

Use of the cefepime-clavulanate ESBL Etest for detection of extended-spectrum beta-lactamases in AmpC co-producing bacteria

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Abstract

Background: Extended-spectrum beta-lactamases (ESBLs) may not always be detected in routine susceptibility tests. This study reports the performance of the cefepime-clavulanate ESBL Etest for the detection of ESBLs in Enterobacteriaceae, including those producing AmpC enzyme.

Methodology: Consecutive non-duplicate isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolated from bloodstream infections from January to June 2008 were tested for ESBL by both the standard CLSI double-disk diffusion method using ceftazidime and cefotaxime disks and Etests using ceftazidime/ceftazidime-clavulanate, cefotaxime/cefotaxime-clavulanate and cefepime/cefepime-clavulanate gradients. Isolates were also tested for the presence of transferable AmpC beta-lactamase by AmpC disk test and the efficacies of the different Etests in detecting ESBL production were compared.

Results: A total of 113 bacterial isolates (61 *K. pneumoniae*, 50 *E. coli*, and 2 *P. mirabilis*) were recovered. Respectively, 42 (37.2%) and 55 (48.7%) isolates were positive for ESBL by the ceftazidime-clavulanate and cefotaxime-clavulanate combined disk tests. The cefepime/cefepime-clavulanate Etest strip detected the maximum number of isolates (70/113, 61.9 %) as ESBL-positive compared to the ceftazidime/ceftazidime-clavulanate and cefotaxime/cefotaxime-clavulanate strips, which detected 57 (50.4%) isolates each as ESBL-positive. All three ESBL Etest strips were equally effective in detecting ESBL in the isolates that were AmpC negative. In the 66 (58.4%) isolates that co-produced AmpC in addition to the ESBL enzymes, cefepime/cefepime-clavulanate Etest strip detected ESBL in an additional 13 (11.4%) isolates as compared to the other ESBL Etest strips.

Conclusions: Cefepime-clavulanate ESBL Etest is a suitable substitute to test for ESBL production, especially in organisms producing AmpC beta-lactamases.

Key words: antimicrobial resistance, AmpC beta-lactamase, ESBL Etest, extended-spectrum beta-lactamases

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Introduction

Since their first description more than twenty years ago, pathogens producing extended-spectrum beta (β) lactamases (ESBLs) have become an increasing cause of clinical concern for several reasons [1-3]. First, systemic infections due to ESBL-producing Enterobacteriaceae are associated with severe adverse clinical outcomes. Second, initially restricted to certain geographical areas, these enzymes have spread globally and their prevalence varies by geographic region. Third, primarily characterized in limited bacteria such as *Escherichia coli* and *Klebsiella* spp., ESBLs have been spreading and reaching other genera, principally *Enterobacter* and *Proteus* spp. Finally, besides the growing species diversity, ESBL phenotypes have become more complex due to the production of multiple enzymes

including inhibitor-resistant ESBL variants, plasmid-borne AmpC, production of ESBLs in AmpC - producing bacteria, production of ESBLs in KPC-producing bacteria, enzyme hyperproduction and porin loss [1-4].

The ESBLs are typically plasmid-mediated enzymes that hydrolyse penicillins, third-generation cephalosporins and aztreonam [5]. They are not active against cephamycins (cefotaxime and ceftazidime), but are susceptible to β -lactamase inhibitors (clavulanic acid). In contrast, AmpC β -lactamase usually is chromosomally encoded, poorly inhibited by clavulanic acid, reversibly inhibited by boronic acid, and can be differentiated from ESBLs by its ability to hydrolyse cephamycins as well as other third-generation cephalosporins [5,6]. Plasmid-

mediated AmpC β -lactamases have arisen through the transfer of chromosomal genes for the inducible AmpC β -lactamase onto plasmids. This transfer has resulted in plasmid-mediated AmpC β -lactamases in isolates of *E.coli*, *Klebsiella pneumoniae*, *Salmonella* spp., *Citrobacter freundii*, *Enterobacter aerogenes*, and *Proteus mirabilis* [5]. Recently, Gram-negative organisms that produce both ESBLs and AmpC β -lactamases are being increasingly reported worldwide [7,8]. These organisms usually exhibit multidrug resistance that is not always detected in routine susceptibility tests. The inability to detect such complex resistance phenotypes is a serious challenge facing clinical laboratories and may have been a major factor in the uncontrolled spread of ESBL-producing organisms and related treatment failures. Hence, there is a need for better detection of ESBLs in the clinical laboratory.

The Clinical and Laboratory Standards Institute (CLSI) recommendations for phenotypic confirmation of ESBL still relies on the minimum inhibitory concentration (MIC) difference test, in which a β -lactamase inhibitor is used to protect the activity of an indicator drug against an ESBL-producing strain [9]. Laboratory tests that have been developed include double-disk diffusion using cefotaxime and ceftazidime disks with or without clavulanic acid, microdilution, and MIC using Etest or automated systems such as Vitek [10]. Etest is a convenient method for detection of ESBL by MIC reduction. Two different Etest gradient formats have been in use based on reduction of ceftazidime or cefotaxime MICs by ≥ 3 two-fold dilutions in the presence of clavulanic acid and have been used successfully for ESBL detection [10,11]. However, in isolates that co-produce both ESBL and AmpC β -lactamase, high-level expression of AmpC may mask recognition of ESBL by the inhibitor-based method. Cefepime, a fourth-generation cephalosporin, is known to be a poor substrate for AmpC β -lactamases making this drug a more reliable agent for ESBL detection in the presence of an AmpC enzyme [11].

Recently, a new Etest ESBL strip based on clavulanate synergy with cefepime has been reported to be a valuable supplement to current methods for detection of ESBLs in Enterobacteriaceae [12]. In this study, we aim to report on the performance, in our laboratory, of cefepime-clavulanate ESBL Etest for detection of ESBLs in Enterobacteriaceae, including those producing AmpC enzyme.

Materials and methods

Bacterial strains

The study was conducted on consecutive non-duplicate isolates of *E. coli*, *K. pneumoniae* and *P. mirabilis* isolated from bloodstream infections over a six-month period from January to June 2008. The study was limited to these organisms since CLSI recommends ESBL testing and reporting only for these organisms [9]. Isolates from bloodstream infections were chosen for the study since they reflect systemic infections and inadequate detection of ESBLs may lead to inappropriate therapy resulting in therapeutic failure [1,2]. Organism identification was performed by conventional biochemical tests using standard microbiological techniques [13].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing and interpretation for all isolates was conducted on Mueller Hinton agar (HiMedia, Mumbai, India) by the standard disk diffusion method per CLSI guidelines using disks of standard concentration [9]. The antibiotics tested were (concentrations in μg) as follows: ceftazidime (30), cefotaxime (30), cefepime (30), ceftazidime (30), piperacillin (100), amikacin (30), netilmicin (30), gentamicin (10) ciprofloxacin (5), piperacillin/tazobactam (100/10), cefoperazone/sulbactam(75/10), meropenem (10), imipenem (10), and ertapenem (10).

Minimum inhibitory concentration (MIC) to ceftazidime, cefotaxime and cefepime was determined for all isolates by the Etest (AB Biodisk, Solna, Sweden).

ESBL Detection

All isolates showing reduced susceptibility to ceftazidime (zone diameter of ≤ 22 mm and/or MIC ≥ 2 mg/L) and cefotaxime (zone diameter of ≤ 27 mm and/or MIC ≥ 2 mg/L) were selected for ESBL production. Isolates were tested for ESBL by both the standard CLSI double-disk diffusion method and Etests using ceftazidime/ceftazidime-clavulanate, cefotaxime/cefotaxime-clavulanate and cefepime/cefepime-clavulanate gradients. The tests were quality controlled using standard strains *E. coli* ATCC 25922 (ESBL negative), *Pseudomonas aeruginosa* ATCC 27853 (ESBL negative) and *K. pneumoniae* 700603 (ESBL positive).

Table 1. ESBL test results for the Enterobacteriaceae isolates studied.

Organism	AmpC	ESBL combined disk method result with		ESBL Etest result with		
		CAZ	CTX	CAZ-CLA	CTX-CLA	PM-CLA
<i>K. pneumoniae</i> (n = 61)	Positive (n = 49)	19	26	27	27	36
	Negative (n = 12)	1	1	1	1	1
<i>E. coli</i> (n = 50)	Positive (n = 40)	19	25	26	26	30
	Negative (n = 10)	2	2	2	2	2
<i>P. mirabilis</i> (n = 2)	Positive (n = 0)	0	0	0	0	0
	Negative (n = 2)	1	1	1	1	1
Total (n = 113)		42	55	57	57	70

CLSI disk method [9]

For the CLSI method, ceftazidime (30 μ g) and cefotaxime (30 μ g) disks were used, each with and without clavulanate (10 μ g). ESBL production was indicated by an increase in zone size of ≥ 5 mm in the disk with clavulanic acid.

Etests for ESBLs [12]

The ceftazidime/ceftazidime-clavulanate (CAZ-CLA) ESBL Etest strip generates a stable concentration gradient of ceftazidime (MIC test range, 0.5-32 mg/L) on one end and the remaining end generates a gradient of ceftazidime (MIC test range, 0.064–4mg/L) plus 4 mg/L clavulanic acid. Similarly, the cefotaxime/cefotaxime-clavulanate (CTX-CLA) Etest ESBL strip contains cefotaxime (MIC test range, 0.25 – 16 mg/L) and cefotaxime (MIC test range, 0.016 – 1mg/L) plus 4 mg/L clavulanic acid. The recently introduced cefepime/cefepime-clavulanate (PM-CLA) Etest ESBL strip contains cefepime (MIC test range, 0.25-16 mg/L) and cefepime (MIC test range, 0.064 – 4 mg/L) plus 4 mg/L clavulanic acid. The Etest procedure, reading, and interpretation were performed according to the manufacturer's instructions. Isolated colonies from an overnight plate were suspended in saline (0.85% NaCl) to achieve an inoculum equivalent to 0.5 McFarland standard. This suspension was swabbed on a Mueller-Hinton agar plate and allowed to dry completely. An ESBL Etest strip was then applied to the agar surface with sterile forceps and the plate was incubated at 35°C overnight. ESBL results were read either as MIC values or observation of "phantom zones" or deformation of inhibition ellipses. Reduction of MIC by ≥ 3 two-fold dilutions in the presence of

clavulanic acid is indicative of ESBL production. Deformation of ellipses or the presence of a "phantom zone" is also indicative of ESBL production even if the MIC ratio is < 8 or cannot be read.

Test for transferable AmpC β -lactamase Detection

After screening with cefoxitin (30 μ g disk), all isolates were tested for the presence of transferable AmpC enzyme by AmpC disk test [14]. The test was performed by preparing a lawn of 0.5 McFarland suspension of *E. coli* ATCC 25922 on Mueller-Hinton agar plates. Sterile disks (6 mm) were moistened with 20 μ l of a 1:1 mixture of saline and 100X Tris –EDTA and inoculated with several colonies of the test organism. The inoculated disk was placed beside a 30 μ g cefoxitin disk on the inoculated plate. After overnight incubation at 37°C, a positive test appears as a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk.

Results

A total of 113 bacterial isolates were recovered during the study period, which included 61 *K. pneumoniae*, 50 *E. coli*, and 2 *P. mirabilis*. Forty-one isolates were from the neonatal unit, 38 from the pediatric unit, 13 from the intensive care unit, and 21 were from the adult medical unit. Forty-two (37.2 %) and 55 (48.7%) isolates were positive for ESBL by the ceftazidime-clavulanate and cefotaxime-clavulanate combined disk tests respectively (Table 1). When the ESBL Etest results were compared, it was observed that the cefepime-clavulanate Etest strip detected the maximum number of isolates

Table 2. Number of ESBL- and AmpC-producing isolates with different MICs to ceftazidime, cefotaxime and cefepime.

Antimicrobial agent	No. of ESBL- and AmpC-producing isolates (n = 66) with MIC (mg/L) (%)		
	≤ 8	8-16	≥ 32
Ceftazidime	1 (1.5)	27 (40.9)	38 (57.6)
Cefotaxime	0 (0)	0 (0)	66 (100)
Cefepime	22 (33.3)	20 (30.3)	24 (36.4)

(70/113, 61.9 %) as ESBL-positive compared to CAZ-CLA and CTX-CLA strips, which detected 57 (50.4%) isolates each as ESBL-positive (Table 1).

Among the 70 ESBL-positive isolates detected by PM-CLA, 66 also tested positive for transferable AmpC β -lactamases and 4 were lone ESBL producers. Thus, co-production of ESBL and AmpC β -lactamases were observed in 66 (58.4%) isolates. AmpC β -lactamase alone was detected in an additional 23 isolates, the total number of AmpC producing isolates thus being 89 (78.7%). All AmpC producers were found to be cefoxitin resistant.

It was further observed that in all four isolates that were AmpC negative, all three ESBL Etest strips were equally effective in detecting ESBL. However, in the 66 isolates that co-produced AmpC in addition to the ESBL enzymes, the PM-CLA Etest strip detected ESBL in an additional 13 (8 *K. pneumoniae* and 5 *E. coli*; 11.4%) isolates as compared to the CAZ-CLA and CTX-CLA ESBL Etest strips (Table 1). Thus the PM-CLA ESBL Etest strip was found to be particularly useful for detecting ESBLs in AmpC producing bacteria, whereas the CAZ-CLA and CTX-CLA strips yielded a high number of non-determinable or negative results and thus showed marked inability to detect ESBL production in this group of isolates (Fig. 1).

When the MICs of ceftazidime, cefotaxime and cefepime in the ESBL and AmpC co-producing isolates (n = 66) were compared, it was observed that the MIC of 22 (33.3%) isolates were in the susceptible range (< 8 mg/L) for cefepime in contrast to one (1.5%) and no (0%) isolates in the susceptible range for ceftazidime and cefotaxime respectively (Table 2). This observation indicates the stability of cefepime in the presence of AmpC beta-lactamases as compared to ceftazidime or cefotaxime.

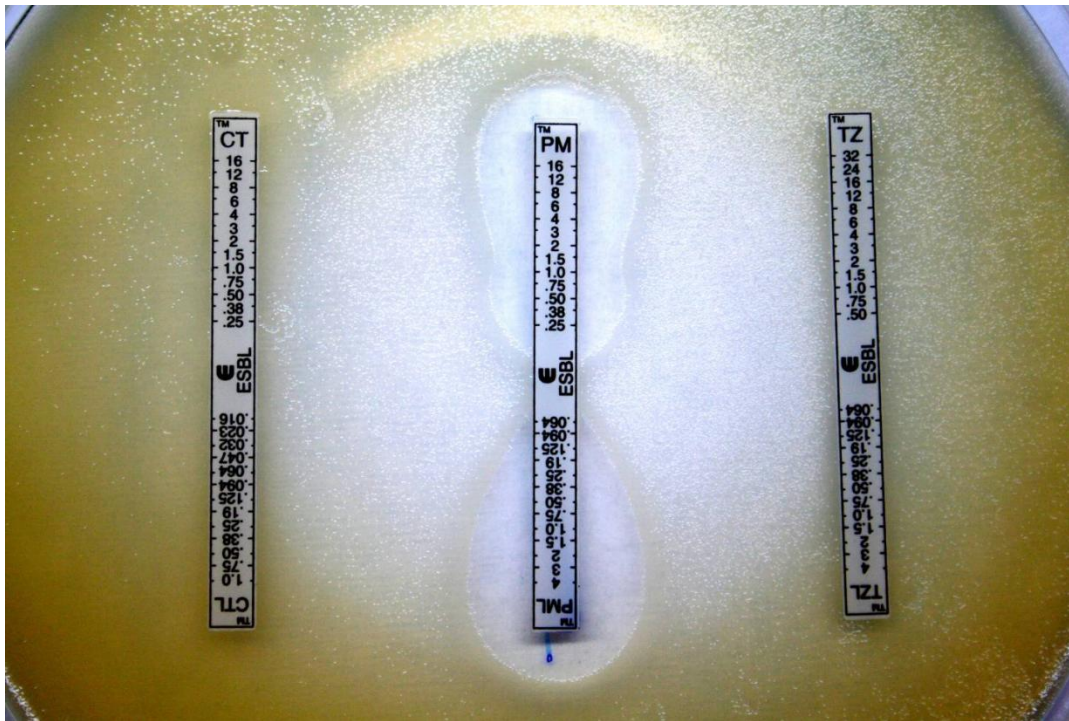
All 70 ESBL-producing isolates were susceptible to imipenem and meropenem. Ertapenem was active against 68 (97.14%) of the ESBL-producing isolates,

with resistance observed in two isolates that were ESBL and AmpC co-producers. Amikacin, netilmicin, gentamicin, ciprofloxacin, piperacillin-tazobactam, and cefoperazone-sulbactam were active against 52.8%, 52.8%, 15.7%, 27.1%, 32.8% and 37.1% of the ESBL-positive isolates, respectively.

Discussion

The present study demonstrated that the new Etest ESBL strip containing cefepime-clavulanate was the most sensitive in detecting ESBL, especially in isolates producing AmpC β -lactamase. Presence of ESBLs can be masked by the expression of AmpC β -lactamase, which can be generated by chromosomal (*eg.*, in most *Enterobacter*, *Serratia*, *C. freundii*, *Morganella*, *Proteus* and *Pseudomonas* species) or plasmid genes (mostly in *E. coli* and *Klebsiella*) [15]. Even though they are not inducible, plasmid-encoded AmpC β -lactamase typically are expressed at median to high levels [16]. Like their counterpart on the chromosome, plasmid-encoded AmpC β -lactamase provide a broader spectrum of resistance than ESBL and are not blocked by commercially available inhibitors [16]. Thus, high-level expression of a plasmid-mediated AmpC enzyme as in *E. coli* and *Klebsiella* may also prevent recognition of an ESBL. In our study, dominant AmpC production also covered and masked underlying ESBL production in 13 additional strains of *E. coli* and *Klebsiella* spp. which were initially labeled as ESBL negative by the CAZ-CLA and CTX-CLA ESBL Etests.

Possible approaches to overcome the difficulty of ESBL detection in the presence of AmpC include the use of tazobactam or sulbactam, which are much less likely to induce AmpC β -lactamases and are therefore preferable inhibitors for ESBL detection tests with these organisms, or testing cefepime as an ESBL detection agent [11]. Cefepime, a fourth-generation cephalosporin, is a more reliable detection

Figure1. Cefepime-Clavulanate ESBL Etest showing deformation of the PM inhibition ellipse indicative of ESBL.

agent for ESBLs in the presence of an AmpC β -lactamase, as this drug is stable to AmpC β -lactamase and will thus demonstrate the synergy arising from the inhibition of ESBL by clavulanate in the presence of AmpC enzyme. This result has also been observed in our study, which shows that the MIC to cefepime was in the susceptible range for 33.3% of isolates producing ESBL and AmpC in contrast to ceftazidime and cefotaxime where one and none of the isolates respectively had MICs in the susceptible range. This reinforces the stability of cefepime in the presence of AmpC enzyme.

Cefepime in double-disk synergy tests was first used for the detection of ESBLs among AmpC producers by Tzelepi *et al.* [17]. In this study [17], the use of cefepime increased the sensitivity of the double-disk synergy test with expanded-spectrum cephalosporin for the detection of ESBLs in enterobacters from 16 to 61% when the disks were applied at the standard distance of 30 mm from clavulanate and from 71 to 90% with closer application of the disks. More recently, the performance of a modified double-disk test (MDDT) utilizing cefotaxime, ceftazidime, cefepime and aztreonam along with a amoxicillin-clavulanate disk was evaluated for the detection of ESBLs in clinical isolates of *E. coli* and *K. pneumonia* [18]. Of the 136

isolates, 112 (82%) and 102 (75%) were positive for ESBL by the MDDT and NCCLS/CLSI methods respectively. Ten (7.4%) isolates (eight *E. coli* and two *K. pneumoniae*), all of which were positive for ESBL by the MDDT, yielded negative results with the NCCLS/CLSI disk method [18]. These strains showed a clear extension of the edge of inhibition produced by cefepime towards the amoxicillin-clavulanate disk, thus revealing the superior activity of cefepime for detecting ESBLs. Similarly, in another study [19], two *K. pneumoniae* isolates out of 100 consecutive isolates of *E. coli* and *Klebsiella* were positive by the double-disk synergy test for ESBL with cefepime only, but not with any of the other third-generation cephalosporins used. With regard to the detection of ESBLs by Etest, Stürenburg *et al.* [12] evaluated the performance of the cefepime-clavulanate ESBL Etest to detect ESBLs in an Enterobacteriaceae strain collection. The ESBL Etest was 98% sensitive with cefepime-clavulanate, 83% with cefotaxime-clavulanate, and 74% with ceftazidime-clavulanate strips. The cefepime-clavulanate strip was observed to be the best configuration for detection of ESBLs, particularly in *Enterobacter* spp. where inducible chromosomal AmpC β -lactamase can interfere with clavulanate synergy [12].

In conclusion, the results of the study indicate that the current CLSI recommended methods to confirm ESBL enzymes by conducting clavulanate synergy tests with ceftazidime and cefotaxime may be insufficient for ESBL detection in clinical isolates of *E. coli* and *K. pneumoniae* since these organisms often produce multiple β -lactamses. In such situations, where AmpC β -lactamase can interfere with clavulanate synergy, the new cefepime-clavulanate strips could be a more sensitive alternative for the detection of ESBL-producing organisms. Thus, in our opinion, cefepime-clavulanate Etest is a suitable substitute to test for ESBL production, especially in organisms producing AmpC β -lactamase. Optimum identification of ESBL-producing isolates would allow clinical microbiologists and infectious disease specialists to formulate policies for empirical antimicrobial therapy, especially in high-risk units where infections due to these organisms are common. It also helps in monitoring the development of antimicrobial resistance and in the implementation of proper hospital infection control measures.

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