Detection of Amp-C type Producing *Escherichia coli*using the Clavulanic acid and Boronic Acid Inhibitor and Multiplex PCR method

Majid Eslami[1], Alireza Nourizadeh[2*], Amir Salek Farrokhi[3], Soghra Fallahi[4]

[1-] M.SC of Medical Bacteriology, Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[2-] M.SC of Applied Genetics, Research Center for Health Information Management, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[3-]Phd Student of Immunology, faculty of medical science, TarbiatModares University, Tehran, Iran.
[4-]Phd by Research student of Molecular Medicine, Research Center for Molecular Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.
Tel: +98-9121835532; Email: Nourizadeh_a@yahoo.com

Abstract: Introduction: Amp-C β-lactamases which belong to Cephalosporins family, in comparison with benzyl penicillin, exhibit stronger activity against Cephalosporins like Cefoxitin, Cefotetan and oximino cephalosporin and hydrolyze them. This study was aimed to determine the prevalence of Amp-C producing *E.coli* and molecular evaluation of 6 sub-groups of respective enzymes among E.coli strains using Multiplex PCR method. Materials and Methods: A total of 200 clinical *E.coli* isolates were collected from clinical specimens. Antibiotic susceptibility was evaluated by disk diffusion method. Amp-C enzyme production was determined using Combined Disk. Subsequently, the Multiplex PCR approach with specific primers was employed to determine the presence of 6 sub-groups of $bla_{\text{Amp-C}}$ genes. Results: Patterns of resistance to 14 antibiotics for isolates was identified. In combined disk test, a ≤5mm increase in zone diameter of FOX tested in combination with Clavulanic acid and a ≤3mm increase in combination with Boronic acid and Clavulanic acid, was considered positive for Amp-C production. With combined disk method 118 strains (59%) were producing Amp-C enzyme. 26 strains with Boronic acid and 92 strains with both inhibitors were considered Amp-C phenotype, among 118 strains. Prevalence of Amp-C enzyme was 2.5% that 4 strains were CITM positive and 1 strain was both DHA and CITM positive. Conclusion: The results of phenotypic tests in this study indicate that Amp-C β-lactamase enzyme has high frequency (59%); however, low frequency of this enzyme is observed when plasmid-mediated Amp-C β lactamase was used in PCR.
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Key words: Combined disk, Amp-C, Boronic acid, Clavulanic acid, *E.coli*, Multiplex PCR

Introduction

Escherichia coli bacterium is one the most important members of Enterobacteriaceae which is found in the form of normal flora in the small intestine of humans and animals. This bacterium is the most common reason of urinary tract infections, and it plays an opportunist role in wound, pneumonia, meningitis and septicemia infections. One of the shared resistance mechanisms of bacteria, especially Escherichia coli, is their resistance to β-lactamase-producing enzymes of β-lactam antibiotics, which break the β-lactam ring in these drugs, which is the most important resistance mechanism of negative gram bacteria in comparison with β-lactames[1]. In the last two decades, various types of β-lactames have been developed and designed which are resistant to the hydrolytic activity of β–lactamases, but using these new class drugs which are used for the treatment of diseases, new types of β-lactams have been emerged; Amp-C βlactamase is one of them[2]. Amp-C β-lactamases are Cephalosporins which have serine in their active part and belong to C class of Ambler classification and in the classification scheme of Bush et al, they belong to group 1. Amp-C β-lactamases are active againstpenicillins but their activity against Cephalosporins like Cefoxitin, Cefotetan, and oximino Cephalosporins like Cephtazidime and cehtarioxin and menobactames is stronger and they can hydrolyze them. The subgroup of this family include β-lactam enzymes of ACC-1, ACT-1, CFE-1, DHA-1, 2, MOX-1, 2, MIR-1 and CMY and FOX families. Amp-C β-lactamases have less activity against benzyl penicillins.Inhibitor enzymes of class A like Clavulanic acid, sulbactam and Tazobactamhave minor effect on Amp-C β-lactamases. However, some of them are inhibited by sulbactam and Tazobactam.Amp-C B-Lactamases are inhibited weakly by P-chloro-mercury benzoate, but notby EDTA at all. Cloxacillin and Oxacillin are also good inhibitors for this enzyme[3]. β-lactam Inhibitors are in fact β-lactames which stop the activity of this enzyme. These compounds have less

antibacterial activity, but when combined with a β-lactam sensitive to hydroliziation, they protect it against dissolution and they allow for antibacterial effects to be applied. The activity of β-lactam inhibitor is tested on the basis of its ability to penetrate easily and rapidly through porin channels of gram negative bacteria, the most common types of β-lactamse inhibitor are Clavulanic, sulbactom and Boronic Acid [3]. Boronic Acid is known as an Amp-C inhibitor. Using Boronic acid-added blank disks which are located close to β-lactam disk or adding Boronic acid to antibiotic disks with 5mm ≥ increase in inhibition zone around cefotaxime and ceftazidime when gμ300 3 - Amino Phenyl acid had been added, is used, but in the case of ESBL andCarbapenemaz, it was negative. Thetest can detect non-Amp-C β-lactamase of class A KPC.

In most species, β lactamases are produced at very lower rates, but in the presence of β-lactames, their production becomes higher. Functional Amp-C β-Lactamases also belong to these types of enzymes. Amoxicillin – is compound Clavulanic acid compound that is commonly used to control pathogenic bacteria and it functions as an inhibitor for most of β-lactamases. But in the case offunctional Amp-C β-Lactamases, these types of drugs can be harmful rather than helpful. If β-lactamase-producing bacteria are not diagnosed timely, great serious health failures can be caused. Although clinicianstreat infections based on allergic resultsat hand, various infections caused by β-Lactamase-producing Amp-C β-lactamasesare increasing and it is considered as a threat for treatment of patients,even as treatment failure [4]. The purpose of this cross-sectional and descriptive study was to investigate β-Lactamase Amp-Cβ-Lactamase Plasmid genes in clinical samples using both phenotypic and genotypic methods and studying effective antibiotics to treat these types of bacteria in the above samples.

Materials and methods

A total of 200 Escherichia coli isolates were collected from clinical specimens in early 1389 for 8 months in Tehran's hospitals (Children's' Medical Center, Tehran Heart Center, Bagiatallah, Milad and Mehr). The bacteria were isolated from various clinical samples such as skin, blood, tissue, secretions, urine and feces and confirmed by biochemical tests.

Disk Diffusion test

This test is the most common test used to evaluate the antibiotic resistance on Agar which was introduced in 1966 by de Boer and et al. Antibiotic susceptibility of strains was determined using disk diffusion method (Kirby-Bauer) as recommended by CLSI against 14 antibiotics, namely Cefoxitin μg30 (FOX), Ceftazidime μg30 (CAZ), Cefotaxime μg30 (CTX), Cefepime μg50 (CPM), Aztreonam μg30 (ATM), Erythromycin μg15 (ERY), Gentamicin μg10 (GM), tetracycline μg30 (TE), Cotrimoxazole μg25 (SXT), Coamoxiclav μg30 (AX), Ampicillin Amoxicillin μg25 (AM),Imipenem μg10 (IPM), Amikacin μg30 (AN) and Ciprofloxacin μg30 (CP) (MAST Co, UK, Himedia Co, India).

Combined Disk test for the phenotypic detection of Amp-Cβ-lactamase

Ceftazidime, cefotaxime and Cefoxitin μg30 (FOX) + Clavulanic acid and Cefoxitin μg 10 μg30 (FOX) + Boronic acid with Cefoxitin μg 400 μg30 (FOX) alone and Cefoxitinμg30 (FOX) + Clavulanic acid μg 10+ 400μgBoronic acid together were used to identify Amp-C β-lactames enzyme [3] (materialsprovided from Himedia Co, India),carried out after 24 hours of incubation at 37 ° C was performed. In the combined disk test,increase in the inhibition zone diameter of ≥ 5 mm against cefotaxime in combination with Boronic Acid and increase of ≥ 3 mm against cefotaxime in combination with Clavulanic Acid and Boronic Acid are indicative of Amp-C production.

DNA extraction

For DNA extraction,CinnaGen Kit was used in this method. For this purpose, was taken from an overnight bacteria cultureand it is solved in 500 λ TE Buffer (10 min centrifugation at 7500 g), Then we removed the surface liquid and added 100 ml protease buffer and kept it at 95 ° C for 10 minutes. Then, 400 ml Lysis Solution was added and homogenized thoroughly using vortex. Subsequently, 300 ml Precipitation solution is addedand it is vortexedfor 3 to 5 seconds at - 20 ° C for 10 min. Then, kept in 12,000 g Centrifugation for 10 min, and drain and driedthe micro tubes. 1 ml of wash buffer was added on the bacteria pellet and centrifuged at 12,000 rpm for 5 minutes, and buffer was drained and washed and kept at 65 ° C for 5 minutes. The pellet was shook in 50 ml solvent buffer and kept at 65 ° C for 5 minutes.Finally, we centrifuge for 30 seconds, and the supernatant was poured in sterile micro-tube for PCR and kept it in refrigerator at - 20 ° C d.

Multiplex PCR

Prevalence of Amp-C β-lactamasegenes was investigated usingprimers listed in Table 1. 5 ml of extracted DNA with PCR Master mix with final volume of l μ 25 (each vial contains 1 ml micro Mgcl2 (From 50 mM stock)), 2 micro liter 10X buffer, 2 ml dNTP (from 10 mmol stock), and 1 ml from each primer (from mix primer 10 pM) and 1 ml of Taq Polymeraseenzyme) were added and 100 bp marker(all materials from provided from CinnaGen company)was used to confirm the molecular weight of the amplified products in PCR Used and the results

were analyzed using electrophoresis in 2% Agarose gel. Thermo-cycler programming was carried out for Amp-C gene as follows: 6 subtypes (CITM ،DHA ، MOX ،FOX ،EBC و ACC)were considered using Multiplex PCR method. The initial 4-min de-naturation at 95 ° C, then 35 cycles of de-naturation at 94 ° C for 45 s, the pairing stage of primers55 ° C for 45 seconds, and the primer elongation at 72 ° C for 1 min. Finally, final elongation was done at 72 degrees Celsius for 10 min.

Results

Among200 *E.coli* strains isolated from clinical specimens, 63 samples (31.5%) were related to urine samples, 38 samples to (19%) to waste, 35 sample (17.5%) to wound, 28 samples to tissue, 21 samples to excretions and 15 samples (7.5%) were related to blood. Resistance of the isolated strains against various antibiotics is as follows:

The highest percentage of antibiotic resistance belongs to Erythromycin and Ampicillin by about 93.5% and 91% respectively, and the lowest percentage of resistance belongs to Imipenem by 0.5% and Amikacinby15.5%. Disc diffusion results in *E. coli* are indicated by resistant intermediate and susceptible strainsand are given in table 2. (The analysis was carried out using SPSS 20).

Table 1. Nucleic sequence, Gene name and Product size

Product size	Nucleic sequence	Gene name
190	AAC ATG GGG TAT CAG GGA GAT G	FOX (F)
	CAA AGC GCG TAA CCG GAT TGG	FOX (R)
302	TCG GTA AAG CCG ATG TTG CGG	EBC (F)
	CTT CCA CTG CGG CTG CCA GTT	EBC (R)
346	AAC AGC CTC AGC AGC CGG TTA	ACC (F)
	TTC GCC GCA ATC ATC CCT AGC	ACC (R)
405	AAC TTT CAC AGG TGT GCT GGG T	DHA (F)
	CCG TAC GCA TAC TGG CTT TGC	DHA (R)
462	TGG CCA GAA CTG ACA GGC AAA	CITM (F)
	TTT CTC CTG AAC GTG GCT GGC	CITM (R)
520	GCT GCT CAA GGA GCA CAG GAT	MOX (F)
	CAC ATT GAC ATA GGT GTG GTG C	MOX (R)

Table 2.Antibiotic resistance in clinical strains of *E.col*

Row	Antibiotic	Number/Percent		
		Resistance	Intermediate	Sensitive
1	Ceftazidime	90(45%)	9(4.5%)	101(50.5%)
2	Cefotaxime	144(72%)	13(6.5%)	43(21.5%)
3	Cefepime	72(36%)	14(7%)	114(57%)
4	Cefoxitin	108(54%)	31(15.5%)	61(30.5%)
5	Aztreonam	79(39.5%)	30(15%)	91(45.5%)
6	Gentamicin	73(36.5%)	25(12.5%)	102(51%)
7	Erythromycin	187(93.5%)	12(6%)	1(0.5%)
8	Tetracycline	150(75%)	19(9.5%)	31(15.5%)
9	Coamoxiclav	170(85%)	13(6.5%)	17(8.5%)
10	Cotrimoxazole	114(57%)	8($%)	78(39%)
11	Ampicillin	182(91%)	7(3.5%)	11(5.5%)
12	Imipenem	1(0.5%)	3(1.5%)	196(98%)
13	Amikacin	31(15.5%)	34(17%)	135(67.5%)
14	Ciprofloxacin	78(39%)	6(3%)	116(58%)

Combined Disk test for the detection of Phenotype Amp-C

Cefoxitin Antibiotic Disk (FOX) was used to detect Amp-C. the increase of zone diameter by ≤5 mm in combination with and without Boronic acid and the increase of inhibition zone diameter by ≤3 mm with both inhibitors of Boronic Acid and Clavulanic are indicative of Amp-C. In figure 1 Combined Disk test using FOX(30µg) in the presence of Clavulanic Acid 10 µg and Boronic Acid 400 µg, and the number and the antibiotic resistance percentage in isolates with Amp-C Phenotype are given in table 3.

Table 3. Antibiotic resistance percentage in isolates with Phenotype Amp-C

Antibiotic Name	Total	blood N=6	tissue N=22	faces N=24	secretions N=10	urine N=35	wound N=21
Ampicillin	102(92.3)%	5(4.5%)	20(18.3%)	21(19.2%)	9(8.2%)	33(30.2%)	21(19.2%)
Amikacin	19(16.1%)	0%	3(15.7%)	1(5.2%)	2(10.3%)	9(47.3%)	4(21%)
Imipenem	0%	0%	0%	0%	0%	0%	0%
Ciprofloxacin	50(42.3%)	3(6%)	11(22%)	5(10%)	3(6%)	16(32%)	12(24%)
Ceftazidime	62(52.5%)	4(6.4%)	14(22.5%)	11(17.7%)	6(9.6%)	15(24.3%)	12(19.3%)
Tetracycline	88(81.4%)	4(4.5%)	18(20.4%)	18(20.4%)	7(7.9%)	29(32.9%)	12(13.6%)
Cefotaxime	66(61.1%)	1(1.5%)	13(19.6%)	13(19.6%)	5(7.5%)	19(28.7%)	15(22.7%)
Erythromycin	110(93.2%)	5(4.5%)	22(20%)	21(19%)	9(8.1%)	33(30%)	20(18.1%)
Aztreonam	62(52.5%)	3(4.8%)	15(24.1%)	7(11.2%)	5(8%)	17(27.4%)	15(24.1%)
Cotrimoxazole	77(65.2%)	2(2.5%)	15(19.4%)	18(23.3%)	7(9%)	23(29.8%)	12(15.5%)
Gentamicin	50(42.3%)	4(8%)	14(28%)	2(4%)	6(12%)	13(26%)	11(22%)
Cefepime	57(48.3)%	2(3.5%)	10(17.5%)	10(17.5%)	3(5.2%)	17(29.8%)	15(26.3%)
Coamoxiclav	104(88.1%)	5(4.8%)	20(19.2%)	22(21.1)	9(8.6%)	29(27.8%)	19(18.2%)

In this table,antibiotic resistance percentage in insolates which have identified in Combined Disk test have been investigated. In total, 118 isolates had Amp-C phenotype, from which 26 isolates had Phenotype in using Boronic Acid and 92 were also contained Amp-C Phenotype in using two types of Boronic Acid and Clavulanic Acid inhibitors. The highest percentage of resistance belongs to urine and wound samples. The lowest percentage belongs to blood samples. No resistance was observed against antibiotic Amikacin in blood samples. However, resistance against Amikacinwas the highest in urinary samples with 47.3 % and the lowest resistance against cefotaxime was in blood samples with 1.5% among samples.

PCR test for the detection of Amp-C gene

Using Multiplex PCR test for the detection of Amp-C gene, it was observed that 4 samples contained CITM β-lactamase gene 462bp and one sample contained two CITM and DHA β-lactamase genes 405 bp. Resistance against Ampicillin, Erythromycin, Tetracycline, and Cefoxitin and sensitivity to Imipenem antibiotic were characteristics of 5 samples. The sample which contained two CITM and DHA -βlactam genes is related to the urine sample. This bacterial strain shows strong resistance to ampicillin, ciprofloxacin, tetracycline, erythromycin, Cotrimoxazole, gentamicin, Cefepime and Cefoxitin, average resistance to ceftazidime, cefotaxime, Imipenem, coamoxiclav and Aztreonam, and susceptibility against ceftazidime, cefotaxime, coamoxiclav and Aztreonam. The mentioned samples exhibited 9 mm inhibition diameter zone in Combined Disk test using cefotaxime with Clavulanic Acid and exhibited 12 mm inhibition diameter zone with Clavulanic acid + Boronic Acid. Also, each of 4 samples in which CITM gene was indentifiedwas isolated from different hospitals. 2 samples related to faces and 2 related to urine and wound. All of the 4 samples were resistant to ce,ampicillin, arit and were sensitive to impinem and gentamaysin. PCR reaction to Amp-C βlactamase gene is shown in figure 1.

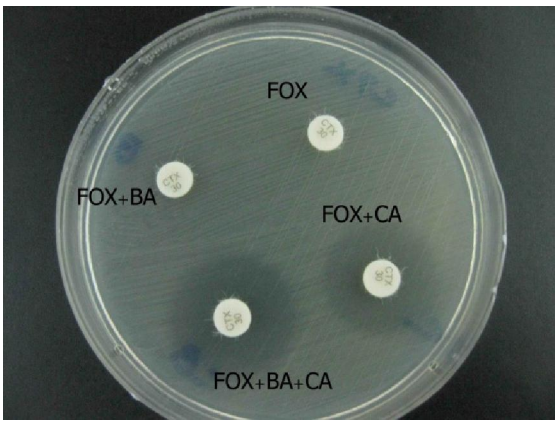

Figure 1. Combined Disk test using FOX 30 µg with Clavulanic Acid and Boronic Acid

Discussion

There have numerous studies on Amp-C β-lactames enzyme in different countries such as the one carried out by SuranjanaArona at Kolkata hospital in India. In this study, among 284 isolates which were collected from urinary and phlegm samples, 27 were identified to be resistant against Cefoxitin (i.e. Alpha methoxybetalactame). Among identified isolates, 14 were amp-c β-lactamase producer, 4 functional amp-c β-lactamse and 4 did not contain Amp-C β-lactames enzyme. Among 23 β-lactamases-producing Amp-C, 11 strains 47.8 % were *E.coli*, 4 *Pseudomonas aeroginosa*, three strains (13%) were *Klebsiella pneumoniae,* and 1 strain of

(3/4%) and *Klebsiella* Oaks Bird [6]. Encodingβ-lactamase using plasmid was reported for the first time in 1989 from South Korean Seul in Klebsiella pneumoniae isolates. In 1989, 19 subgroups of β lactamase amp-c enzyme were reported from different countries like Aljazeera, France, Germany, Greece, India, Pakistan, Taiwan, Turkey, England and the United States of America [7]. The prevalence of Amp-C enzyme in Escherichia coli strains and K.pneumoniae in China was 2% and 17.1% respectively [8]. Plasmid-Encoded Amp-C β-Lactamases are rarely found. DHA-1 is a fictional Amp-C β-lactamase reported for the first time in Saudi Arabia(9) and then in 2002 in Taiwan. In a study conducted in South Korea on 51 isolates of Enterobacteriaceae,6 –isolates contained plasmid-encoded β-lactamase. Also, in the Richmond state of US 6/2 % of Klebsiella pneumoniae contained Amp-C β-lactamase enzyme. Plasmid-encoded Amp-Cswere detected from 5.8 % K. pneumoniae, 6.9 % from Klebsiella oxy Bird, and of 4 % Escherichia coli isolates collected from 25 states in the USA [9]. In 2003, 2.7 % of the gram-negative bacteria isolates in Guru TeghBahadorhospitals in Delhi, India contained the desired enzyme, andin the same year, in a study conducted by Subha and his colleagues inChennai, India, 24.1 % of the isolates wereK.pnuemonia and 37.5 % of the isolates were Escherichia coli isolates contained Amp-C gene. In another study conducted by Shahid and colleagues in Aligra, India, on Pseudomonas aeroginosa bacterium, the prevalence of this enzyme was reported to be 20 % [10]. In a study conducted byNeil Woodford and colleagues in 2006 in England on 173 Escherichia coli and Klebsiella isolates, 67 isolates (49 %) of strains of Escherichia coli and 21 isolates (55 %) of Klebsiella strains contained Amp-C β-lactamase enzyme [11], which shows the high percentage of prevalence of this enzyme in the country. 60 isolates contained CIT type enzyme, 14 types contained ACC subgroups (reported for the first time in 1999 in Germany), 11 types contained FOX and 3 isolates contained DHA type 3. In a number of studies by Woodford, 24 enzyme-producing isolatesof CIT subgroup from isolated from a hospital, and 20 isolates were also simultaneously contained CTX-M-1 βlactamase enzyme[11]. In our samples only CIT and CIT subgroups were isolated, from which 4 samples contained CIT βlactamase gen and only one samples contained DHA subgroup which simultaneously contained Amp-C CIT. The sample which also contained both DHA and CIT genes were both collected from the same hospital and were taken from blood samples. In a another study conducted by Robert in 2008 in Minnesota, USA, Amp-C β-lactamase enzyme production which was measured in terms of phenotypic Boronic, 20 isolates contained the enzyme [12]. The studies conducted in Iran are limited to two works by Chitsaz in 1388 at Tehran University and Soltan Dalal in 1389 in the university of public health, and it was found that in the Chitsaz study EBC, DHA and CITM subgroups were identified and 3 three isolates contained CITM gene and in one isolate contained both the DHA and EBC genes (13). But in their study Dalal and et al on 200 isolates of E.coli DHA and Fox were investigated and 5 isolates (9/3 %) contained DHA gene and MOX was not detected in any isolate (14). Therefore, considering the sestudies, we reported a strain which simultaneously contained both of the CITM and DHA for the first time.

Conclusion

Phenotypic tests showed that the production of Amp-C β-lactamase enzymein the strains is high (59%) because β-lactams enzyme is both controlled by plasmids and bacterial chromosome. But because we investigated plasmidsubgroups, the prevalence of Amp-C B-Lactamase plasmids were low which is indicative of its low prevalence in our country. ESBL production is also considered as a major threat to the widespread use of extended-spectrum Cephalosporins. Therefore, we should be careful in choosing the appropriate antibiotic to treat infections suspected of β-lactamases-producingorganisms. Also, strains whose sensitivity was reduced against ceftazidime and cefotaxime should be investigated in terms of ESBL gene, and under treatment isolates should investigated continuously as well.

Corresponding author:
Ali Nourizadeh Research Center for Health Information Management, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.
Tel: +98-9121835532.
Email: Nourizadeh_a@yahoo.com

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