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Original Research Article

Detection of Plasmid Mediated AmpC Beta-Lactamases in Clinical Isolates of *Escherichia Coli* and *Klebsiella Pneumoniae*

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Abstract: The objective of present study is to investigate the prevalences of AmpC enzyme and compared to the phenotypic tests in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae.* A total of 226 isolates (cefoxitin-insucceptible: 96, cefoxitin-susceptible: 130) were included in this study. Screen positive isolates were selected for boronic acid-clavulonic acid inhibition test (BA-CA test), AmpC disk test (Tris-EDTA impregnanted), modified three dimensional test (M3DT), spot inoculation test, three dimensional extract test(3DET), and multiplex PCR were used. BA-CA test yielded positive result in 53 (55,2 %) of 96 cefoxitin-insusceptible isolates and 17 (13 %) of 130 cefoxitin-insusceptible clinical isolates. Fourty-three (44,7 %) of 96 cefoxitin-insusceptible clinical isolates were confirmed as AmpC producers. Fourty-three (81,1 %) of 53 BA-CA positive isolates harbored *ampC* genes as demostrated by multiplex PCR. When used multiplex PCR as reference test; the sensitivities of AmpC disk test and modified three dimensional test were 67,5 % and 70 % respectively. The specifiticies of both of them were 60 %. Spot inoculation test, yielded positive result in 1 (2,3 %) of 43 AmpC enzyme producers. Three dimensional extract test yielded positive result in 2 of 4 AmpC enzyme producers. Used combination disk method of CLSI for detection of Extended Spectrum Beta Lactamase (ESBL) yielded positive result in 21 isolates. When BA was added to this test, the number of positive isolates of ESBL increased to 25 due to inhibition of AmpC with BA. BA-CA inhibition test is simple to perform and easy to interpret for the detection of AmpC beta-lactamases and ESBL.
Keywords: AmpC beta-lactamases, ESBL, *E. coli*, *K. pneumoniae*.

INTRODUCTION

Plasmid mediated ampC genes are found in nosocomial isolates of *E. coli* and *K. Pneumoniae* [1, 2, 3, 4]. Organisms with plasmid mediated AmpC enzymes are generally resistant to broad spectrum penicillins, extended-spectrum cephalosporins, monobactam and cephamycins but are susceptible to cefepime, cefpirome and carbapenems [5]. These newer beta-lactamases cause resistance to a wide range of antibiotics (e.g. aminiglycosides, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole) leaving few therapeutic choices. Both types of enzymes (ESBL and AmpC beta-lactamases) are associated with potentially fatal laboratory reports of false susceptibility to cephalosporins [6].

According to the report of Pai *et al* [5] demonstrated a high rate of clinical failure among patients who were infected in the bloodstream with AmpC beta-lactamase producing *K. pneumoniae* and who received initial antimicrobial therapy, especially cephalosporin treatment, the failures included deaths and failures until therapy was switch to a carbapenem. For this to happen it is essential that clinical laboratories accurately detect isolates producing a plasmid mediated AmpC beta-lactamases. Clearly detection of AmpC producing organisms is important to ensure effective therapeutic intervention and optimal clinical outcome [7]. Methods to screen for and confirm production of class A (ESBL) and class B metallobeta-lactamases (MBL) have been established and utilized clinically [8]. But currently no guidelines for detection of plasmid mediated AmpC beta-lactamase producing organisms to date. For this reason a study designed to compared to the phenotypic tests with some modifications that proposed by some authours.

MATERIALS AND METHODS

Bacterial isolates

A total of 96 cefoxitin-insusceptible clinical isolates of *Escherichia coli* (n=70) and *Klebsiella pneumoniae* (n=26) were selected in 2009 from two

hospitals in Turkey, 50 cefoxitin-insusceptible clinical isolates of *E. coli* and *K. pneumoniae* were selected between June to September 2009 from Yüksek İhtisas Education and Research Hospital laboratory. Fourty-six cefoxitin-insusceptible clinical isolates of *E. coli* and *K. pneumoniae* were selected between March to November 2009 from Ankara Numune Education and Research Hospital. For the detection of ACC-1 type of AmpC beta-lactamase, a total of 130 cefoxitin-susceptible clinical isolates of *E. coli* and *K. pneumoniae* resistant to any cephalosporines and amoxycilin-clavulonate, but not to cefoxitin collected from Ankara Numune Education and Research Hospital, were included in this study.

Screening of AmpC beta-lactamases.

Isolates were tested for susceptibility by the standard disk diffusion method and the results were interpreted according to the guidelines of the CLSI. İsolates with cefoxitin zone diameters less than 18 mm and ceftazidime and cefotaxime zone diameters respectively less than 27 mm and 22 mm were considered positive for the AmpC beta-lactamase screening test [9, 10].

Detection of ESBL and AmpC Enzymes by BA-CA İnhibition Disk Test

Disks containing Benzeneboronic acid (BA) (Merck) or 3-aminophenylboronic acid (CA) (Sigma-Aldrich-Germany) were prepared as follows: 120 mg boronic acid was dissolved in a 3 ml of dimethylsulphoxide. Benzeneboronic acid was used with isolates that yielded negative tests with 3-aminophenylboronic acid Three ml of sterile distilled water was added to this solution. Twenty microliters (400 µg) of the stock BA solution was dispensed onto cefoxitin (30 µg), cefotaxime (30 µg) and ceftazidime (30 µg) disks and were allowed to dry for 30 min. And used immediately by inoculating Mueller Hinton agar by standard disk diffusion method (CLSI). Then dispensed 10 µl (10 µg) of CA (prepared by Bioanalyse) onto these of cephalosporin disks. Placed of 9 disks were included cefoxitin-BA, cefoxitin-CA cefoxitin-CA-BA, cefotaxime-CA, ceftazidime-CA, cefotaxime-BA, ceftazidime-BA, cefotaxime-CA-BA, ceftazidime-CA-BA. The plates were incubated overnight at 35 ºC. An organisms that demonstrated a zone diameter around the disk containing cefoxitin-CA-BA, cefotaxime-CA-BA, ceftazidime-CA-BA diameter zones were >5 mm or greater than the zone diameter around the disk containing cefoxitin-CA cefotaxime-CA, ceftazidime-CA was considered an AmpC producer. An organisms that demonstrated a zone diameter around the disk containing cefoxitin-CA-BA, cefotaxime-CA-BA, ceftazidime-CA-BA diameter zones were >5 mm or greater than the zones diameter around the disk containing cefoxitin-BA cefotaxime-BA, ceftazidime-BA was considered an ESBL producer. For the detection of ACC-1 type of AmpC beta-lactamase, cefotaxime-CA /cefotaxime-CA-BA and ceftazidime-CA/ceftazidime-CA-BA were used [11, 12].

AmpC Disk Test and Modified Three Dimensional Test (M3DT) for Detection of AmpC Beta-Lactamases

AmpC disks were prepared by applying 20 µl of a 1:1 mixture of saline and 100X Tris-EDTA to sterile filter paper disks, allowing the disks to dry and storing them at 2 -8 ºC. The surface of a Mueller Hinton agar plate was inoculated with a lawn of cefoxitin susceptible *E. coli* isolate according to the standard disk diffusion method [8]. A 30 µg cefoxitin disk was placed on the inoculated surface of the Mueller Hinton agar. The disk of impregnanted Tris-EDTA was then placed in contact with the agar surface and the cefoxitin disk AmpC disks were rehydrated with 20 µl of saline and several colonies of test organisms were inoculated onto disk [13]. For the modified three dimensional disk test we applied Coudron's [9] method with some modification, a blank disk was placed on the other side of the cefoxitin disk of the same plate of AmpC disk test then inoculated with 20 µl of 5 McFarland cell suspension of BA positive isolates. The plates were incubated overnight at 35ºC. After incubation plates were examined for either enhanced growth of the surface organism around the blank disks and AmpC disk or intersected the zone of inhibition was considered a positive result [7].

Preparation of template DNA

A single colony of each organisms was inoculated from a blood agar plate into 5 ml of Muller Hinton broth and incubated for 20 h at 37 ºC with shaking cells from 1,5 ml of the overnight culture were harvested by centrifugation at 17,310 g for 5 min. After the supernatant was decanted, the pellet was resuspended in 500 µl of distilled water. The cells wee lysed by heating at 95 ºC for 10 min. And cellular debris was removed by centrifugation at 17,310 g for 5 min. the supernatant 3 µl of the total sample was used as the source of template for amplification.

PCR protocol

The primers used for PCR amplification are listed in (Table 1). PCR was performed with a final volume of 50 µl in 0.5 ml thin-walled tubes. Each reaction contained Buffer (5x Green GoTaq Flexi Buffer; containing blue and yellow dye; pH:8,5) (Promega, USA); 0,2 mM each deoxynucleoside triphosphate (Fermentas, Canada); 2 mM $MgCI_2$; 0,5 µM primers MOXMF, MOXMR, CITMF, CITMR, DHAMF, DHAMR, ACCMF, ACCMR, EBCMF, EBCMR, FOXMF, FOXMR. Template DNA (3 µl) was added to master mixture.

PCR program

The PCR program consisted of initial denaturation step at 94ºC for 3 min, followed by 25 cycles of DNA denaturation at 94ºC for 30s., primer annealing at 64 ºC for 30s and primer extension at 72ºC for 1 min. After the last cycle a final extension at 72ºC for 7 min was added. Five microliters aliquods PCR product were analyzed by gel electrophoresis with 2 % agarose with Tris asetat EDTA solution at 90 V for 2 h and visualised by UV transillumination a 100 bp DNA ladder used as a marker. Negative controls were PCR mixtures with the addition of distilled water in place of template DNA [14].

Spot Inoculation Test

The surface of the Müller Hinton agar plate was inoculated with a lawn of cefoxitin susceptible *E. coli* isolate. Then cefoxitin disk was placed on inoculated plates. Five to six colonies were spot inoculated at a distance of 4-5 mm from the edge of the cefoxitin disk and overnight incubation at 37 ºC was carried out. After incubation plates were examined for distortion of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result) or the absence of distortion, indicating no inactivation of cefoxitin (negative result), absence of distortion but the enhanced growth of inoculated test organism in the inhibition zone of disk indicating weaker positive [15].

Three Dimensional Extract Test(3DET)

Briefly, 50 µl of a 0,5 Mc Farland bacterial suspension prepared from an overnight blood agar plate was inoculated into 12 ml of triptic soy broth and the culture was grown for 4 h at 35 ºC. The cells were concentrated by centrifugation and crude enzyme preparations were made by freezing-thawing the cell pellets five times. The surface of a Müller Hinton agar plate was inoculated with a lawn of cefoxitin susceptible *E. coli* isolate as described for the standard disc diffusion method. A 30 mg cefoxitin disk was placed on the inoculated agar, with a steril scalpel blade a slit beginning 5 mm from the edge of the disk was cut in the agar and moving outward radial direction. By using a pipet, 30 µl of enzyme preparation was dispensed into the slit, begininnig near the disk and moving outward. The inoculated media were incubated overnight at 35 ºC. Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition was considered a positive three dimensional test result and was interpreted as evidence for presence of AmpC beta-lactamase [9].

RESULTS

Antibiotic susceptibility data

All of the PCR-AmpC positive isolates of *E. coli* and *K. pneumoniae* were resistant to the penicillins, cefoxitin and amoxycilin-clavulonate. İmipenem was more active than the other cephalosporins against AmpC producers. 29 (67,4 %) of 43 PCR-AmpC positive isolates were resistant to ceftazidime, 26 (60,4 %) to cefotaxime, 22 (51,2 %) to ciprofloxacin, 21 (48,8 %) to cefepime, 20 (46,5 %) to trimethoprim-sulfamethoxazole, 17 (39,5%) to piperacillin-tazobactam, 12 (30 %) to gentamicin.

The results of BA-CA inhibition test

Fourty-three (44,7 %) of the 96 cefoxitin-insusceptible clinical isolates were AmpC producers. BA-CA test yielded positive test with 53 (% 55,2) of 96 cefoxitin-insusceptible clinical isolates (50 of them with 3-APB, 3 of them with benzeneboronic acid). Fourty-three (81,1 %) of 53 BA positive isolates harbored plasmid-mediated AmpC beta-lactamase genes, as demonstrated by multiplex PCR. Benzeneboronic acid was used instead of 3-APB to detect of ACC-1 type enzyme. This test yielded positive tests with 17 (13 %) of 130 cefoxitin-susceptible clinical isolates but PCR test yielded negative tests with these isolates.

FOX-BA-CA disk yielded positive test with 15 (83,3 %) of 18 AmpC and non ESBL producers whereas FOX-BA-CA disk yielded positive AmpC test with 14 (56 %) of 25 AmpC enzyme and ESBL coproducers. In this study to detection of AmpC enzyme in ESBL and AmpC coproducers we used CTX-CA-BA versus CTX-CA and CAZ-BA-CA versus CAZ-CA. Used combination disk method of CLSI for detection of ESBL yielded positive result in 21 isolates. When BA was added to this test, the number of positive isolates of ESBL increased to 25 due to inhibition of AmpC with BA.

The results of AmpC disk and M3DT

Thirteen isolates that AmpC disk test negative but BA test positive, yielded positive test with multiplex PCR. Six isolates that BA and AmpC disk test were positive, yielded negative test with multiplex PCR. Twelve isolates that 3MDT negative but BA test positive, yielded positive test with multiplex PCR.

The results of multiplex PCR

In this study, a plasmid-mediated AmpC enzyme was found in 43 (44,7 %) of 96 cefoxitin-insusceptible isolates of *E. coli* and *K. pneumoniae*. CIT family was the most common AmpC enzyme with 36 (83,7 %) and followed by 5 (11,6%) were EBC and 1 (2,35 %) of them had both CIT and EBC, 1 (2,35 %) of them had both CIT and MOX fam.

The results of spot inoculation test

yielded positive result in 1 (2,3 %) of 43 PCR-AmpC enzyme producers. In the spot inoculation test, except one isolate of *E. coli* 5, all the AmpC positive isolates shows enhanced growth in the inhibition zones (weaker positive). The bacterial isolate of *E. coli* 5 had

a distortion of the inhibition zones of the cefoxitin disk and also had ESBL.

The results of three dimensional extract test

Two (50 %) of 4 PCR-AmpC positive isolates yielded a positive three dimensional extract test with a surface organism. The extract of one of PCR-AmpC positive isolate was dropped on the blank disk in place of the slit. All of the isolates that used in 3DET were ESBL negative.

Table1: Primer used for amplification

Primer	Sequence	Amplicon size
MOXMF MOXMR	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	520
CITMF	TGG CCA GAA CTG ACA GGC AAA	462
CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
DHAMR	CCG TAC GCA TAC TGG CTT TGC	
ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
ACCMR	TTC GCC GCA ATC ATC CCT AGC	
EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
FOXMR	CAA AGC GCG TAA CCG GAT TGG	

Table 2a: The results of AmpC disk, 3MDT and ESBL in PCR positive and negative isolates

BA positive isolates	AmpC disk test	PCR-AmpC negative isolates	M3DT	ESBL
K. pneumoniae23	-	-	-	+
K. pneumoniae 26	+	-	+	-
K. pneumoniae 22	+	-	+	-
K. pneumoniae 28	+	-	+	-
K. pneumonia 15	+	-	+	-
K. pneumonia 21	+	-	+	-
E.coli 4	-	-	-	-
E.coli 33	+	-	+	+
E.coli 49	-	-	-	+
E.coli 37	-	-	-	+

Table 2b: The results of AmpC disk, M3DT and ESBL in PCR positive and negative isolates ilies and had ESBL as well (Fig 1).

BA positive isolates	AmpCdisk negative isolates	PCR-AmpC positive isolates	M3DT	ESBL
E.coli 29	-	+	-	-
E.coli 38	-	+	-	-
E.coli 7	-	+	-	+
E.coli 36	-	+	-	+
E.coli 46	-	+	-	+
E.coli 45	-	+	-	+
E.coli 11	-	+	-	-
E.coli 30	-	+	-	+
E.coli 32	-	+	-	+
E.coli 34	-	+	-	+
K. pneumoniae 52	-	+	-	-
K. pneumoniae 53	-	+	-	+
K. pneumoniae 18	-	+	+	+

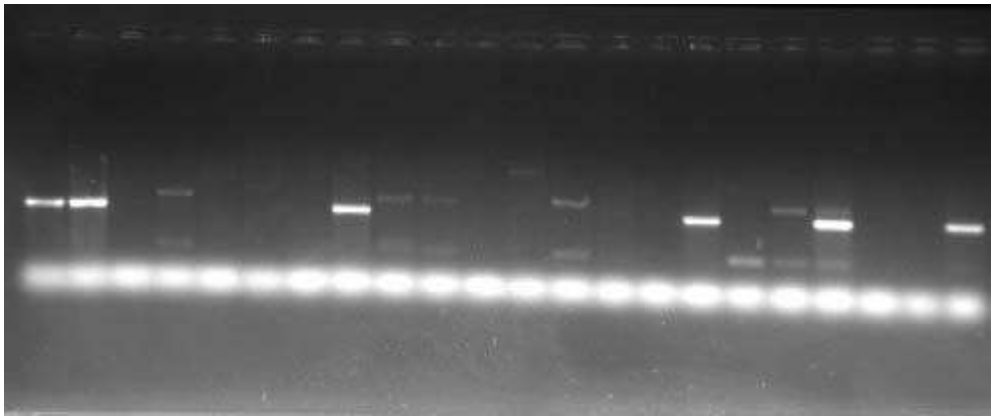

Fig. 1: CIT spesific family lanes from 1 to 22. E.c.2 (+), E.c.31(+), E.c.29 (+), E.c.39((++), K.p.26 (-), K.p.24 (-), K.p.23 (-), E.c.1 (+), E.c.9 (++), E.c.38 (++), K.p.27 (-), E.c.43 (+), E.c.34 (++), K.p.19 (-), Negative control, E.c.14 (+), K.p.16 (+), E.c.50 (++), E.c.51 (+++), K.p.15 (-),K.p.21 (-), E.c.13 (+).

DISCUSSION

The prevalences of the AmpC beta-lactamases; in the course of this study, a plasmid mediated AmpC enzyme was found in 43 (44,7 %) of 96 cefoxitin-insusceptible isolates of *Escherichia coli* and *Klebsiella pneumoniae* compared to a frequency of 17,7 % found in USA, 6,5 % in China with somewhat different selection criteria [9, 16].

In China, a plasmid mediated AmpC enzyme was found in 54 (2,8 %) of 327 cefoxitin -insusceptible *Escherichia coli* and *Klebsiella pneumoniae* and 75,9 % of these isolates were DHA family, 22,2 % were CIT family [17]. In USA from 2001 to 2002 FOX family of AmpC enzyme was found in 92,5 % of 28 *K. pneumoniae* of AmpC producers [6].

In this study, 36 (83,7 %) of 43 AmpC-PCR positive isolates were CIT family, 5 (11,6 %) of them were EBC, 1 (2,35 %) of them were CIT and EBC, 1 (2,35 %) of them were CIT and MOX families. And we detected different bands that hybridized to the CIT family primer, though the size of each band differed in each isolate, could presumably represent another gene that has homology to CIT or partial or complete duplications of CIT group and we interpreted of them as positive.

The substrats of the AmpC beta-lactamases; Song *et al*. [11] reported that FOX-BA versus FOX disk yielded positive test with 42 (97,7 %) of 43 AmpC and non ESBL producers. But the combination of FOX-BA and CTT-BA disks yielded positive AmpC result with 19 (90,5 %) of 21 AmpC and ESBL coproducers. In our study, FOX-CA versus FOX-BA-CA disk test yielded positive test with 15 (83,3 %) of 18 AmpC and non ESBL producers whereas FOX-CA versus FOX-BA-CA disk yielded positive AmpC result with 14 (56 %) of 25 AmpC and ESBL coproducers. In this study, decreased the positive results in AmpC and ESBL coproducers maybe due to the outer membrane protein mutations with beta-lactamases.

The prevalences of ESBL and AmpC coproducers; there are numerous reports in which *E. coli* and *K. pneumoniae* isolates have been found to produce both AmpC and ESBLs. In Korea, 8,7 % found by Song *et al*. [18] in China 24 % found by Ding *et al*. [16] in China % 68,5 found by Li *et al*. [17] in USA 24 % found by Alvarez *et al*. [19] of AmpC producers also produce ESBLs. In this study, 58,1 % of AmpC producers also produce ESBLs.

AmpC disk test and M3DT; Black and *et al*. [13] reported that 44 (31,4 %)of the 140 cefoxitin insusceptible *K. pneumoniae* isolates yielded positive AmpC disk test and 42 of 44 isolates were PCR-AmpC positive.

Ingram *et al* [20] reported that the performance of confirmatory tests varied from widely, ranging in senstivity from 19 % to 97 % and in specificity from 88 % to % 100. The Tris-EDTA disk test had sensitivity and specificity above %. 90 %. In this study we applied Tris-EDTA AmpC disk methods to BA positive isolates. Thirteen isolates that AmpC disk test negative but BA test positive, yielded positive test with multiplex PCR. Six isolates that BA and AmpC disk test were positive, yielded negative test with multiplex PCR. But 12 isolates that 3MDT negative but BA test positive, yielded positive test with multiplex PCR. It means that 3MDT is more sensitive than AmpC disk test.

The detection of AmpC by the BA disk test; BA disk test is most user-friendly and simple than AmpC disk test and three dimensional extract tests. Because of requirements for equipment, labour intensiveness and high costs, the use of multiplex PCR has been limited to mainly reference laboratories. And when ESBL production is suscepted but the

confirmatory tests are negative the strains should be screened for the presence of class C beta lactamase, especially in species lacking chromosomal AmpC beta-lactamase. But one of the disadvantage of BA disk test for detection of AmpC beta-lactamase is reported by Robberts *et al.* [21] and in this study APB screening results were negative for two PCR-AmpC positive isolates, in one (CMY-2 producers) of these isolates concurrent ESBL genes were detected but the second false AmpC negative isolate did not contained any additional beta-lactamase gene. In our study, we found that 3 of APB screen negative but PCR-AmpC positive isolates yielded positive test with benzeneboronic acid. Because of that there is need more investigation with boronic acid derived (benzene boronic acid and 3-APB).

The detection of ESBL and AmpC beta-lactamase by the BA-CA disk; the present study demonstrated that BA-CA test was the most sensitive in detecting AmpC beta-lactamases. Presence of AmpC enzymes can be masked by the expressing of ESBL which can be producing by plasmids. BA-CA test also enhances detection of isolates that harbor both ESBL and AmpC beta-lactamases [11, 22]. In our study, when BA was added to ESBL confirmatory test, the number of positive isolates of ESBL increased from 21 to 25 due to inhibition of AmpC with BA. Whereas 2 of 4 isolates yielded positive ESBL result with CAZ-BA versus CAZ-BA-CA, 2 of 4 ESBL positive isolates yielded positive ESBL result with FOX-BA versus FOX-BA-CA. The last 2 isolates may be cefoxitin insusceptible ESBL.

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