Utility of the Ceftazidime-Imipenem Antagonism Test (CIAT) to Detect and Confirm the Presence of Inducible AmpC Beta-Lactamases Among Enterobacteriaceae

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Detection of AmpC beta-lactamase production by enterobacteria has been problematic. Contrary to ESBLs, no specific guidelines are available for detection and confirmation of AmpC production by clinical relevant microorganisms. Moreover, some bacterial species may produce inducible AmpC beta-lactamases that can be easily overlooked by routine susceptibility tests. We reported here a new test based on the strong inducible effect of imipenem on AmpC genes and the consequent antagonism with ceftazidime. This test is very simple and proved to be helpful in detecting AmpC-inducible enzymes among several species of clinical isolates.

Key-Words: AmpC, inductible beta-lactamases, screening tests, imipenem, cefoxitin screening.

Production of enzymes that hydrolyze beta-lactam antibiotics is one of the major mechanisms of bacterial resistance [1,2]. Several members of Enterobacteriaceae are naturally resistant to ampicillin and first generation cephalosporins due to the production of chromosomally encoded beta-lactamases, collectively called class C or AmpC beta-lactamases [3]. Furthermore, point mutation in these *ampC* genes may confer resistance to virtually all cephalosporins and monobactams, with the possible exception of cefepime, cefpirome and the carbapenems [4]. Cefepime resistance, however, has already been reported among AmpC-producing strains [5,6]. Moreover, plasmids containing derivatives of the chromosomally encoded AmpC cephalosporinases are becoming disseminated among enterobacteria, thus providing a new mechanism of resistance for those originally AmpC-deficient bacterial strains [4,7]. Chromosomal AmpC beta-lactamases are usually inducible, while, except for DHA enzymes, plasmid-mediated AmpC enzymes are not [4,8,9].

Clinical microbiology laboratories should be able to detect bacterial strains producing AmpC enzymes, since these strains may appear susceptible to a particular beta-lactam antibiotic *in vitro*, but show no clinical response when used to treat serious infections [10]. The former National Committee for Clinical Laboratory Standards (NCCLS, now CLSI) guidelines for performing *in vitro* susceptibility tests have included procedures for screening and confirmation of ESBL-producing microorganisms [11]; however, it does not contain any information on AmpC detection or confirmation. Recently proposed tests for AmpC beta-lactamases include: use of new beta-lactamase inhibitors [12], phenotypic tests [8], PCR for plasmid-mediated *ampC* genes [13], and even DNA chips [14]. These tests either depend on drugs not widely available or are still too cumbersome or technically demanding to be widely used by clinical microbiology laboratories.

We reported here the utility of a phenotypic test to detect and confirm the presence of inducible AmpC beta-lactamases among enterobacterial strains, based on the strong inducing effect of imipenem on these enzymes [15,16].

Materials and Methods

Bacterial strains isolated from clinical samples such as urine, blood, sputum, and swabs from hospitalized patients were represented by several members of Enterobacteriaceae, showing resistance to a 30 µg-cefuroxime disk [15]. Identification at species level was performed using the Vitek system (bioMérieux, France) or 16S rDNA sequencing [17]. Susceptibility tests were done by a standard disk diffusion method (Oxoid, Hampshire, UK), and screening for extended spectrum beta-lactamase (ESBL) was performed following the NCCLS guidelines [11], and then confirmed using both ceftazidime plus cefotaxime disks, both with and without clavulanate [11] and the double-disk approximation test [18]. Thirty-four cefuroxime-resistant strains were negative for ESBL and they all showed evidence of AmpC beta-lactamase production, as judged by the ceftazidime-imipenem antagonism test (CIAT), which consisted of a imipenem disk (10 µg) placed 20 mM apart (edge-to-edge) from a ceftazidime disk (30 µg) on a Mueller-Hinton agar plate previously inoculated with a 0.5 McFarland bacterial suspension, and incubated for 24 h at 35°C. For comparison, a cefoxitin disk was also placed 20 mM apart from the ceftazidime disk (Figure 1A). Antagonism, indicated by a visible reduction in the inhibition zone around the ceftazidime disk adjacent to the imipenem or cefoxitin disks, was regarded as positive for inducible AmpC beta-lactamase production, and further confirmed using molecular testing. Whenever necessary, the presence of ESBL (e.g. bla_{TEM} and bla_{SHV}) or AmpC were confirmed by PCR amplification followed by DNA sequencing using the BigDye Terminator kit (Applied Biosystems) [9,13,19,20,21]. Multiple PCR runs and sequencing were performed for each enzyme to insure that a Taq polymerase error had not occurred.

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Results

A total of 34 cefuroxime-resistant and ESBL-negative enterobacterial strains were CIAT positive, indicating the production of inducible AmpC beta-lactamases. Among them, 1 *K. pneumoniae* strain and 1 *Proteus mirabilis* strain were CIAT positive, and the respective enzymes were identified as bla_{DHA} by PCR with specific primers [9]. Further characterization of these genes by nucleotide sequencing analysis showed 100% homology with bla_{DHA-1} for *K. pneumoniae*. The *P. mirabilis* sequence revealed only 95% nucleotide homology with bla_{DHA-1}, resulting in 97.3% identity with bla_{DHA-1} and bla_{DHA-2} (Fig. 1B) and was provisionally called DHA-3. All the other 32 CIAT positive strains consisted of species known to naturally produce inducible AmpC beta-lactamases. Twenty-one of these strains belonged to the genus *Enterobacter*. Homology search (DDJB) showed that 2 *Enterobacter* strains harbored *ampC* genes that were 99.3 and 100% homologous to ACT-1, respectively, suggesting that they may be in fact *E. asburiae* species [22]. All other *ampC* genes could be arranged into 2 subclasses, as previously described for *Enterobacter* spp. [23]: (i) 7 strains containing a*mpC* genes close to (≥99.8% homology) bla_{MNH-1} from *E. cloacae* (GenBank accession number X08082), and (ii) 12 strains with *ampC* genes highly homologous (≥99.6%) to the chromosomal *ampC* gene of *E. cloacae* Q908R (X08081).

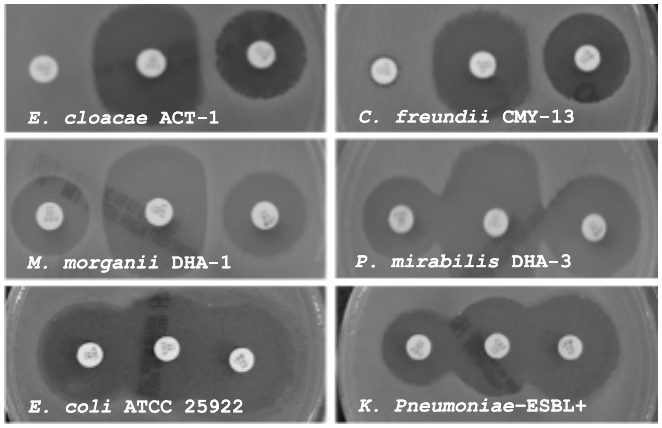
M. morganii was the second most common pathogen harboring inducible AmpC beta-lactamase. DNA sequencing revealed 100% homology with bla_{DHA-1} for 8 of these strains, in accordance with previous reports [24,25]. One *M. morganii* strain harbored an *ampC* gene that was 100% homologous to the sequence of a *M. morganii ampC* gene described as MOR-2 in the DDJB databank (accession number AY235804). The *ampC* gene from 1 *Citrobacter freundii* strain was similar to bla_{CMY-13} (97.6% identity).

Discussion

In this study, we used imipenem, a potent inducer of *ampC* genes, together with ceftazidime to detect inducible AmpC beta-lactamases [9,15,16]. Obviously, the CIAT test can not be used to

Figure 1. Detection of Amp-C beta-lactamases using CIAT. (A) Bacterial strains and the respective enzymes are indicated. *E. coli* ATCC 25922 is a negative control and the *K. pneumoniae* strain shows that the test is negative (i.e. not affected) for ESBL. For some strains, the blunting of ceftazidime zone (center) by imipenem-induced enzyme (right side) is more pronounced, compared with the cefoxitin-induced enzyme (left side). Note the intermediate resistance for cefoxitin (disk on the left) for DHA-producing organisms. (B) Comparison of the deduced amino acid sequences of AmpC beta-lactamase of *P. mirabilis* with DHA-1, DHA-2, and MOR-2 from *M. morganii*. Distinct amino acids found in this *P. mirabilis* beta-lactamase (here designated DHA-3) are shown in bold.

A



B

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DHA-1   MKKSLSATLI SALLAFSAPG FSAADNVAAV VDSTIKPLMA QQDIPGMAVA VSVKG KPYYF
DHA-2   ---------- ---------- ---------- ---------- ---------- ----------
MOR-2   ---------- ---------- ---------- ---------- ---------- ----------
DHA-3             - ---------- ---------- ---------- ---------- E---------
DHA-1   NYGFADIQAK QPVTENTLFE LGSVSKTFTG VLGAVSVAKK EMALNDPAAK YQPEL ALPQW
DHA-2   ------V--- ---------- ---------- ---------- --M-----E- ----------
MOR-2   ------V--- ---------- ---------- ---------- --T-----E- ----------
DHA-3   ------V--- --A------- ---------- -------G-- -T-------- ----------
DHA-1   KGITLLDLAT YTAGGLPLQV PDAVKSRADL LNFYQQWQPS RKPGDMRLYA NSSIG LFGAL
DHA-2   ---------- --T------- -----N--E- -H-------- ---------- ----------
MOR-2   ---------- ---------- ---------- -H-------- ---------- ----------
DHA-3   ---------- ---------- ---------- ---------- W--------- ----------
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test strains showing no inhibition zone for ceftazidime or for those strains bearing plasmid-mediated AmpC enzymes that are not typically inducible. However, such strains are readily identified by their resistance profile, e.g. being susceptible only to cefepime and carbapenems [26]. On the other hand, detection of inducible AmpC enzymes is much more challenging. Susceptibility tests often do not detect resistance to third generation cephalosporins, and the clinical use of these cephalosporins could segregate resistant mutants that would ultimately result in therapeutic failure [26-28]. Resistance to cefoxitin alone (≤18 mM inhibition zone) was reported to overestimate the presence of AmpC beta-lactamases among *Enterobacteriaceae* [8,18]. To aggravate the problem, it is also known that *E. coli* harboring bla_{ACC-1} can be susceptible to cefoxitin, while strains with bla_{DHA-2} can have intermediate resistance for this drug [4]. This was the case of all strains harboring bla_{DHA} genes in this study, with the exception of one cefoxitin-resistant *K. pneumoniae* bearing bla_{DHA-1}. A combination of mechanisms, such as loss of membrane permeability or hyper-production of the AmpC enzyme could explain this finding. On the other hand, decreased susceptibility to cefoxitin is also found among non-AmpC producers, which makes screening with cefoxitin unreliable to detect AmpC production [8,18].

The CIAT is a simple test that can be used to confirm the presence of known, as well as new, inducible ampC enzymes, among enterobacterial strains. The test can be done directly on the initial susceptibility test or in combination with other disks used to detect ESBL production.

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