

Characterization of TEM-, SHV-, CTX- and AmpC-type β -lactamases from Cephalosporin Resistant *Escherichia coli* Isolates from Northwest of Iran

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Escherichia coli isolates that produce Extended-Spectrum β -Lactamase (ESBL) and AmpC β -lactamases not only cause treatment failure, but usually carry resistance genes for other antibiotics, which limits the choice of therapy. This study focused on the prevalence, detection and comparison of ESBL-type resistance genes (TEM, SHV, and CTX-M) with plasmid-mediated AmpC-producing genes (MOX, CIT, DHA) from *E. coli* isolates from Northwest of Iran. A total of 146 ceftazidime-resistance *Escherichia coli* clinical isolates were collected from hospitalized cases of urinary tract infection from Northwest of Iran. Phenotypic and genotypic examination by PCR was done for the presence of ESBL and AmpC genes by PCR. One hundred and twenty eight (87.8%) and 18 (12.3%) isolates were detected possibly as ESBLs and AmpC producers respectively. Of these, 135(92.5%) of the isolates harbored *bla*_{CTX-M}, 105(71.9%) *bla*_{TEM}, 111(76%) *bla*_{SHV}, 47(32.2%) CIT, 8(5.5%) MOX and 2 (1.4%) DHA gene. Findings of the present study indicates high rate of resistance to beta-lactam antibiotics due to CTX-M, TEM and SHV genes. Secondly, in 32% of AmpC isolates CIT genes was detected, which indicates high prevalence of this gene. This findings shows importance of developing local stewardship program for antibiotic consumption and screening isolates for the presence of ESBL and AmpC genes.

Keywords: *Escherichia coli*, ESBL, AmpC, Resistance, Phenotypic, Genotypic.

Resistance to β -lactam antibiotics is predominantly mediated by β -lactamases. Over two hundred β -lactamases are well known, classified into four basic groups and eight subgroups according to their functional and structural characteristics.¹ Ambler class A Extended-Spectrum β -Lactamase (ESBL) genes in *Escherichia coli* (*E. coli*) are well documented. Possible ESBL production has been reported to

arise in up to 9% of European *E. coli* isolates.² ESBLs are plasmid-mediated enzymes³ and have the ability to hydrolyze diverse types of the newer beta-lactam antibiotics, including extended-spectrum cephalosporins of the 3rd and 4th generation (e.g. cefotaxime, ceftriaxone, ceftazidime) and monobactams (e.g. aztreonam), which were evaluate as “critically important antimicrobials” by the WHO.^{4,5} At the present, the predominant ESBL-gene families encountered are *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV}.⁶ Also chromosomal Ambler class C (AmpC) genes have been documented and are now being distributed on

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plasmids, reminiscent of the early dissemination and evolution of ESBLs.⁷ AmpC β -lactamases decompose cephamycins (e.g. ceftiofur and cefotetan), oxyimino-cephalosporins (e.g. ceftazidime, cefotaxime, and ceftriaxone), and monobactams (e.g. aztreonam) and are not inhibited by commercially available β -lactamase inhibitors.⁸ P'erez-P'erez and Hanson have recognized six groups of plasmid mediated AmpC by using PCR. These recognition of groups are ACC, DHA, CMY, EBC, FOX, and MOX⁹ which the CIT-type AmpC beta-lactamases are universally detected.⁹

Progressively reports documented the detection of plasmid-mediated AmpC resistance (pAmpC) in *E. coli*.¹⁰ Data from the SENTRY antimicrobial surveillance program for North America showed that 19/65 ESBL screen-positive *E. coli* isolates harbored pAmpC.¹¹ Infections caused by ESBL and/or AmpC- positive bacteria are of particular clinical and epidemiological importance and cause higher patient unhealthiness and mortality^{12, 13}, that *Escherichia coli* rank highest among Gram-negative pathogens causing significant infections worldwide, they are the reason of nearly 50% of sepsis cases, greater than 70% of urinary tract infections (UTI) and even a significant percentage of intestinal infections⁹.

E. coli that produce ESBLs and AmpC β -lactamases not only cause treatment failure, but usually also carry resistance genes for other antibiotics, which limits the choice of therapy.¹⁴ Many clinical laboratories currently test *Escherichia coli* and *Klebsiella* spp. for production of ESBLs but do not intention to distinguish plasmid mediated AmpC β -lactamases (also known as imported, conveyable, strange, or ambulatory AmpC B-lactamases). Resistance to third-generation cephalosporins (3GCs) in clinical *E. coli* has been on the rise all over the world¹⁵ subsequently in Iran¹⁶. The selection of proper antibiotic therapy is a key factor in the control of infections.¹⁷ With over 700 β -lactamases described, and an increase in the number of isolates capable of producing multiple β -lactamases, laboratory testing is becoming more complex.¹ This study focused on the prevalence and detection and comparison of ESBL-type resistance genes (TEM, SHV, CTX-M) in plasmid-mediated AmpC-producing gens (MOX, CIT, DHA) from *E. coli* isolates from Northwest of Iran.

MATERIALS AND METHODS

Collection of Isolates

A total of 146 ceftazidime-resistance *Escherichia coli* clinical isolates were collected from hospitalized cases of urinary tract infection from North west of Iran that isolates recognition was based upon colonial attribute and conventional biochemical tests¹⁸. Isolates were screened by disk diffusion method according to the criteria published by the CLSI¹⁹, *Escherichia coli* ATCC 25922 were used as quality control strain, the collection period was from January 2014 to June 2014.

Phenotypic Tests for recognition of ESBL and AmpC

The phenotypic confirmation double disc synergy test (DDST) according to Dalela *et al.*,²⁰ was used for recognition of ESBL in the studied isolates, when was ESBL negative, isolates could be AmpC positive.¹⁰ Confirmatory tests for AmpC β -Lactamase included the AmpC E-test (Liofilchem, Italy), which was performed according to the producer's guidance. The test principle comprises a strip impregnated with a focusing gradient of cefotetan on one half of the strip and cefotetan with cloxacillin on the other half of the strip. MICs of cefotetan alone and cefotetan with cloxacillin were determined as recommended by the producer. Ratios of cefotetan versus cefotetan/cloxacillin of ≤ 8 were considered positive for AmpC beta lactamase production.²¹

Molecular Recognition of ampC and ESBLs Genes Arrangement of Template DNA

Plasmid DNA extraction and purification were done using the Nano-plus Plasmid Mini Extraction Kit (bioneer, Korea). Also DNA extraction was done by Tissue buffer method. 20 μ l of Tissue Buffer (0.25% SDS + 0.05M NaOH) was mixed with single colony of bacterial isolate and the mixture was incubated for 10 minutes in 95°C, after incubation mixture centrifuged for 1 minute in 13000g and finally 180 μ l of MilliQ water was added and extracted DNA freeze in -20°C for long time period.²² PCR Protocol: The recognition of MOX, CIT, DHA that families of plasmid mediated AmpC β -lactamases (pAmpC) was done using the primers organized by P'erez-P'erez and Hanson, primers were synthesized and provided by Ferments (Carlsbad, Canada). Annealing

temperatures were used comprising 64! for enlarging of genes belonging to the gens. Five-microliter aliquots of PCR product were analyzed by gel electrophoresis with 2% agarose. Gels were stained with ethidium bromide at 10 μ g/ml and visualized by UV transillumination.^{23,24} Detection of the *bla* genes was performed by PCR using a panel of specific primers for *bla*_{CTXM}, *bla*_{TEM}, and *bla*_{SHV}²⁴⁻²⁶. All protocols for PCR was done according to the protocol previously described by Simpolinsky *et al.*²⁵. Standard strains for quality control of each gene were provided by Drug Applied Research Center (Institute Microbial Collection) and were used for each gene. PCR products were electrophoreses in 1.5% agarose gels and after staining with 0.5 μ g/ml ethidium bromide visualized under UV light²⁷. The size of fragments was determined in comparing with 100bp DNA ladder plus size marker (Fermentas., Germany).

Statistical analysis

Chi-square test (or Fisher exact test) was performed for data analysis. P values below 0.05 were considered to be significant. Statistical analysis was done by SPSS 21 software. The performances of phenotypic test method were evaluated by comparing their results to those of PCR method.

RESULTS

In our study, among 146 ceftazidime resistant clinical isolates of *E. coli*, antimicrobial resistant pattern to 14 antimicrobial agents are shown in Table 1. Isolates were almost resistant to cephalosporin family of antibiotics, 58% were resistance to gentamicin and the lowest rate of resistance belonged to imipenem (5.5%) and nitrofurantoin (13%) (Table 1). In combined disk assay, among 146 screened isolates, 128 (87.8%) and 18 (12.3%) isolates were detected possibly as ESBLs and AmpC producers respectively. Confirmatory phenotypic tests with cefotetan/cloxacillin combined E-test for AmpC β -Lactamase detected 19 (13%) isolates as AmpC producers. PCR was performed on all 146 resistant isolates and the results among these isolates showed that for ESBLs: 135(92.5%) isolates had *bla*_{CTXM}, 105 (71.9%) *bla*_{TEM}, 111(76%) *bla*_{SHV} respectively. For PAmpC: 47(32.2%) of isolates was positive for CIT, 8(5.5%) of isolates harbored MOX and 2 (1.4%) of isolates harbored DHA gene (Figure 1, 2).

DISCUSSION

Prevalence of beta-lactamase-producing *E. coli* are different in various geographical areas

Table 1. Pattern of Resistance to 15 Antimicrobial agents Among 146 *E. coli* Isolates.

	Resistance Number (%)	Intermedi-ate Number (%)	Sensitive Number (%)
Cephalexin	146(100%)	0	0
Cefpodoxime	144(98.6%)	0	2(1.4%)
Aztreonam	138(94.5%)	3(2.1%)	5(3.4%)
Cefixime	138(94.5%)	5(3.4%)	3(2.1%)
Ceftriaxone	137(93.8%)	3(2.1%)	6(4.1%)
Cefotaxime	133(91.1%)	10(6.8%)	3(2.1%)
Nalidixic acid	118(80.8%)	15(10.3%)	13(8.9%)
Cefepime	112(76.7%)	22(15.1%)	12(8.2%)
Trimethoprim/ sulfamethoxazole	101(69.2%)	3(2.1%)	42(28.8%)
Ciprofloxacin	88(60.3%)	20(13.7%)	38(26%)
Gentamicin	85(58.2%)	11(7.5%)	50(34.2%)
Amikacin	24(16.4%)	10(6.8%)	112(76.7%)
Nitrofurantoin	19(13%)	11(7.5%)	116(79.5%)
Imipenem	8(5.5%)	12(8.2%)	126(86.3%)

and Because of the Irregular use and self-medication in developing countries this ratio is more prevalent. As well as, studies conducted in recent years Indicates that the AmpC and ESBLs genes frequency is increasing in Iran²⁸ nowadays, the significant obstacle for diagnostic laboratories is detection of ex-tended-spectrum β -lactamases in the presence of AmpC β -lactamase enzymes because there is no guideline (CLSI) for identification of AmpC which leads to a diagnostic problem for ESBLs via phenotypic methods and occur-rence of ESBLs false negative²⁹. Present study aimed to investigate rate of AmpC related resistance in β -lactamase producing isolates and compare it with presence of ESBL genes in *E. coli*



Fig. 1. CTX: cefotaxime, CAZ: ceftazidime, CV: clavulanic acid first: Positive ESBLs phenotypic test second: negative AmpC Confirmatory phenotypic test with mutant colonies third: positive AmpC Confirmatory phenotypic test

isolates from Northwest of Iran. Findings of the present study indicate high prevalence of CTX-M, TEM and SHV with 95%, 71% and 76% respectively. These data shows importance of studies on ESBL resistance. Also pAmpC PCR for CIT, Mox and DHA showed high prevalence of CIT with 32% and then Mox with 5.5% and DHA with 1.4%. this high prevalence of CIT indicates importance of AmpC genes in β -lactamase producing isolates of *E. coli* from Northwest of Iran. Unfortunately these data can indicate high pressure of selection for drug resistance isolates of *E.coli* because of high rate of beta-lactam antibiotics consumption in our hospitals and also a wrong culture of self- consumption of antibiotics. This high rate of AmpC prevalence can conclude carbapenem resistance as well as β -lactam resistance. Studies in other parts of Iran indicated increasing prevalence of AmpC mediated resistance in gram negative bacteria^{30,31}. Mirsalehian *et al.*, found AmpC as the main cause of resistance to cephalosporin and carbapenems in *P. aeruginosa* isolates from Tehran³⁰.

In our results 32% of isolates had ESBL and AmpC in the same time and they were co-producer of ESBL and AmpC. Previous studies indicated that coproduction of ESBL and AmpC can lead to false negative in phenotypic methods^{9,32}, but in our study co presence of these two mechanisms did not cause any false negative phenotypic results, however there is need to investigate new methods for distinguishing ESBLs and AmpC resistance by phenotypic methods.

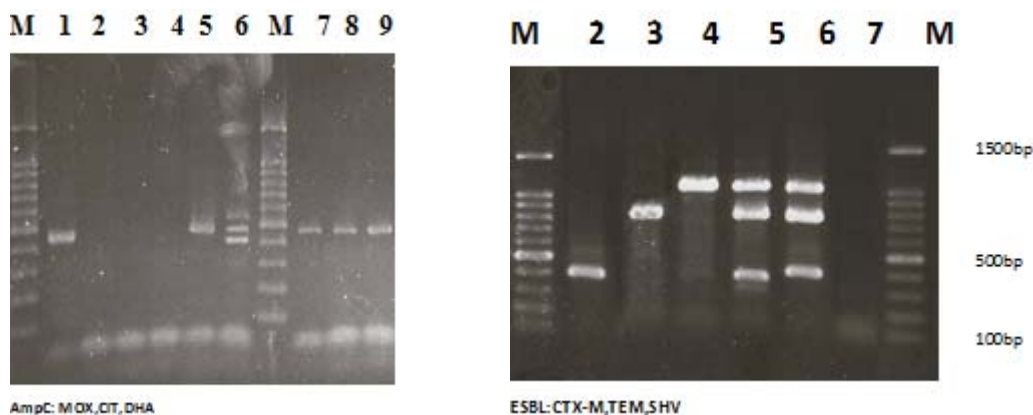


Fig. 2. PCR results for identification of AmpC genes (MOX,CIT,DHA) and multiplex PCR for ESBL genes (CTX,TEM,SHV)

Findings of the present study indicates increasing rate of resistance to beta-lactam antibiotics in results of high rate of consumption. Unfortunately our countries have not any general stewardship or survey for controlling antibiotic resistance. Today beside of ESBLs, there is high number of reports of carbapenemase resistance in our country. Fortunately results of our study show low level of resistance to carbapenems in *E.coli* isolates, but we need to develop protocols and control antibiotic consumption to support carbapenems as the last option of treatment of such infections.

In conclusion, findings of the present study indicates high rate of resistance to beta-lactam antibiotics due to CTX-M, TEM and SHV genes, also in 32% of isolates we had CIT genes of AmpC which indicates high prevalence of this gene. This findings shows importance of developing local stewardship program for antibiotic consumption and screening isolates for the presence of ESBL and AmpC genes.

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