RESEARCH ARTICLE

Investigation of plasmid mediated AmpC beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* isolates by phenotypic and genotypic

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Abstract

Objective: To investigate the susceptibility and specificity of the phenotypic methods to determine plasmid-mediated AmpC.

Methods: The cross-sectional study was conducted at Duzce University Faculty of Medicine, Microbiology Laboratory from January 2015 to June 2016, and comprised *Escherichia coli* and *Klebsiella pneumonia* isolates intermediate susceptible or resistant to cefoxitine. Combined disk diffusion test, double disc synergy test, agar gradient test and polymerase chain reaction were used to detect plasmid-mediated AmpC.

Results: Of the 2024 *E. coli* samples, 44(2.17%), and of the 792 *K. pneumoniae* samples, 16(2%) were included. Combined disk diffusion test had susceptibility of 68% and specificity of 50%; double disc synergy test 24% and 82%; and agar gradient test 40% and 68%. Of the isolates positively detected by polymerase chain reaction method, more than one gene region positivity was detected in 15(25%) isolates.

Conclusion: All three phenotypic methods were found to be insufficient to detect plasmid-mediated AmpC positivity.

Keywords: Plasmid-mediated AmpC beta-lactamases, PCR, *Escherichia coli, Klebsiella pneumoniae.* (JPMA 69: 834; 2019)

Introduction

Beta-lactamases are one of the important resistance mechanisms that bacteria use against beta-lactam antibiotics. The first beta-lactamase enzyme was identified against penicillin and after that various beta-lactamases have emerged due to intensive use of antibiotics, and, therefore, they need to be classified.[1] Beta-lactamases are also divided into four groups according to their biochemical properties and substrate profiles, and AmpC beta-lactamases are defined as group C or group I.[2] AmpC beta-lactamases are not inhibited by clavulanic acid and cause resistance to a broad spectrum of beta-lactam antibiotics except carbapenem. Plasmid-mediated AmpC (pAmpC) beta-lactamases are important in transferring resistance in *Escherichia (E.) coli, Klebsiella (K.) pneumoniae* and salmonella species which do not have chromosomal AmpC gene.[3]

Because of the high cost and time-consuming nature of genetic tests, their routine applicability is not possible. Therefore, routine phenotypic validation tests must be performed to distinguish cefoxitin resistance due to loss of porin from the presence of AmpC enzyme and to prevent masking of AmpC's presence of extended-spectrum beta-lactamase (ESBL). Many methods such as cefoxitin hodge test, Boronic acid (BA) inhibition disc test, LN-2-128, RoP 48-1220 inhibition disc test, Syn2190 inhibition disc test, piperacillin and piperacillin / tazobactam (TZP) inhibition disc test, cloxacillin double disc synergy test, agar gradient test (AGT), isoelectric focussing (IF) method, multiplexed polymerase chain reaction (PCR) and conjugation experiments have been mentioned in studies to demonstrate the presence of pAmpC.[4,5]

The current study was planned to determine the susceptibility and specificity of the combined disk diffusion test (CDDT), double disc synergy test (DDST) and AGT in determining pAmpC.

Materials and Methods

The cross-sectional study was conducted at Duzce University Faculty of Medicine, Microbiology Laboratory from January 2015 to June 2016 and comprised *E. coli* and *K. pneumoniae* isolates that were cultured in various clinical samples sent from polyclinic, service and intensive care unit (ICU) patients to the microbiology laboratory. Phenotypic validation tests included CDDT, DDST and AGT, while genotypic analysis was done through conventional PCR. These methods were applied to resistant and intermediate susceptible strains where the

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cefoxitin disc inhibition zone diameter was >18mm. Strains susceptible to cefoxitin were not excluded since pAmpC positivity is not usually found in cefoxitin-susceptible strains. An *E. coli* isolate known to carry blaCMY-2 gene, and a *K. pneumoniae* isolate known to carry the blaACT-1 gene were used as positive controls.

Piperacillin / *TZP*, imipenem, gentamycin, ciprofloxacin and trimethoprim / sulfomethoxazole (Oxoid, England) susceptibility of the isolates were examined using Kirby-Bauer disc diffusion method and according to Clinical and Laboratory Standards Institute (CLSI) standards.[6]

For CDDT, suspensions were prepared from *E. coli* and *K. pneumoniae* strains with eosinemethylen blue (EMB) agarose passage in 0.5 Mcfarland turbidity standard and inoculation to Mueller Hinton agar (MHA) (Oxoid, UK). After ceftazidime (CAZ 30 μg) and cefotaxime (CTX 30 μg) discs with CAZ / BA, 30/400 μg and CTX / BA, 30/400 μg antibiotic discs (Bioanalyse, Turkey) were placed on the plate surface, they were incubated aerobically at 37°C for 18-24 hours. The inhibition zone diameter around the CAZ / BA or CTX / BA discs was considered pAmpC-positive in strains with a diameter of 5mm or more according to CAZ or CTX zone diameters.

For DDST, suspensions were prepared from bacterial colonies at 0.5 Mcfarland turbidity standard and inoculation to MHA. This test was done by placing CAZ and CTX discs at a distance of 2.5cm from centre to centre to 500μg of cloxacilin (CA) (Bioanalyse, Turkey) disc. The zone enlargement of any of the cephalosporins towards CA was evaluated as pAmpC presence.

For AGT, suspensions were prepared from bacterial colonies at 0.5 Mcfarland turbidity standard and inoculation to MHA. Cefotetan ± CA (0.5-32) strips (Liofilchem, Italy) were placed on the agar surface. The test principle comprises a strip impregnated with a concentration gradient of cefotetan on one half of the strip and cefotetan with CA on the other half of the strip. Minimum inhibitory concentrations (MICs) of cefotetan alone and cefotetan with CA were determined as recommended by the manufacturer. Ratios of cefotetan versus cefotetan/CA of ≥8 were considered positive for AmpC beta-lactamase production.

In the conventional PCR method, 60 isolates resistant to cefoxitin were incubated in brain heart infusion Broth medium for 24 hours at 37°C for deoxyribonucleic acid (DNA) extraction. DNA isolation was performed from bacterial isolates using the mini-prep DNA isolation kit (Gene All Exgene™ Cell SV DNA Isolation Kit).[7] The resulting bacterial DNA was amplified in a thermal cycler (Perkin Elmer Cetus, DNA Thermal Cycler 480) by adding to PCR mixture in which appropriate primers were used and displayed on the transilluminator by running in the agarose gel.[3]

Taq polymerase enzyme required for amplification and PCR mixes containing the required components were used (Gene All Amp MasterTM Taq, Cat. No: 541-005, Lot. No: TM115L03015). Primers were resolved by pipetting with 1X Tris-Ethylenediaminetetraacetic acid (TE) buffer according to the amount of dilution in the documents accompanying the primers. The sequences of the primers used in the PCR amplification (Table-1) were noted.

PCR conditions were: initial denaturation at 95°C for 5min; 35 cycles of denaturation at 95°C for 45s, 1m of primer attachment at 59°C, 2m of extension at 72°C and a further 7m extension at 72°C in addition to the final cycle. The obtained PCR products were coloured with 3μl (10μg / ml) of ethidium bromide (Biotium Gel Red Nucleic Acid Gel Stain, 10,000 × in water, Cat No. 41003, Lot No. 15G0629) and run at 90 volts for 120 minutes in 1% Lysagarose gel (NzytechAgarose Cat. No. MB14402, 0.5X Tris/Borate/EDTA [TBE] buffer) to obtain images after gel electrophoresis. Sequence analysis was performed by Atlas Biotechnology for validation to gene amplification products of isolates that were found to be positive in conventional PCR.

Kappa statistics was used for comparison of pAmpC positivity and negativity detection rates of applied phenotypic methods with the PCR method. Pearson chi-square test was used for susceptibilities of pAmpC-positive and -negative isolates in some antibiotics. Pearson chi-square test was used for the comparison of gender and sample types with regard to pAmpC beta-lactamase positivity. Independent samples t-test was used for difference in the average of ages of pAmpC-positive and negative patients.

Results

Of the 2024 *E. coli* samples, 44(2.17%), and of the 792 *K. pneumoniae* samples, 16(2%) were included. Of the 50 samples, 26(43%) belonged to male patients and 34(57%) to female patients (p>0.05). The mean age of the patients was 54 ± 24, 7 years (p>0.05). Overall, 32(53%) samples were from the outpatient department (OPD), 16(27%) from the services and 12 (20%) from the ICU (p>0.05). Among the isolates 38(63%) were produced in urine culture, 8(13.5%) in deep tracheal aspirate, 4(7%) in phiegm, 4(7%) in wound, 3(5%) in blood, 1(1.5%) each in peritoneum, abscess and tissue cultures (p>0.05).

Table-1: Primer sequences used in the study.

Primer	Target gene	Base sequence	Expectedamplicon size
MOXM F		5'-GCTGCTCAAGGAGCACAGGAT-3'	
MOXM R	CMY1,8,9,10,11,MOX1,2	5'-CACATTGACATAGGTGTGGTGC-3'	520 bp
CITM F		5'-TGGCCAGAACTGACAGGCAAA-3'	
CITM R	LAT1-4, CMY2-7, BIL1	5'-TTTCTCCTGAACGTGGCTGGC-3	462 bp
DHAM F		5'- AACTTTCACAGGTGTGCTGGGT-3'	
DHAM R	DHA1, DHA2	5'- CCGTACGCATACTGGCTTTGC-3	405bp
EBCM F		5'- CCGTACGCATACTGGCTTTGC-3	
EBCM R	ACT1, MIR1	5'-CTTCCACTGCGGCTGCCAGTT-3	302 bp
FOXM F		5'- AACATGGGGTATCAGGGAGATG-3'	
FOXM R	FOX1-5, FOX5b	5'- CAAAGCGCGTAACCGGATTGG-3'	190 bp

F: Forward, R: Reverse, bp: basepair.

Table-2: Comparison of the plasmid-mediated AmpC positivity and negativity detection rates of applied phenotypic methods with the PCR method.

Phenotypic methods		PCR					
		Positive		Negative		Total	
		n	%	n	%	n	%
CDDT	Positive	26	70	11	30	37	67
	Negative	12	52	11	48	23	33
DDST	Positive	9	69	4	31	13	22
	Negative	29	62	18	38	47	78
AGT	Positive	15	68	7	32	22	37
	Negative	23	60	15	40	38	63

CDDT: Combined disc diffusion test, DDST: Double disk synergy test, AGT: Agar gradient test, PCR: polymerase chain reaction.

Table-3: Susceptibilities of plasmid-mediated AmpC-positive and -negative isolates in some antibiotics.

		?PM	GN	C?P	SXT	TZP
pAmpC positive	Susceptible	32(84)	25(66)	13(34)	14(37)	16(42)
	Intermediate	5(13)	-	-	-	10(26)
	Resistant	1(3)	13(34)	25(66)	24(63)	12(32)
pAmpC negative	Susceptible	22(100)	16(73)	11(50)	7(32)	9(41)
	Intermediate	-	-	-	-	12(54)
	Resistant	-	6(27)	11(50)	15(68)	1(5)
p value		0.149	0.578	0.229	0.694	0.021

pAmpC: Plasmid-mediatedAmpC, IPM: Imipenem, GN: Gentamicin, CIP: Ciprofloxacin, SXT: Trimetoprim / sulfomethoxazole, TZP: Piperacillin / tazobactam.

Of all the isolates, pAmpC positivity was found in 37(67%) with CDTT, 13(22%) with DDST and 22(37%) with AGT (Figure-1).

Using PCR, PAmpC positivity was detected in 38(63.3%) isolates; 12(32%) *K. pneumoniae* and 26(68%) *E. coli* of n=60 isolates by PCR method. The pAmpC positivity rate in *K. pneumonia* was 12(75%) and 26(59%) in *E. coli* ($p<0.05$).

PCR confirmed CDDT as having susceptibility 68%, specificity 50%, a positive predictive value (PPV) 70%, negative predictive value (NPV) 48%. Corresponding values for DDST were 24%, 82%, 69% and 38%, and for AGT 40%, 68%, 68% and 40% (Table-2).

In terms of antibiotic susceptibility, *piperacillin / TZP* resistance was significantly higher in pAmpC positives and TZP moderate susceptible was significantly higher in pAmpC negatives ($p= 0.021$). Susceptibility of the other antibiotics was similar for pAmpC positive and negative isolates ($p>0.05$) (Table-3).

Of the isolates positively detected by PCR, blaDHA group gene region in 7(18%), blaFOX group gene region in 4(10%), blaMOX group gene region in 2(5%), blaCIT group gene

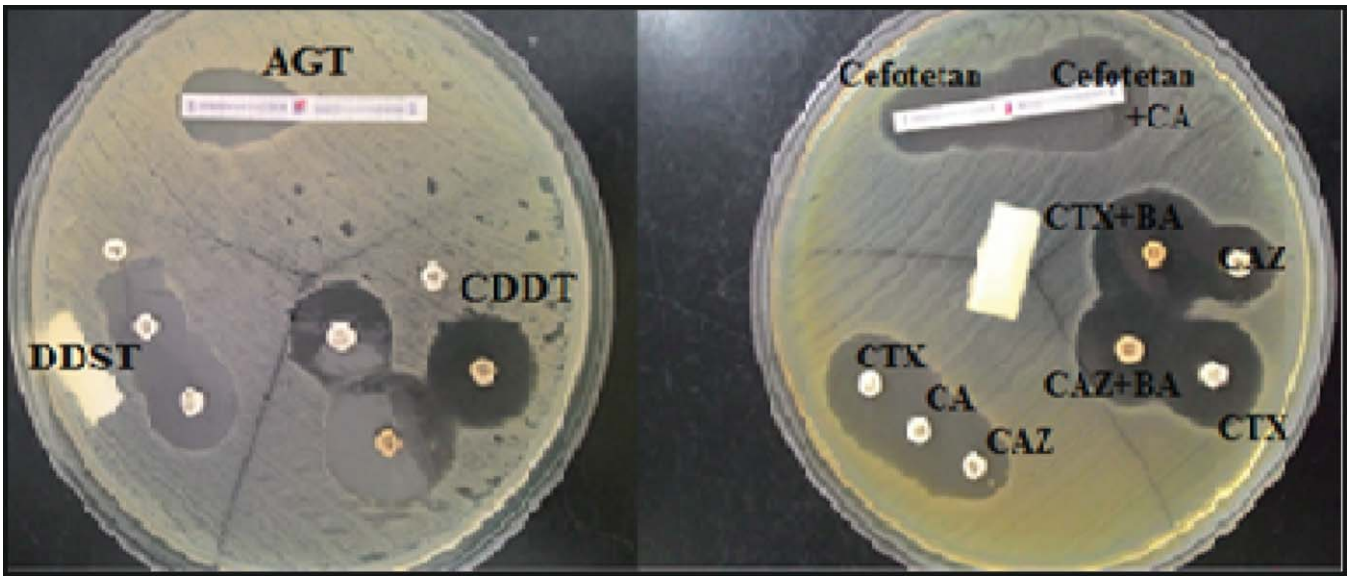

Figure-1: The examples of isolates in which all three phenotypic tests determine the pAmpC positivity (CA: Cloxacillin, CAZ: Ceftazidime, CTX: Cefotaxime, BA: Boronicacid, CDDT: Combined disc diffusion test, DDST: Double disk synergy test, AGT: Agar gradient test).

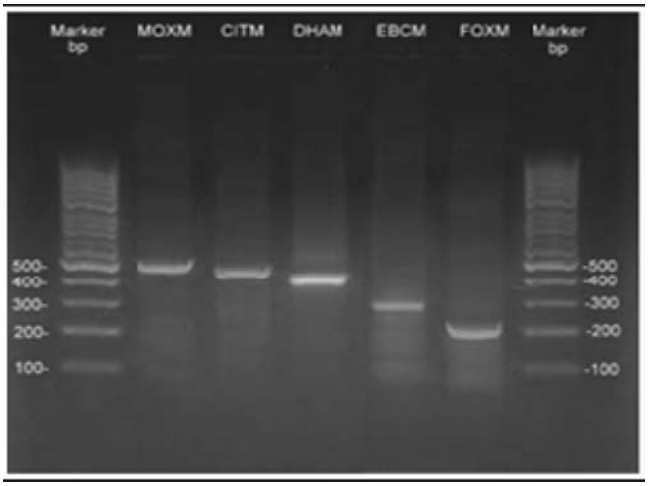

Figure-2: The agarose gel electrophoresis images of some of the isolates that gave positive results in the PCR study with MOX, CIT, DHA, EBC, FOX primers.

region in 3(8%) and blaEBC group gene region in 5(13%) were found alone. Also, of the isolates positively detected, blaDHA+blaCIT group gene region in 4(10%), blaDHA+blaEBC group gene region in 1(3%), blaMOX+blaFOX group gene region in 1(3%), blaMOX+blaCIT group gene region in 1(3%), blaFOX+blaCIT group gene region in 1(3%), blaCIT+blaEBC group gene region in 2(5%), blaDHA+blaCIT+blaEBC group gene region in 1(3%), blaDHA+blaMOX+blaFOX group gene region in 1(3%), blaMOX+blaFOX+blaEBC group gene region in 1(3%), blaDHA+blaMOX+blaCIT group gene region in 1(5%) were found positive together (Figure-2).

Discussion

Plasmid-mediated AmpC beta-lactamases have been identified since the late 1980s and are known to be a threat to the treatment of infectious diseases, particularly those caused by *E. coli* and *K. pneumoniae* isolates, as they cause resistance to broad-spectrum beta-lactam antibiotics.[8] The pAmpC frequency varies. In a study[9] in Egypt, pAmpC positivity was found with multiplex PCR in 7(38.9%) of the 18 cefoxitin-resistant *K. pneumoniae* isolates and in 2(66.7%) of the three cefoxitin-resistant *E. coli* isolates. Maleki et al.[10] found pAmpC positivity with multiplex PCR in 5(14%) of the 28 cefoxitin-resistant *E. coli* isolates in Iran. Liu et al.[11] reported pAmpC positivity with multiplex PCR in 12(28%) of 43 *E. coli* isolates which were resistant and intermediate susceptible to cefoxitin in China. Cherif et al.[12] found pAmpC positivity in 78(73.8%) of 107 bacteria (*E. coli, K. pneumoniae, Proteus mirabilis)* resistant to third-generation cephalosporin in Tunisia, and 11 of them were detected to have multiple resistance gene region. Abdalhamid B et al.[13] found pAmpC positivity in 36(18 %) of 200 cefoxitin-resistant bacteria (*E. coli, K. pneumoniae, Proteus mirabilis)*.

In a small number of studies conducted in Turkey, Koldas et al.[14 found with PCR method pAmpC positivity in cefoxitin-resistant *E. coli* and *K. pneumoniae* isolates to be 34.8% between 2006 and 2008; Yilmaz et al.[15] reported a ratio of 8.1% in 2009; Sari et al.[3] found it to be 8.8% between 2007 and 2012; and Balikci et al.[5] reported it to be 27.7% between 2008 and 2011. In the current study,

pAmpC positivity was 63.3% with PCR method in *E. coli* and *K. pneumoniae* isolates which were moderate susceptible and resistant to cefoxitin. It is believed that resistance to antibiotics may have increased rapidly over the years as data in the current study related to 2015-16, and that antibiotic usage policies applied by hospitals may be the reason for the difference in pAmpC positivity among regions.

Phenotypic methods are needed to confirm pAmpC positivity in laboratories due to the difficulty and high cost of applying molecular methods. Yilmaz et al.[15] found that the susceptibility of CDDT was 100% and the specificity of CDDT was 66%. Reuland et al.[16] found that the susceptibility of DDST was 91% and the specificity was 93%; the susceptibility of AGT was 79% and the specificity 98%. Helmy et al.[17] showed DDST susceptibility as 78.3% and the specificity 100%. Balikci et al.[5] found zone extension with BA in only half of the 10 isolates in which pAmpC gene could be detected, and false negativity was 50% in the BA infusion test with aminophenyl-BA and FOX. For this reason, it is stated that using it alone is not suitable. In our study, susceptibility and specificity of all three phenotypic methods were found to be inadequate in detecting pAmpC positivity, as they can lead to false positives and false negatives.

The pAmpC beta-lactamases can be detected in both community-acquired and hospital-acquired infections. Helmy et al.[17] found that 40% of pAmpC positivity was community-based. Also, Rordiguez et al.[18] reported that pAmpC-positive enteric bacteria were present in community-acquired infections. In our study, there was no difference in inpatients or outpatient in terms of pAmpC positivity. The fact that this resistance has been shown in studies on foodstuffs, such as chicken meat, suggests that pAmpC positivity will be increasingly detected in community-acquired infections.[19,20]

AmpC beta-lactamase-producing bacteria often cause treatment difficulties because of resistance also to other antibiotic groups, such as fluoroquinolones and aminoglycosides.[21] Also, increased resistance to carbapenems has been reported in pAmpC beta-lactamase-positive bacteria.[22] In our study, one of the pAmpC-positive bacteria was imipenem-resistant and five were intermediate susceptible. Also, TZP resistance was significantly higher in pAmpC-positive isolates. It is believed that use of ciprofloxacin, gentamicin and trimetophrim / sulfomethoxazole (SXT) in the treatment of *E. coli* and *K. pneumoniae* isolates should be limited since they have high resistance rates in both pAmpC positive and negative isolates; that the use of TZP in the treatment of infections with pAmpC-positive isolates is not suitable because of the high resistance rate in pAmpC-positive isolates; that in spite of being a suitable option for our region, imipenem should be used carefully because of the increased resistance rates.

MOX, CIT, DHAM, EBC, FOX and ACC primers are used to detect pAmpC beta-lactamases by PCR.[3,5] Nakaye et al.[23 detected ACC, CIT, EBC, FOX, DHA gene regions in pAmpC-positive isolates and found more than one gene regions in 30 isolates. Manoharan et al.[24] showed there was EBC positivity in 10 isolates, FOX positivity in four, CIT positivity in three and DHA positivity in one; CIT+FOX in 25, EBC+ACC in two, EBC+DHA in one, FOX+DHA in one and FOX+EBC+DHA in one. Liu et al[11] showed there was DHA positivity in 11 isolates, ACC positivity in one. In Turkey, Balikci et al.[5] detected isolates carrying the CMY-2 gene region; Sari et al.[3] detected isolates carrying ACT-1 and CMY-2 gene regions; Koldas et al.[14] detected isolates carrying EBC, CIT, FOX, MOX gene regions but they did not detect co-positivity in gene region. The current study detected that EBC, CIT, FOX, MOX and mainly DHA gene regions had positivity alone and co-positivity. It is thought that there is variation in resistance genes as parallel to the high pAmpC positivity in the region.

Conclusion

It is important to determine the efficacy of different phenotypic methods in detecting pAmpC positivity and to search for appropriate tests for routine use in laboratories. In addition, the identification of pAmpC resistance genotypes is thought to contribute to epidemiological data.

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