoxazole [3, 4].

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Multidrug-resistant microorganisms (MDR), in particular, Gram-negative MDR bacteria, are an increasing problem in hospital care around the world. The rate of antibiotic resistance among enterobacteria has accelerated dramatically in recent years and has reached a pandemic scale, due to the great diversity of mechanisms of resistance to existing antibiotics. It is no longer uncommon to find infections caused by bacteria with multiple resistance mechanisms. In recent years, new types of ESBL, AmpC, and carbapenemases β-lactamases have emerged [4].

Beta-lactamases are enzymes that cause resistance to β-lactams antibiotics, specifically by hydrolysis of the β-lactam ring. Ambler and Bush-Jacoby-Medeiros classified β-lactamases according to its function and molecular structure. AmpC β-lactamases belong to Class C and Group 1, according to Ambler and Bush-Jacoby-Medeiros, respectively. These enzymes confer resistance to cephamycin as cefoxitin to the oxyimino-cephalosporins (ceftazidime and cefotaxime, for instance), and these are not inhibited by clavulanate, sulbactam, and tazobactam. Boronic acid and cloxacillin are considered AmpC inhibitors used in phenotypical tests to confirm the enzyme presence [5–9].

Chromosomal AmpC is naturally found in group II Enterobacteriaceae (like *Enterobacter* spp., *Serratia* spp., *Providentia* spp., *Morganella morganii*, *Citrobacter freundii*, and *Hafnia alvei*). The most common plasmid-encoded AmpC (pAmpC) enzymes are CIT, CMY, and DHA. Other pAmpC can also found in Enterobacterales as EBC, FOX, and MOX. Originally, these genes were transferred by natural producers as described previously to another bacteria. Actually, these genes are disseminated between non-natural producers by plasmids or other mobile elements. Some authors describe that pAmpC can also confer resistance to carbapenems associated to the loss of outer membrane porin proteins in clinical isolates of *Klebsiella pneumoniae* and *Salmonella enterica* [7, 9, 10].

The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have designated β-lactamase-producing Gram-negative bacteria some of the world's most serious or critical threats [9].

AmpC-producing Enterobacterales studies have become important to understand the possibilities to treatment failures. Until 2012, AmpC production was rarely reported in the literature in both community and hospital cases [11]. Currently, this study has demonstrated that plasmid-mediated AmpC (pAmpC) is most detected in community-acquired infections, while cromossomal AmpC (cAmpC) producers are mainly involved in healthcare-associated infections [12–14].

The main goal of this work was to confirm AmpC production in clinical strains isolated from patients in hospitals in the north of Portugal before 2010 [15] and demonstrate the possibility of this mechanism of resistance being mediated by conjugative plasmids, leading to treatment failure in nosocomial infections.

Materials and methods

Identification and antimicrobial susceptibility test

Five strains previously identified and resistant to cefoxitin were used in this study. These bacteria were isolated from hospitals in the north of Portugal in 2009. They were maintained to − 80 °C until the use in this work. The isolates were grown on brain and heart infusion agar (BHIA) (Liofilchem) and incubated at 35 °C for 24 h. After this period, they were seeded on MacConkey agar (Liofilchem) and incubated in the same conditions for subsequent phenotypic confirmation of colonies characteristics. From this growth, the isolates were inoculated in trypticase soy broth (TSB) (Liofilchem). Identification was confirmed using Chromagar Orientation (CHROMagar) and ID32GN (Biomérieux).

These isolates were submitted to disk diffusion test to confirm phenotypic characteristics and confirm AmpC production using amoxicillin (AMO 10 μg), amoxicillin with clavulanate (AMC 20/10 μg), aztreonam (AZT 30 μg), cefepime (CEF 30 μg), cefotaxime (CTX 30 μg), cefoxitin (FOX 30 μg), ceftazidime (CAZ 30 μg), imipenem (IMI 10 μg), and streptomycin (STP 10 μg) (Oxoid; Sensidisc) [16].

AmpC inhibition by cloxacillin and boronic acid

The cloxacillin inhibition method was performed using Muller-Hinton agar (MHA) added to cloxacillin (200 μg/mL Sigma-Aldrich) to disk diffusion method, as described previously. It was positive when isolates demonstrated susceptibility to the antimicrobials to which were resistant in the method without cloxacillin [6, 8].

In boronic acid test, 400 μg of boronic acid was added to cefoxitin disks. The isolates were inoculated in the MHA plate, and one disk with and one without boronic acid were added on each plate. Those isolates in which an increase in the diameter of the disk halo containing boronic acid was a value equal to or greater than 5 mm was observed, the isolate was considered to produce AmpC [5] (Fig. 1).

Conjugation method

This methodology was standardized by Mota (17) and was carried out with minor modifications. Firstly, the donor is prepared to conjugation method. This preparation involves three steps of bacterial culture, one in solid medium and two in liquid medium. The donor isolates were inoculated in CLED agar, and after 24 h, 35 °C, one colony of each isolate was suspended in 2 mL of TSB containing one disk of cefoxitin 30 μg. This broth was incubated at 35 °C for 24 h and after this period 200 μL was added to 2 mL of TSB without antimicrobial disk and incubated to the same period and temperature mentioned before.

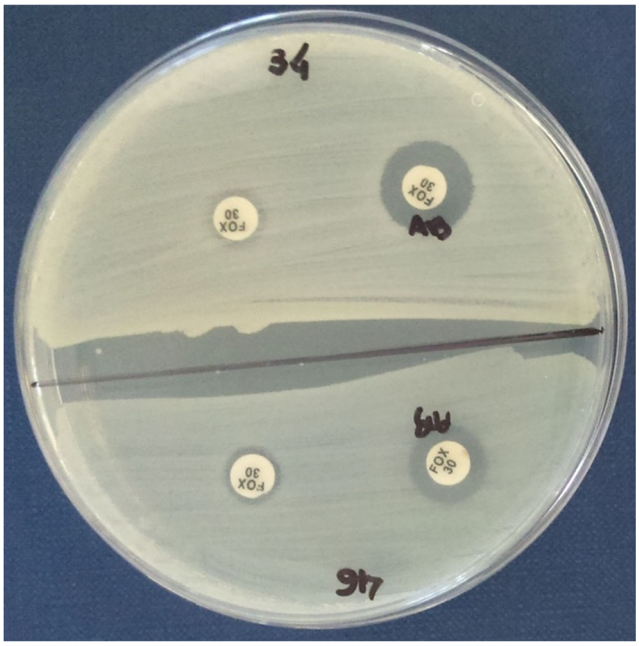

Fig. 1 Phenotypic test using boronic acid to detection of AmpC producing in Enterobacteriales. Example of isolates tested to AmpC inhibition by boronic acid (AB). Right disks of cefoxitin were added boronic acid (AB) solution and left ones, no. It is possible to note increase in halo around AB + cefoxitin disk comparing with no added disk

The receptor strain used in the conjugation is *Escherichia coli* HB101. This strain was inoculated in CLED agar and after 24 h, 35 °C, one colony was suspended in 5 mL of TSB without cefoxitin disk. These culture mediums were inoculated at 35 °C for 24 h to be used in mating.

In 2 mL of TSB without cefoxitin were added 200 μL of donor bacteria broth (the second growth) and 400 μL of receptor bacteria broth. The donor broth and the receptor broth used were prepared as mentioned previously. This suspension was incubated at 35 °C for 24 h without shaking. The presence of transconjugants was observed in selective MacConkey agar plates with cefoxitin (10 μg/mL Labesfal) and streptomycin (100 μg/mL Sigma-Aldrich)—presenting lactose non-fermenting colonies after incubation at 35 °C for 24 h.

From this bacterial growth in the selecting agar for transconjugants, five colonies for each tested strain were selected to verify the phenotypic and genotypic characteristics using the same methodologies described to detection and confirmation of AmpC performed in donor strains.

PCR assays

To obtain genomic DNA, a loopful of each pure isolate culture was suspended in 300 mL of sterile distilled water and was boiled for 10 min. After 5 min of centrifugation, the supernatant was used as a DNA template for PCR amplification [17, 18].

The detection of *ampC* was performed firstly to universal *bla*AmpC [19]. The specific groups of AmpC were tested in multiplex PCR using primers to CIT, ACC, EBC, FOX, and MOX genes [20]. The PCR for DHA [21] and CMY [22] were performed using uniplex PCR.

Each reaction for uniplex PCR contained 20 mM Tris-HCl, pH 8.4; 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$; 0.6 μM of each primer; and 1.25 U of Taq DNA polymerase (Kapa Biosystems) in a total reaction volume of 25 μL containing 2 μL of the extracted DNA [19, 21, 22]. The concentrations of primers used for multiplex amplification were as described by Pérez-Pérez and Hanson [20]. The thermal cycler T100TM Thermal Cycler (BIO-RAD) was used for PCR reactions and the products were evaluated by electrophoresis on agarose gel (2%) (GeneON), revealed with Midori Green Advance DNA Stain (Nippon Genetics) and the molecular weight markers of 100 pb (GRISP) and 1000 pb (Bioron). The gels were registered using a BIO-RAD photo-documenter (Molecular Imager Gel Doc TM XR + System with Image LabTM Software, BIO-RAD).

Control strains and receptor strain to conjugation method

Escherichia coli CMY-2 positive was used as a control to tests performed in this work [23], *Escherichia coli* NCTC 13451 were used as negative control in this work, and *Escherichia coli* HB101 was used as receptor in conjugation.

Results and discussion

The isolates studied in this work were collected from clinical samples obtained in hospitals in north of Portugal in 2009 to evaluate other β-lactamases types. These five isolates demonstrate resistance to cefoxitin, characteristic of AmpC producers according to CLSI [16], but this enzyme was not evaluated at the time. The species presenting this resistance have been considered important species to nosocomial infections: *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* and these bacteria are commonly associated with multidrug-resistance [3, 4, 15]. *K. pneumoniae* (3/5) was obtained from pus and urine, *E. coli* (1/5) also was obtained from urine, and *P. mirabilis* (1/5) was isolated from a blood catheter. According to dates described by Goossens and Grabein (2005) [24], the prevalence of AmpC in the Europe was observed in *Enterobacter* spp. and *Citrobacter* spp. Both species are natural producers of AmpC, different from the species mentioned in this work.

The bacteria selected with AmpC characteristics were submitted to the disk diffusion using β-lactams antibiotics to confirm the profile evaluated in 2009. The resistance profile was confirmed; the isolates presented resistance to

amoxicillin, amoxicillin with clavulanate, cefoxitin, ceftazidime, ceftriaxone, cefepime, and imipenem (Table 1). AmpC enzyme confers resistance to oxyimino-cephalosporins and cephamycin, and it is not inhibited by clavulanate. Cefepime and imipenem resistance detected in *Proteus mirabilis* can be caused by the association of AmpC production and loss of outer membrane porin proteins [25]. *Proteus* spp. have been reported as a nosocomial bacteria arboring multidrug resistance gene in addition to the natural mechanisms found in this type of bacteria. The increase in multidrug resistance in Enterobacterales and limitation of options for antibiotic treatment is a significant public health issue [26, 27].

Coudron, Rodríguez-Martínez et al., and Pires et al. [5, 6, 8] describe that the AmpC enzyme is inhibited by boronic acid and cloxacillin. It was tested in our isolates, and boronic acid demonstrated to be a suitable inhibitor of resistance. Considering cloxacillin, it was not possible to observe AmpC inhibition in *Escherichia coli* and *Proteus mirabilis*, respectively. This observation was reported in transconjugants as described posteriorly.

Genotypically, the isolates amplified to bla_{ampC} (5/5), bla_{CIT} (3/5), bla_{CMY} (2/5), and bla_{DHA} (2/5). These genes are described in plasmids in *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. Reuland et al. [28] described in their study pAmpC (bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{DHA}, bla_{ACT}, bla_{MIR}, and bla_{ACC}) in community strains in Amsterdam. Ribeiro et al. [15] and Kazemian et al. [29] identified bla_{DHA} bla_{CMY} and bla_{CIT} pAmpC in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* in different types of nosocomial infections in Portugal and Iran, respectively.

As has been observed by several previous studies, there is difficulty identifying AmpC production and identifying the specific type of AmpC enzyme produced by the isolate. This is maybe due to the different AmpC type results in similar phenotypical resistance. By way of explanation, AmpC producers demonstrate cephamycins resistance and other cephalosporins resistance may be present due to enzyme coproduction. *Klebsiella pneumoniae* and *Escherichia coli* studied in this work present the same resistance profile (AMX - AMC, FOX, CAZ) (Table 1), for instance, but they have a different genotype profile. This observation reinforces the importance of associating phenotypic and genotypic methods for the diagnosis of antimicrobial resistance.

The clinical strains were used as donors in the conjugation method in a liquid medium to verify the location of bla_{ampC} genes. All bacteria tested were able to transfer bla_{ampC} genes to *Escherichia coli* HB101. The receptor bacteria were a lactose non-fermenter and resistant to streptomycin. Before conjugation, all donors were submitted to streptomycin susceptibility tests, and they were susceptible to it.

Escherichia coli lactose non-fermenter obtained in MacConkey agar with streptomycin and cefoxitin were subjected to identification confirmation by Chromagar Orientation and ID32GN. Then, five colonies of each strain transconjugant were used to disk diffusion method to confirm

Table 1 Phenotypic and genotypic characteristics of isolates producing plasmid AmpC

Inhibition						
Isolate	Species	Sample	Resistance profile	Boronic acid	Cloxacillin	Genotypic profile (*bla*)
1	*Escherichia coli*	Urine	AMX - AMC - FOX - CAZ	Yes	*	AmpC - CIT - CMY
1T	*Escherichia coli*	–	AMX - AMC - FOX - CAZ	Yes	*	AmpC - CIT - CMY
2	*Proteus mirabilis*	Blood catheter	AMX - AMC - FOX - CAZ - CTX - CEF - IMI	Yes	*	AmpC - CIT - CMY
2T	*Escherichia coli*	–	AMX - AMC - FOX - CAZ - CTX	Yes	*	AmpC - CIT - CMY
3	*Klebsiella pneumoniae*	Pus	AMX - AMC - FOX - CAZ	Yes	Yes	AmpC - CIT
3T	*Escherichia coli*	–	AMX - AMC - FOX - CAZ	Yes	*	AmpC - CIT
4	*Klebsiella pneumoniae*	Urine	AMX - AMC - FOX - CAZ	Yes	Yes	AmpC - DHA
4T	*Escherichia coli*	–	AMX - AMC - FOX - CAZ	Yes	*	AmpC
5	*Klebsiella pneumoniae*	Urine	AMX - AMC - FOX - CAZ	Yes	Yes	AmpC - DHA
5T	*Escherichia coli*	–	AMX - AMC - FOX - CAZ	Yes	*	AmpC
CMY-2 Control	*Escherichia coli*	–	AMX - AMC - FOX - CAZ - CEF	Yes	Yes	AmpC - CIT - CMY
CMY-2 ControlT	*Escherichia coli*	–	AMX - AMC - FOX - CAZ - CEF	Yes	*	AmpC - CIT - CMY

T transconjugant, *AMX* amoxicillin, *AMC* amoxicillin plus clavulanate, *FOX* cefoxitin, *CAZ* ceftazidime, *CTX* ceftriaxone, *CEF* cefepime, *IMI* imipenem

*There was no visible growth in culture medium

the phenotypic characteristics observed in donors' bacteria. Only the transconjugant from isolate 2, *Proteus mirabilis*, do not present the same resistance profile observed in donor. Isolate 2 presented resistance to cefepime and imipenem, but 2T did not present this resistance (Table 1), suggesting other mechanism of resistance, such as lack of porins, commonly reported in *P. mirabilis* [25].

All transconjugants had pAmpC inhibited by boronic acid. In the cloxacillin inhibition test, it was not possible to determine if this substance could inhibit the pAmpC once all the transconjugants did not grow in MHA with cloxacillin.

The genes bla_{ampC}, bla_{CIT}, and bla_{CMY} were transferred to the receptor, demonstrating that these genes are located in a conjugative plasmid. Nevertheless, bla_{DHA} was not identified in transconjugants 4T and 5T. This can be justified by the fact that the gene is not inserted in a conjugative plasmid or it can be in other mobile genetic element since it is not a chromosomal AmpC in this specie [5, 10].

ESBL and carbapenemase are widely studied; however, it is necessary to evaluate the AmpC production in order to understand the risks of the prevalence of this enzyme in and out of hospitals [28]. In the case of pAmpC production by bacteria, it is necessary to study the best option for antimicrobial therapy [26]. Ribeiro et al. [15] reported pAmpC in nosocomial isolates collected between 2010 and 2013 in north of Portugal. Thus, these genes circulated in hospital environments before 2010 according to our data. These studies demonstrate the importance of more studies to understand epidemiology of pAmpC in Portugal.

Conclusion

The bla_{CIT} and bla_{CMY} pAmpC genes were present in hospitals in the north of Portugal since 2009, and this fact is important because these mobile genes can be passed to other bacteria, including non-nosocomial bacteria. This data is epidemiologically relevant to public health demonstrating the circulation of AmpC genes. Furthermore, it is necessary to implement cAmpC and pAmpC detection tests as routine to select the best option to antimicrobial therapy in these environments, once the bla_{AmpC} genes decrease options for antimicrobial therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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