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Original Article

Detection of Different β-Lactamases and their Co-existence by Using Various Discs Combination Methods in Clinical Isolates of *Enterobacteriaceae* and *Pseudomonas* spp.

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ABSTRACT

Background: Resistance to broad spectrum beta-lactams mediated by extended spectrum β-lactamase (ESBL), AmpC, and metallobetalactamase (MBLs) enzymes are an increasing problem worldwide. The study was aimed to detect occurrence rate and to evaluate different substrates and inhibitors by disc combination method for detecting varying degree of β-lactamase enzymes and their co-production.

Materials and Methods: A disc panel containing imipenem (IMP), IMP/EDTA, ceftazidime (CA), ceftazidime-tazobactum (CAT), CAT/cloxacillin (CLOX), ceftazidime-clavulanic acid (CAC), CAC/CLOX, cefoxitin (CN), and CN/CLOX in a single plate was used to detect presence of ESBLs, AmpC, and MBLs and/or their co-existence in 184 consecutive, nonrepetitive, clinical isolates of *Enterobacteriace* (*n* = 96) and *Pseudomonas* spp. (*n* = 88) from pus samples of hospitalized patients, resistant to 3rd generation cephalosporins.

Results: Out of a total of 96 clinical isolates of *Enterobacteriaceae*, 18.7, 20.8, and 27% were pure ESBL, AmpC, and MBL producers, respectively. ESBL and AmpC were co-produced by 25% isolates. Among 88 *Pseudomonas* spp. 38.6, 13, and 6% were pure MBL, ESBL, and AmpC producers, respectively. ESBL/AmpC and MBL/AmpC co-production was seen in 20% and 18% isolates, respectively. Among ESBL and AmpC co-producers, CA/CAC/CLOX disc combination (DC) missed 7 of the 24 ESBL producers in *Enterobacteriace* and 4 of the 18 ESBL in *Pseudomonas* spp., which were detected by CA/CAT/CLOX DC. No mechanism was detected among 8.3% *Enterobacteriaceae* and 2.3% *Pseudomonas* isolates.

Conclusion: Diagnostic problems posed by co-existence of different classes of β-lactamases in a single isolate could be solved by disc combination method by using simple panel of discs containing CA, CAT, CAT/CLOX, IMP, and IMP/EDTA.

Key words: AmpC, co-production, extended spectrum β-lactamase, metallobetalactamase

INTRODUCTION

Gram-negative bacteria can cause serious infection in hospitalized patients. Treatment of these infections is often complicated because of the increasing bacterial resistance mediated by varying degrees of beta-lactamase enzymes. It is not unusual to find single isolate that express multiple beta-lactamase enzymes, further complicating the treatment option.[1]

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The ESBL confirmation method has been established by Clinical and Laboratory Standards Institute (CLSI).[2] Currently, there is no CLSI-recommended method to detect AmpC beta lactamases. Several phenotypic methods of AmpC detection have been described.[1,3,4] However, these methods are labor intensive and subjective and lack sensitivity and/or specificity. PCR has high sensitivity and specificity, but the test is costly and limited to few reference laboratories.

The CLSI-recommended phenotypic confirmation test would fail to detect ESBL in presence of AmpC, as clavulanic acid may induce high level of expression of AmpC, masking synergy arising from the inhibition of an ESBL.[5] Induction of these enzymes may not only obscure the recognition of the ESBL status but also affect adversely the treatment of clinical

conditions caused by such strains. Hence, the present study is designed to investigate the different β-lactamases and their co-existence by using different substrates and inhibitors by disc combination method in *Enterobacteriaceae* and *Pseodomonas* spp. from pus samples of hospitalized patients.

MATERIALS AND METHODS

A total of 184 consecutive, nonrepetitive, clinical isolates of *Enterobacteriace* (n = 96) and *Pseudomonas* spp. (n = 88) from pus samples of hospitalized patients, resistant to 3rd generation of cephalosporins were tested for the presence of ESBLs, AmpC, and MBLs and/or their co-existence. Samples were processed and identified by standard laboratory methods.[6]

Antibiotic susceptibility testing was performed according to CLSI recommended Kirby-Bauer disk diffusion method.[2] The following antibiotics were tested for *Enterobacteriaceae:* Amoxycillin-clavulanic acid (AMC 30 µg/10 µg), Gentamicin (G 30 µg), Amikacin (AK 30 µg), Ceftazidime (CA 30 µg), Cefepime (CPM 30 µg), Cefoxitin (CN 30 µg), Levofloxacin (LE 5 µg), Imipenem (IMI 10 µg), and Polymyxin (PB 300 µg) and for *Pseudomonas* spp: Tobaramycin (TB 30 µg), Amikacin (AK 30 µg), Ceftazidime (CA 30 µg), Cefoxitin (CN 30 µg), Levofloxacin (LE 5 µg), Piperacillin (PT 100 µg), Imipenem (IMI 10µg), Aztreonam (AO-30 µg), and Polymyxin (PB 300 µg).

Isolates were tested for ESBLs production. Disc combination methods by using CA/CAC and CA/CAT were compared for their ability to detect ESBL production phenotypically. MBLs were detected by the IMP/EDTA disc combination method as described by Yong *et al.*[7] To detect the AmpC production CN/CN-CLOX, CAC/CAC-CLOX, and CAT/CAT-CLOX disc combinations were evaluated. This method is based on inhibitory effect of cloxacillin on AmpC. The increase in zones size of >4 mm in presence of cloxacillin was considered indicative of AmpC producer.[3]

Briefly, 5 µl of freshly prepared cloxacillin (obtained from Aristo Pharmaceuticals Pvt. Ltd., Daman) was added to each disc of CAT (30 µg/10 µg), CAC (30 µg/10 µg), and CN (30 µg) (Hi Media Laboratory). The final concentration of cloxacillin on each disc was 200 µg. The discs were allowed to dry for 60 min and used immediately. Discs were placed as shown in Figure 1a.

A novel template for disc placement was designed, which includes IMP, IMP/EDTA, CA, CAT, CAT/CLOX, CAC, CAC/CLOX, CN, and CN/CLOX in a single plate [Figure 1a]. Internal quality control strains obtained from our previous study[8] were used.

The interpretation of results are as follows

1. A ≥5 mm increase in the zone of the CA in the presence of clavulanic acid[2] or tazobactum indicative of ESBL production [Figure 1b].
2. A ≥4 mm increase in the zone of the CAT or CAC or CN in the presence of cloxacillin indicative of AmpC production [Figure 1c].[3]
3. A ≥7 mm increase in the zone of the IMP in the presence of EDTA indicative of MBL production. [Figure 1d].
4. A ≥5 mm increase in zone diameter of CA disc in presence of clavulanic acid or tazobactum and further increase in diameter of ≥4 mm in presence of CLOX in CAC or CAT indicate co-production of ESBL and AmpC [Figure 1e].

RESULTS

Of total of 96 clinical isolates of *Enterobacteriaceae,* 18.7, 20.8, and 27% were pure ESBL, AmpC, and MBL producers, respectively. ESBL and AmpC were co-produced by 25% isolates, whereas none of the isolate co-produced MBL and AmpC or ESBLs and MBLs. No mechanism was detected among 8.3% isolates, which were multidrug resistant [Table 1].

CAT/CLOX DC method detected the maximum number of AmpC [Table 2]. One isolate of pure AmpC that could be detected by CN/CLOX DC was missed by CAT/CLOX DC method.

Among *Pseudomonas* spp., out of 88 isolates, 38.6% (34/88), 13% (12/88), and 6% (06/88) were pure MBL, ESBL, and AmpC, respectively. ESBL/AmpC and MBL/AmpC co-production was seen in 20% (18/88) and 18% (16/88) isolates, respectively [Table 1].

There was 100% concordance by CA/CAC and CA/CAT DC for detecting ESBL producers. However, in combined (ESBL/AmpC) producers, CAC DC method failed to detect 7 of the 24 ESBL in *Enterobacteriaceae* and 4 of the 18 ESBL in *Pseudomonas*, which were detected by CA/CAT DC method [Tables 2 and 3].

All clinical isolates of *Enterobacteriaceae and Pseudomonas* were resistant to ceftazidime, cefepime, and cefoxitin. Imipenem

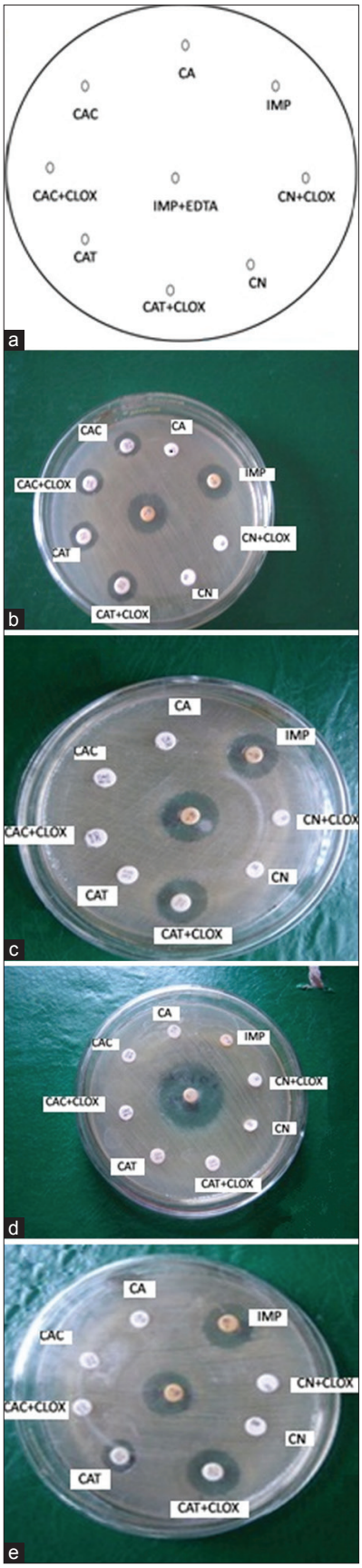

Figure 1: (a) Disc panel, (b) pure ESBL, (c) pure AmpC, (d) pure MBL, (e) ESBL and AmpC co-producer

resistance was seen in 14 isolates of *Enterobacteriaceae* and 32 isolates of *Pseudomonas* spp. For Polymyxin, 100% sensitivity was seen.

DISCUSSION

In the present study, total ESBL production was seen in 39% isolates (43.7% of isolates of *Enterobacteriaceae* and 34% isolates *Pseudomonas* spp.). Among *Enterobacteriaceae,* 18.7% were pure ESBL and 25% were ESBL and AmpC co-producers. Study from Karnataka[9] has shown high level of pure ESBL producers (41%). However, ESBL/AmpC co-producers were 27.5%, close to that in our study. At a teaching hospital of Taiwan, 35% of the clinical *Klebsiella pneumoniae* isolates co-produced AmpC and ESBL, while only 9% produced pure ESBLs.[10] In present study, pure ESBLs production by CAC and CAT disc showed 100% concordance. However, in the presence of AmpC enzyme, CA/CAT DC detected 7 ESBLs producing isolates more in *Enterobacteriaceae* and 4 ESBL in *Pseudomonas* spp., which were missed by CA/CAC DC method. This may be due to potent inducer effect on AmpC by clavulanic acid. Christopher *et al.*,[11] in their experiment also found that increasing concentrations of tazobactam are effective in inhibiting AmpC b-lactamases present in a representative sample of ceftazidime resistant *Escherichia coli*.

Boronic acid disc potentiation test has been used by various workers for detection of AmpC.[9,12] Boronic acid based tests are sometime less sensitive because boronic acid also inhibits KPC enzyme and sometimes certain ESBLs and OXA-12 inhibit some bacterial strains, making it necessary to interpret results with care.[12] These issues have not been reported with cloxacillin. Hence, we used cloxacillin in DC method.

AmpC enzymes do not have action on 4th generation cephalosporin, but, in the present study, all isolates of pure AmpC producers were resistant to cefepime. It may be due to extended spectrum of AmpC enzyme for this substrate or some other mechanism may be responsible for resistance in 4th generation cephalosporin. AmpC enzyme can hydrolyze cephamycin, this makes these drugs better screening agents for AmpC. However, in the present study, all isolates were resistant to cefoxitin. However, AmpC production was seen in only 45% isolates of *Enterobacteriaceae* and *Pseudomonas* spp., respectively, suggesting that cefoxitin resistant could be due to some other enzymatic mechanism (ESBLs, MBLs) or nonenzymatic mechanism like porin channel mutation.[8,11]

Table 1: Organism-wise distribution of different beta-lactamases and their co-production

Organisms	Pure ESBL (%)	Pure AmpC (%)	Pure MBLs (%)	ESBL and AmpC (%)	MBL and AmpC (%)	ESBL and MBL (%)	No mechanism (%)	Total
E. coli-52	12 (24)	04 (07.6)	18 (15.3)	14 (27)	00	0 0	04 (07.6)	52
Klebsiella spp-22	02 (09)	12 (54.5)	00	06 (27.7)	00	00	02 (09)	22
Citrobacter spp-08	00	02 (25)	06 (75)	00	00	00	00	08
Proteus spp-12	04 (33.3)	02 (16.6)	00	04 (33.3)	00	00	02 (16.6)	12
Enterobacter spp-2	00	00	02 (100)	00	00	00	00	02
Pseudomonas spp-88	12 (13)	06 (6.8)	34 (38.6)	18 (20.4)	16 (18.8)	00	02 (2.3)	88
Total-184	30 (16.3)	26 (14.1)	60 (32.6)	42 (22.8)	16 (08.6)	00	10 (5.4)	184

ESBL: Extended spectrum β-lactamase, MBLs: Metallobetalactamase, AmpC

Table 2: Comparisons of disc combination method by using CA/CAC and CA/CAT for ESBL detection and CN-CLOX, CAT-CLOX, and CAC-CLOX for AmpC detection in *Enterobacteriaceae*

Disc combinations	Pure ESBL total-18	Pure AmpC total-20	ESBL and AmpC total-24
CA/CAC	18	-	17
CAC/CLOX	-	16	
CA/CAT	18	-	24
CAT/CLOX	-	20	
CN/CLOX	-	18	-

CA: Ceftazidime, CAC: Ceftazidime and clavulanic acid, CAT: Ceftazidime and tazobactum, CN: Cefoxitin, CLOX: Cloxacillin, ESBL: Extended spectrum β-lactamase, AmpC

Table 3: Comparisons of disc combination method by using CA/CAC and CA/CAT for ESBL detection and CN-CLOX, CAT-CLOX, and CAC-CLOX for AmpC detection in *Pseudomonas* spp.

Disc combinations	Pure ESBL total-12	Pure AmpC total-06	ESBL and AmpC total-18
CA/CAC	12	-	14
CAC/CLOX	-	04	
CA/CAT	12	-	18
CAT/CLOX	-	06	
CN/CLOX	-	05	-

CA: Ceftazidime, CAC: Ceftazidime and clavulanic acid, CAT: Ceftazidime and tazobactum, CN: Cefoxitin, CLOX: Cloxacillin, ESBL: Extended spectrum β-lactamase, AmpC

In our study, CAT/CLOX was found better than CN/CLOX for detecting AmpC production [Tables 2 and 3].

In the present study, 24.4% (20/82) demonstrated MBL activity in IMP sensitive strain of *Enterobacteriaceae*. Among the 14 IMP resistant isolates of *Enterobacteriaceae* in 8 isolates, we could not find any of these mechanisms of resistance. However, due to constrain of resources, we could not perform assay to detect all mechanism of resistance. Remaining 6 (42.8%) IMP resistant isolates were MBL producer. Yan *et al.*,[10] in their study found that 2% isolates of *Klebseilla pneumoniae* were MBL producer and, in 11% isolates, no mechanism was detected. Rai *et al.*,[13] observed 61.7% isolates of *Enterobacteriaceae* demonstrate MBL activity despite *in vitro* sensitivity to IMP, while 89.7% of their IMP resistant isolates were MBL positive. Among *Pseudomonas* spp., 93.7% (30/32) IMP resistant isolates were MBL producers and, in 6.25% isolates, no mechanism was found. Also, 35.5% (20/56) IMP sensitive isolates were MBL producers. A pervious study from this area has also reported 33.3% MBL production in IMP-sensitive gram negative nil fermenter.[14] None of the *Enterobacteriaceae isolates* co-produced MBL/AmpC, whereas 18.8% *Pseudomonas* spp. produced MBL/AmpC.

Surprisingly, we found that, in 6 isolates of *E. coli* and 4 isolates of *Pseudomonas* spp., ESBL and AmpC co-existed, and there was a decrease in zone of inhibition with IMP/EDTA combined disc than with IMP disc alone. This could be attributed to the presence of EDTA, which permeabilizes gram-negative cells and releases β-lactamases other than MBL to participate in the test, as a result, zone size decreases in IMP/EDTA disc. However, to confirm this molecular tests are required. As our Institute does not have molecular set-up, we could not confirm these finding by a molecular method, which is the limitation of our study. Also, we do not have any referral center for detection of antibiotic resistance mechanism, which is also required.

In a previous study from this area on diabetic individuals, co-existence of beta-lactum was seen in only 15.8% (4/63) of them.[8] However, in the present study, higher level of co-producers among beta-lactum suggests that there is an increase in horizontal transfer of resistance gene. This re-enforces the importance of continuous surveillance.

As the ability of clavulanic acid to induce AmpC production may interfere with ESBL detection, tazobactum is likely to be preferred over clavulanic acid for ESBL and AmpC co-producers. With coproduction of ESBL and AmpC, concomitant detection of enzymes was far better by our novel combination method of CA/CAT and CAT/CLOXA in comparison to individual reference method for ESBL detection by CA/CAC and for AmpC (CN/CLOX) detection, respectively. Diagnostic problems posed

by co-existence of different classes of β-lactamases in a single isolate could be solved by using disc combination method in a simple panel of discs containing CA, CAT, CAT/CLOX, IMP, and IMP/EDTA.

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