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Original Article

Evaluation of three methods for detection of AmpC β-lactamase in clinical isolates of Escherichia coli and Klebsiella pneumoniae at a tertiary care hospital"

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ABSTRACT

Aims: Detection of AmpC β-lactamases is a great concern as phenotypic methods are misleading and results in treatment failure. There are no recommended guidelines for detection of this resistance mechanism and it is important to address this issue as much as the detection of extended spectrum beta lactamases (ESBLs) since both may co-exist and mask detection of later. Though resistance to cefoxitin is used as a screening test, it does not reliably indicate AmpC production and several phenotypic and genotypic methods were studied. We have undertaken this study to evaluate three different phenotypic methods for detection of AmpC β-lactamase in Escherichia coli (E.coli) and Klebsiella pneumonia (K.pneumoniae). **Mthods:** A total number of 190 consecutive, non-repetitive, imipenem sensitive clinical isolates of E.coli (n=118) and K.pneumoniae (n=72) were obtained over a period of six months, were screened for AmpC β-lactamase by using cefoxitin disk and confirmed by boronic acid (BA) inhibitor based test, modified three dimensional test (M3DT) and novel fashion method. **Results:** Out of 190 isolates, 84 (44.21%) were cefoxitin resistant, 76 (40%) were AmpC β-lactamase positive by M3DT and BA inhibitor based test, while 64 (33.68%) were positive by novel fashion method. **Conclusion:** Inhibitor based method using boronic acid is a practical and efficient method for detection of plasmid-mediated AmpC β-lactamases and clinical microbiology laboratories should consider testing the presence of AmpC β-lactamase using this method.

1. Introduction

AmpC β-lactamases, which belong to group 1 according to classification of Bush et al[1], have gained importance since the late 1970s as one of the mechanisms of antimicrobial resistance in Gram negative bacilli. These enzymes are cephalosporinases capable of hydrolyzing all β–lactams to some extent [2]. Amp C β-lactamases can be chromosomal or plasmid-mediated. Chromosomal AmpC enzymes are seen in organisms such as Citrobacter freundii, Enterobacter cloacae,Morganella morganii, Hafnia alvei and Serratia marcescenes which are inducible by β -lactam antibiotics such as cefoxitin [3]. These inducible chromosomal genes were also detected on plasmids in 1988[4]. The transfer of AmpC genes to plasmid has resulted in their wide spread among Enterobacteriacae, with consequences they are now present in Klebsiella spp., Escherichia coli, Proteus mirabilis or Salmonella spp

Detection of AmpC mediated resistance in Gram-negative organisms is a great concern for clinical microbiologist as phenotypic tests may be misleading, which results in misinterpretation of results and ultimately treatment failure. Although there are recommendations of Clinical Laboratory Standard Institute (CLSI) for detecting Extended spectrum beta-lactamase (ESBL)-producing isolates of E.coli and Klebsiella spp., there is no recommendation for detecting plasmid-mediated AmpC β-lactamase (pAmp)-producing organisms or organisms having both ESBL and pAmpC [6]. These isolates typically have a negative confirmatory test for ESBLs and thus laboratories may report AmpC-producers as susceptibles to broad-spectrum cephalosporins. This may result in serious consequences if physicians use broad-spectrum cephalosporins for treating critical infections like bacteremia. [7]

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Several methods have been developed for detection of the AmpC β-lactamases. Screening with cefoxitin disc is recommended for initial detection.However, it does not reliably indicate AmpC production [5]. Phenotypic methods like the Kirby-Bauer disk potentiation method with some beta-lactamase inhibitors or the three dimensional method, modified double disk test, AmpC disk test, inhibitor based method using boronic acid (BA) compounds; cloxacillin, newer inhibitor such as Syn2190 and the cefoxitin-Hodge test have been performed. But these methods are labour-intensive, technically intricate, and not suitable for routine clinical use in clinical microbiology laboratories and may not detect all AmpC beta-lactamases [5, 8,9]. Also phenotypic tests are not able to differentiate between chromosomal AmpC genes and plasmid mediated AmpC genes [10]. In spite of many phenotypic tests, isoelectric focusing and genotypic characterization such as Polymerase Chain Reaction (PCR) are considered as gold standard as compared to phenotypic tests which can give ambiguous results. But these are expensive, requires time-consuming techniques and expertise; which are not yet available for routine diagnostic clinical laboratories [5]. Therefore, there is need for practical and simple method to detect the resistance mediated by pAmpc β-lactamase.

The aims of this study were to investigate the presence of the AmpC β-lactamases with different methods and compare the results of BA inhibitor based test with the other phenotypic tests such as Modified three-dimensional test (M3DT) and novel fashion method.

Materials & Methods:

The study was conducted for a period of 6 months (January – June 2009). A total number of 190 consecutive, non-repetitive, imipenem sensitive, but showing resistance to one or more extended-spectrum cephalosporins clinical isolates of E.coli (n= 118) and Klebsiella pneumoniae (n=72) were isolated from different clinical specimens e.g. urine, pus, sputum, blood, stool and other body fluids, which were received for Culture and Sensitivity test at Dept. of Microbiology from various OPD, hospital wards and ICU patients at a tertiary care hospital; were selected for the study. These organisms were confirmed using standard biochemical identification tests[11,12]. Antibiotic susceptibility testing is performed by modified Kirby Bauer method on MHA according to CLSI protocols [13]. The drugs tested were Ampicillin-sulbactam, Cefuroxime, Ceftazidime, Ceftriaxone, Cefoxitin, Cefepime, Ciprofloxacin, Levofloxacin, Gentamicin, Amikacin, and Cefoperazone-Sulbactam. Detection of AmpC B-lactamase: Screening: Isolates which showed cefoxitin zone diameter < 18 mm were considered screen positive for AmpC beta-lactamase production [9]. Confirmatory tests:

Inhibitor based method: A disk containing 30 µg of cefoxitin and another containing 30 µg of cefoxitin with 400 µg of boronic acid (BA) were placed on the agar. Similarly, discs of ceftazidime (30 µg) and ceftazidime-clavulanic acid (30/10 µg) were placed on the medium at a distance of 20 mm. Inoculated plates were incubated overnight at 35°C. An organism demonstrating 5 mm or greater zone size increase around the ceftazidime -clavulanic acid disk compared to the ceftazidime disk was considered indicative of ESBL production. Likewise, an organism exhibiting a zone diameter around the disk containing cefoxitin and boronic acid 5 mm or greater zone diameter around the disk containing cefoxitin alone was considered an AmpC β-lactamase producer[9, 14].

Modified three-dimensional test(M3DT): The presence of AmpC B-lactamase in isolates producing ESBLcan be detected by this method. Fresh overnight growth from MHA is transferred to preweighed sterile microcentrifuge tube. The tube was weighed again to determine the weight of bacterial mass to obtain 10-15 mg of bacterial weight. The bacterial mass is suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 mins. Crude enzyme extract is prepared by freezing and thawing the bacterial pellet. Lawn culture of E.coli ATCC 25922 is prepared on MHA plates, a cefoxitin (30 µg) disc is placed on the surface of the medium. Linear slits (3 cm long) are cut using sterile surgical blade upto a point 3 mm away from the edge of the cefoxitin disc. Wells of 8 mm diameter are made on the slits at a distance 5 mm inside from the outer end of the slit using a sterile pasteur pipette. The wells are loaded with enzyme extract in 10 µl increment until the wells are full. Approximately 30-40 µl of extract is loade in a well. The plates are incubated at 37ºC overnight. Three different kinds of results are recorded. Isolates that show clear distortion of zone of inhibition of cefoxitin are taken as AmpC producers. Isolates with no distortion are taken as AmpC non-producers, and isolates with minimal distortion are taken as intermediate strains. A known AmpC positive isolate of Klebsiella pneumoniae are used as control reference strain [15].

Novel fashion method: As per the study by Rodrigue et al [16], by using Kirby-Bauer disk diffusion method as per CLSI guidelines, disk placement was arranged in the novel fashion to assess AmpC as shown in the figure. The ceftazidime and ceftazidime+clavulanic acid disks were kept 15-20 mm apart from each other (center to center). Imipenem, an inducer, was placed in the center and on either side of it, at a 15 mm distance, were placed ceftazidime and cefotaxime (indicators of induction). In addition, another inducer cefoxitin was placed at 15 mm from from cefotaxime (indicator). This was placed opposite to that of ceftazidime + clavulanic acid to avoid any effect of inducible B-Lactamase on the zone of inhibition of the latter. The remaining disks were placed as shown in the figure.

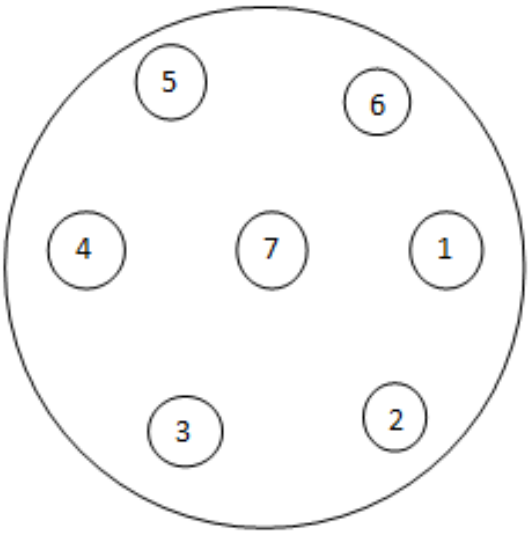

Fig 1: A novel scheme of disk placement to assess AmpC production
1– Cefotaxime, 2 – Cefoxitin, 3 – Ceftriaxone, 4- Ceftazidime, 5 – Ceftazidime + Clavulanic acid, 6 – Aztreonam, 7 – Imipenem.

ESBL detection [6]:

1) Zone diameters of various 3rd generation cephalosporins are: Aztreonam (30 µg) ≤27 mm, Cefotaxime (30 µg) ≤27 mm, Cefpodoxime (10 µg) ≤21 mm, Ceftazidime (30 µg) ≤22 mm, Ceftriaxone (30 µg) ≤25 mm.
2) Susceptible to cefoxitin
3) Increase in zone diameter with addition of inhibitor by ≥5 mm

AmpC detection:

1) Blunting of zone towards inducer
2) No increase in zone size with addition of an inhibitor
3) Susceptible to cefepime

Multiple mechanisms:

1) Resistant to cefoxitin
2) Blunting of zone diameter towards inducer
3) Increase in zone diameter with addition of an inhibitor by ≥5 mm

For the ESBL detection, in the presence of AmpC β-lactamase, ESBL confirmation test was carried out with and without (using CLSI confirmatory test, here while performing novel fashion method) BA solution.

Results & Discussions:

A total of 190 clinical isolates of E.coli (n=118) and Klebsiella pneumoniae (n=72) were analysed. Distribution of different clinical specimens among isolates are shown in Table 1.

Table 1:Distribution of E.coli and Klebsiella pneumoniae among various clinical specimens

Specimens	Urine		Swab		Blood		Sputum		Stool		Pus		Others	
GNB	No.	%	No	%	No.	%	No.	%	No.	%	No.	%	No.	%
E.coli	29	49.15	6	10.17	1	1.69	4	6.78	9	15.25	5	8.47	5	8.47
Klebsiella	6	16.66	13	36.11	5	13.89	9	25	1	2.77	1	2.77	1	2.77

Of 190 isolates, 84 (44.21%) isolates were cefoxitin resistant (E.coli: 48, K.pneumoniae:36); 76 (40%) were positive by M3DT and BA inhibitor test (E.coli:44, K.pneumoniae:32) and 64 (33.68%) were positive by novel fashion method E.coli:38, K.pneumoniae:26). The incidence of pAmpC-producing isolates of E.coli was higher than that of K.pneumoniae isolates.

ESBL detection by CLSI confirmatory test and BA inhibitor based test:

Sixty four isolates were only ESBL positive (E.coli:36; K.pneumoniae:28). Twelve isolates harbored both ESBL and pAmpC, which were detected by BA inhibitor based method. As presence of ESBL can be masked by production of pAmpC, we had negative ESBL results by CLSI confirmatory test as a part of novel fashion method. So, this BA inhibitor based test was helpful in detection of all masked ESBL producing isolates.

An increase in the incidence and prevalence of pAmpC resistance in E.coli and K.pneumoniae, which are the most commonly isolated organisms in the routine clinical laboratory, is becoming a serious problem all over the world. High level of AmpC β-lactamase is associated with in vitro resistance to third-generation cephalosporins and cephamycins, which leads to high rate of treatment failures clinically with the use of cephalosporins antibiotics [7]. However, there is documentation of susceptible results of third-generation cephalosporins in isolates producing AmpC β-lactamase [18]. Because of this reason, it is very important to detect AmpC β-lactamase production for the effective treatment of patients as well as for the purpose of infection control.

Exact prevalence of AmpC β-lactamase is unknown due to lack of simple and reliable methods for its detection in the routine clinical laboratories. There are vast differences in the results reported from the various regions of the world and even from the same country/state/geographic location. Prevalence of pAmpC β-lactamase is 40% in current study (E.coli:23%; K.pneumoniae:16.8%); which is much higher than prevalence reported from the other parts of the world [18, 19, 20]. Some Indian studies has reported 8, 43 and 47.3 percent prevalence [9, 17, 21]. The reasons for variations in results may be due to either difference in geographic regions or detection methods (phenotypic or genotypic).

Although there is no CLSI guidelines for phenotypic methods to screen and detect AmpC activity in E.coli and Klebsiella spp.[6], several methods have been developed for the detection of pAmpC β-lactamases. Reduced susceptibility to cefoxitin is one of the screening method for AmpC β-lactamase enzyme production in Enterobacteriacae family. But resistance to cefoxitin is not only due to AmpC enzyme activity; it can be due to altered outer membrane permeability [5].

In the currenct study, out of 84 cefoxitin resistant isolates, 76 were positive for AmpC β-lactamase by M3DT and BA inhibitor based test. The cause of resistance in the remaining 8 isolates probably due to non-enzymatic mechanisms like altered permeability. So, screening of AmpC production by using cefoxitin resistance is less sensitive and specific.

It has been stated that the amp C β-lactamases when present along with ESBLs can mask the phenotype of the later [22]. Thus, the co-existence of pAmpC and ESBL in the same isolate may give false negative results for the detection of ESBL. As per the new CLSI interpretive criteria, ESBL testing in routine clinical laboratory is not required; but still it is necessary for epidemiological as well as infection control purposes. In current study, we had 12 isolates (E.coli:6, K.pneumoniae:6) had negative results for ESBL production as per CLSI guidelines and novel fashion method by Rodrigue et al; but all were AmpC producers along with ESBL, which were detected with BA inhibitor based test and M3DT. If CLSI ESBL confirmatory test is used alone, 12 ESBL-producing organisms were missed.

There are several reports of studies which have used boronic acid compounds to detect the AmpC β-lactamase [8, 9, 16, 18]. It appears to be simple and promising for pAmpC β-lactamase detection; while M3DT is also promising, but procedure is cumbersome, time-consuming and needs technical expertise. A novel fashion method is less sensitive especially when there is co-existence of ESBL and AmpC β-lactamase. Though these tests are unable to distinguish between chromosomal and plasmid mediated AmpC β-lactamase, they are suitable for routine clinical microbiology laboratories. Further confirmation should be done by using genotypic method.

Conclusion:

The exact detection of pAmpC and ESBLs in clinical isolates is important for epidemiological and infection control purposes. Inhibitor based method using boronic acid is a practical and efficient method for detection of plasmid-mediated AmpC β-lactamases in E.coli and K.pneumoniae showing resistance to cefoxitin. In addition, it can also differentiate between ESBL nzymes and AmpC enzymes.

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