ORIGINAL ARTICLE

Phenotypic and Genotypic Detection of AmpC Enzymes in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae*

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Abstract:

Background: AmpC *β*-lactamase enzymes cause multi-drug resistance. *Aim and Objectives:* The purpose of this study is detection of AmpC enzymes in the isolated strains of *E. coli* and *K. pneumoniae* in three educational hospitals in Hamadan (Iran). *Materials and Methods:* One hundred and forty strains of *E. coli* and *K. pneumoniae* were isolated from the hospitals in Hamadan from March to September 2015. To detection of phenotypic AmpC activity, the AmpC detection disks were used. To detect genes encoding for AmpC PCR method was used. *Results:* Sixty eight isolates (48.5%) were resistant to third generation cephalosporin and of these 61 (43.5%) isolates had MIC ≥ 8 µg/mL to cefoxitin. All 68 isolates were analyzed by AmpC detection disks, of which 10 (7.14%) isolates were AmpC- *β*-lactamase producers. By PCR method, 24 (35.2%) isolates had *cit* and 48 (70.5%) were positive for *fox*, 42 (61.7%) for *ebc*, 46 (67.6%) for *mox* genes. None of the *dha* and *acc* genes were identified. *Conclusion:* High resistance to cephalosporins has been observed among the clinical isolates. Due to the possibility of plasmid transferring of *ampC* genes between bacterial, changing consumption patterns of antibiotics and the treatment protocols is necessary.

Keywords: *Escherichia coli, Klebsiella pneumoniae,* AmpC, Multi-Drug Resistance, Beta Lactamase

Introduction:

Enterobacteriaceae included the largest number of gram-negative and facultative anaerobic organisms which can be found in the clinical samples. *Klebsiella* and *Escherichia* bacilli belong to the family of Enterobacteriaceae [1-4]. *E. coli* consist of various serotypes, ranging from highly pathogenic to nonpathogenic strains, and cause several clinical manifestations, including bacteremia, sepsis, meningitis, gastroenteritis and Urinary Tract Infections (UTIs) [5,6].

Various strategies are used by bacteria to remain immune against the deleterious effects of antibiotics. The most important mechanism of resistance is antibiotic hydrolysis mediated by the bacterial enzyme *β*-lactamase [7]. Beta-lactamases are bacterial enzymes which make the *β*-lactam antibiotics inactive by hydrolyzing the *β*-lactam ring [8]. Beta-lactamase enzymes are categorized into four classes: A, B, C, and D. Many new *β*-lactam antibiotics have been produced during the last decades which resist the hydrolysis function of *β*-lactamase enzymes [9]. Although the new antibiotics have been used in curing patients, new kinds of *β*-lactamase enzymes such as AmpC *β*-lactamases and carbapenemases have appeared. Gram negative bacteria could resist the newest *β*-lactam antibiotics by these kinds of *β*-lactamases [10].

AmpC enzymes belong to *β*-lactamases of class C. Many of these enzymes are cephalosporinase, but they are able to hydrolyze all *β*-lactam antibiotics widely. These enzymes are mostly coded by chromosomal genes in many gram negative bacteria [11]. Although, the encoding genes of these enzymes were identified in plasmids in late 1980s which could be transferred among different organisms [12]. The coding genes of the AmpC enzymes exists in *E. coli* chromosome but these enzymes expression is low, although the chromosomes of *Klebsiella* and *salmonella* spp. lack *ampC* genes; if these genes are transmitted to the organisms by plasmids, the enzymes are expressed in high level which causes resistance in them [13].

AmpC enzymes hydrolyze broad-spectrum cephalosporins like ceftazidime, ceftriaxone, cefepime and monobactams such as aztreonam and cephamycins, but they cannot be inhibited by common inhibitors like clavulanic acid [14]. Twenty-nine different plasmid-mediated *ampC* genes have been identified [15]. The phenotypic diagnosis of AmpC positive microorganisms is difficult and clinical laboratories cannot detect these bacteria.

In fact, resistance to beta-lactam antibiotics in those bacteria that typically harbor co-existence of AmpC and ESBL enzymes are not distinguishable and known as *β*-lactamase producers. For this reason, AmpC producing bacteria are often not detected by clinical laboratories [16].

Although Clinical and Laboratory Standards Institute (CLSI) has guidelines to diagnosis of *E. coli* and *Klebsiella* isolates containing Extended Spectrum Beta Lactamases (ESBLs), there are not any guidelines for diagnosis of the organisms which produce AmpC enzymes [17]. The diagnosis of these enzymes in clinical isolates of bacteria is important in epidemiological, research studies and hospital infections control because plasmid genes can be transmitted to other organisms in the hospitals [18].

Due to little studies on AmpC *β*-Lactamase producer microorganisms in our areas, this study aims to detect the phenotypic and genotypic AmpC-beta lactamases in clinical isolates of *E. coli* and *K. pneumonia* in educational hospitals of Hamadan, west of Iran.

Material and Methods:

Sampling and Isolation of Bacteria

In this cross sectional study which was conducted from March to September 2015, various clinical specimens were collected from patients in three educational hospitals in Hamadan, west of Iran. Samples were cultivated on usual cultures of microbiology including Blood agar and MacConkey agar (Merck Co, Germany) and incubated at 37°C for 18 hrs. The isolated gram negative bacteria identified by using common biochemical tests including oxidase, catalase, Triple Sugar Iron Agar (TSI) test, Simon citrate, Indole and H_2S production and Motility in SIM media, Methyl Red/Voges–Proskauer broth (MR/VP), and Urease test [19]. For confirmation some individual colonies were identified as *E. coli* and *K. pneumonia* according to the analytical profile index (API) 20NE protocol (BioMérieux Co, France).

Antibiotics Sensitivity of Isolates

Antibiotic sensitivity of isolated strains was studied by standard disk diffusion method and based on CLSI protocol [20, 21]. The used antibiotic disks (MAST Chemical Co., Bootle, UK) included cefotaxime (30 µg/CTX), ceftriaxone (30 µg/CRO), cefepime (30 µg/FEP), cefoxitin (30 µg/FOX), ceftazidime (30 µg/CAZ),

co-trimoxazole (25 µg/SXT), piperacillin (100 µg/PIP), imipenem (10 µg/IMP), ciprofloxacin (5 µg/CIP) and amikacin (30 µg/AMK).

Determining of MIC to Cefoxitin

Minimum Inhibitory Concentration (MIC) of cefoxitin in clinical isolates which were resistant to ceftriaxone, cefepime, and ceftazidime antibiotics was performed by serial dilution method according to CLSI guidelines [22].

Phenotypic Detection of AmpC *β*-lactamases using AmpC Diagnosis Disks:

The clinical isolates with MIC ≥ 8 µg/ml for cefoxitin were included for evaluation by AmpC diagnosis disks (MAST Chemical Co., Bootle, UK). The disks had three cartridges: A (disks with cefpodoxime 10 µg + AmpC inducer), B (disks with cefpodoxime 10 µg + AmpC inducer + ESBL inhibitor) and C (disks with cefpodoxime 10 µg + AmpC inducer + ESBL inhibitor + AmpC inhibitor).

The isolates were oculated on Mueller–Hinton agar; the disks were put on them and incubated at 37°C for 16-18 h according to manufactures guideline. For the interpretation of results, the diameter of halo of lack of growth around disk A is compared with a halo around the disks B and C, if the diameter of the halos is about 3 mm, that organism is considered negative in terms of producing AmpC *β*-lactamase enzymes. We reduced the diameter of halo around disk A (Z_A) from the diameter of halos of disk B (Z_B) and C (Z_C) and the diameter of halo of disk B (Z_B) from disk C (Z_C).

If $Z_C - Z_A \geq 5$mm and $Z_C - Z_B \geq 5$ mm, the organism has AmpC enzymes and is considered positive. If $Z_C - Z_A \geq 5$mm and $Z_B - Z_A \geq 5$ mm and Z_B and Z_C have maximum of 4 mm difference, this indicates that the organism uses another mechanism for resistance.

Multiplex PCR for *ampC* genes

The bacteria genome was extracted by boiling method. For this purpose, a bacteria colony from fresh culture dissolved in 1.5 ml PBS and centrifuged for 10 min at 1000 rpm and rinsed. The pellets were resuspended in 500 µL distilled water and homogenized. The suspension incubated in boiling water for 10 min and immediately incubated at -4°C, and finally centrifuged for 4 min at 3000 rpm. Subsequently, the supernatant containing bacterial genome was collected and used as DNA template.

The PCR reaction in 25 µl final volume includes: 1.5 µl MgCl2 25Mm, 2.5 µl buffer 10X, 2 µl dNTPs10 pmol, 1 µl of 10 pmol/ µl of each primers (Table1), Taq DNA polymerase 0.2 µl, DNA 1 µl and 16.8 µl sterile distilled water [23].

The amplification program consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of DNA denaturation at 94°C for 30s, annealing at 64°C for 30 s, and extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 7 min was added [15].

Statistical Analysis:

Statistical analysis was performed using chi-square test with SPSS software (version 16). *P* value of ≤ 0.05 was considered to indicate statistical significance.

Results:

During the study period, of clinical samples such as urine, trenches, scar and blood collected from three educational hospitals in Hamadan, 140 microbial strains were isolated, these included 102 (72.9%) *E. coli* and 38 (27.1%) *K. pneumoniae*.

The results showed widespread resistance of the

isolates to the antibiotics. The most resistance to antibiotics was piperacillin (86.6%), co-trimoxazole (84%), ceftriaxone (66.5%) and cefotaxime (66%) respectivelly. The highest sensitivity was related to the imipenem (90%), amikacin (80%), and cefoxitin 63% (Table 2). The minimum inhibitory concentration of 68 isolates resisting against ceftazidime, cefepime and ceftriaxone was evaluated by cefoxitin antibiotic powder (Science Lab Co, Iran). Furthermore, the highest concentration of MIC was 32 µg/mL which could be seen in 49 strains and the minimum MIC was 8 µg/mL which seen in 7 strains and 12 strains had MIC 16-24 µg/mL. It can be said that all 68 isolates have MIC ≥ 8 µg/mL; therefore, they were all suspected to produce AmpC *β*-lactamases. According to the CLSI standard strains contain MIC more than 8µg/ml is considered to be as resistant strains [24].

AmpC diagnostic disks were applied for 68 cefoxitin resistant strains, of which 10 (14.7%) isolates, eight *E. coli* and two *K. pneumonia*, were phenotypically positive in terms of producing AmpC *β*-lactamase enzymes.

The PCR results for the diagnosis of AmpC enzymes encoding genes in 68 cefoxitin resistant isolates of *E. coli* and *K. pneumonia* showed that 60 (88.2%) strains were positive in terms of AmpC enzymes producer genes; of which (n=30) 44.1% were in *E. coli* and (n=38) 55.8% in *K. pneumonia* isolates. The results indicated that most cases of *K. pneumonia* containing *ampC* genes were isolated from ICU and infectious sections. Considering the results of PCR, 24 (35.2%) isolates were positive for *cit* gene and of these 14 (20.5%) were *E. coli* and 10 (14.7%) *K. pneumoniae*, 38 (70.5%) isolates showed the presence of *fox* gene and of these 28 (41.1%) were *E. coli* and 10 (14.7%) *K. pneumoniae*, 32 (61.7%) isolates were positive for *ebc* gene and of these 20 (29.4%) were *E. coli* and 12 (17.6%) *K. pneumonia* and 36 (67.6%) isolates showed the presence of *mox*gene and of these 32 (47%) were *E. coli* and 4 (5.8%) *K. pneumoniae.* The *dha* and *acc* genes were not detected.

Table 1: Primers Used in this Study [10]

Genes Family	Sequence (5' to 3', as Synthesized)	Target(s)	Amplicon (bp)	Nucleotide Positions	Gen Bank Accession No
MOXM-F MOXM-R	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	CMY-1, CMY-8 to CMY-11, MOX-1, MOX-2	520	358–378 877–856	D13304
CITM-F CITM-R	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	BIL-1, CMY-2 to XMY-7, LAT-1 to LAT-4	462	478–498 939–919	X78117
DHAM-F DHAM-R	ACC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	DHA-1, DHA-2	405	1244–1265 1648–1628	Y16410
ACCM-F ACCM-R	ACC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATCATC CCT AGC	ACC	346	861–881 1206–1186	Aj133121
EBCM-F EBCM-R	TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT	MIR-1T, ACT-1	302	1115–1135 1416–1396	M37839
FOXM-F FOXM-R	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	FOX-1 to FOX-5b	190	1475–1496 1664–1644	X77455

Table 2: Antibiotic Resistance and Sensitivity Patterns of *E. coli* and *K. pneumonia*

Antibiotic	Bacteria					
	K. pneumoniae			*E. coli*		
	Resistant No (%)	Intermediate No (%)	Sensitive No (%)	Resistant No (%)	Intermediate No (%)	Sensitive No (%)
Cefotaxime	25(69.4)	0	11(30.5)	67(64.4)	2(1.9)	35(33.6)
Ceftriaxone	27(75)	0	9(25)	67(64.4)	4(3.8)	35(33.6)
Cefepime	21(58.3)	4(11.1)	11(30.5)	43(41.3)	12(11.5)	49(47.1)
Cefoxitin	20(55.5)	6(16.1)	10(27.7)	20(19.2)	6(5.7)	78(75)
Cefatizidime	24(66.6)	2(5.5)	10(27.7)	55(52.8)	7(6.7)	42(40.3)
Co-trimoxazole	31(86.1)	0	5(13.8)	78(75)	1(0.96)	15(14.4)
Piperacillin	27(75)	4(11.1)	5(13.8)	93(89.4)	2(1.9)	98(42.2)
Imipenem	8(22.2)	0	28(77.7)	4(3.8)	2(1.9)	9(8.6)
Ciprofloxacin	26(72.2)	2(5.5)	8(22.2)	61(58.6)	8(7.6)	35(33.6)
Amikacin	20(55.5)	2(5.5)	14(38.8)	4(3.8)	2(1.9)	98(94.2)

Table 3: Characterization of AmpC Positive Isolates

Bacterial Isolates	Ceftriaxone	Cefepime	Cefatizidime	MIC of Cefoxitin (µg/ml)	Identified Genes					
					cit	*fox*	*ebc*	*mox*	*dha*	*acc*
E. coli	R	R	R	≥32	+	+	-	-	-	-
E. coli	R	R	R	≥8	+	+	-	-	-	-
K. pneumoniae	R	R	R	≥128	+	+	-	-	-	-
E. coli	R	R	R	≥8	+	+	-	-	-	-
E. coli	R	R	R	≥8	+	+	+	-	-	-
E. coli	R	R	R	≥16	+	+	-	-	-	-
E. coli	R	R	R	≥8	+	+	-	-	-	-
K. pneumoniae	R	R	R	≥256	+	+	-	-	-	-
E. coli	R	R	R	≥8	+	+	-	-	-	-
E. coli	R	R	R	≥16	+	+	+	-	-	-

R: resistant; +: positive; -: negative

Discussion:

Plasmid-mediated AmpC *β*-lactamases are important clinically, and their recognition will be useful for both surveillance and for epidemiological processes and infection control, in order to avoid nosocomial outbreaks and treatment failures [25]. Statistically, the prevalence of ESBLs in our region was reported than other countries in Asia [26]. One of the most important reasons can be the excessive use of *β*-lactamase especially broad-spectrum cephalosporins; therefore, an effective measure to control the cure-resistant infections is decreasing incorrect use of the antibiotics [27]. Nowadays, there are no considerable directions for the design and implementation of screening tests and the confirmation of AmpC enzymes in bacteria. For example, if an isolate resists against cefoxitin antibiotic, it can potentially have AmpC enzymes, however, this is not certain and resistance can be due to disorder in the permeability of bacteria external membrane and may have no enzymatic sources [28].

Therefore, the molecular diagnosis of *β*-lactamase enzymes, especially AmpC type is necessary for careful and reliable study of antibiotics [29]. One of the best molecular methods is PCR technique. Although plasmids included AmpC *β*-lactamase enzymes encoding genes were discovered many years ago, unfortunately, the clinical importance of these enzymes is not understood properly [30]. The strains containing these enzymes are not separated and diagnosed in clinical laboratories; however, the PCR method is available as an effective research tool to diagnose these enzymes, but it is not a conventional method in the clinical laboratories [31].

In this study, the AmpC diagnostic disks have been used for phenotypic detection of AmpC *β*-lactamase enzymes. This test is a more convenient method for the detection of beta-lactamases *apmC* genes on plasmids. In the present study, the results of determining antibiotic resistance pattern in isolates indicated that imipenem, with 90% sensitivity, is the most effective antibiotic against *K. pneumoniae* and *E. coli*; therefore, imipenem can be a good choice for treatment. The amount of resistance to cefoxitin was 48.5% (68 isolates).

Sixty-eight (48.5%) clinical isolates were tested by PCR for the presence of *β*-lactamase genes. In 8 (11.7%) isolate whit MIC ≥ 8 µg/mL to cefoxitin, which weresuspected to the production of AmpC *β*-lactamases, any *ampC β*-lactamases genes were not found.

This shows that other mechanisms including non-expression of purines in external membrane and carbapenem enzymes play a role in developing resistance toward cefoxitin.

Considering the AmpC diagnostic disks, according to the instructions, only 10 (14.7%) isolates which included 8 *E. coli* and 2 *K. pneumoniae* were positive to produce AmpC enzymes phenotypically. The previous study by Niakan *et al*. (2008) from 168 *K. pneumoniae* isolates, the frequency of AmpC genes (10 isolates) reported 5.9%, whereas in our research, the frequency of AmpC genes was 14.7%, which indicates the increase of resistance due to AmpC enzymes production by now [32]. Beta lactamase genes related to FOX, CIT, and EBC families were reported in both studies, whereas genes related to ACC family were found in none of the studies. Genes related to Fox family had the highest frequency in both studies. Also, the genes related

to MOX family were not reported but in the current study, we showed that the genes were related to MOX; in addition the most identified gene was CIT.

In the research conducted on *E. coli* by Mansoori *et al*, 5 (5.7%) isolates produced AmpC enzymes [33].The previous study by Kalantar *et al*, it was found that of 75 *K. pneumoniae* isolates, 31 (41.3%) had broad-spectrum *β*-lactamases and 11 (14.6%) had AmpC [34]. Also, the similar survey by Shanthi *et al*, revealed thatof the 16 *K. pneumonia* that were positive for AmpC enzymes, 25% containing *ACT, FOX* and *MOX* genes [35].

Another investigation from Egypt revealed that 57.7% isolates were Amp C enzyme producer of which, 22 isolates contained AmpC enzyme encoding genes included MOX, FOX family and CIT genes [36].

In general, microorganisms that have ESBL enzymes should be reported resistant against all broad-spectrum cephalosporins unless their sensitivity to the given antibiotic is proven [37]. Transferring broad-spectrum *β*-lactamase enzymes producer genes is proven from one hospital to another one, from one city to another city and from one country to another country [38]. If effective measures are not taken to prevent making resistance, the issue will increase more and more. Extensive consumption of medicines may facilitate the antibiotic resistance extension; therefore, antibiotic consumption should be done carefully. Laboratories are not able to diagnose the mechanism of resistance [39]. When bacteria simultaneously have different resistance mechanism to antibiotics, the phenotypic diagnosis of AmpC enzymes are very difficult and it is better to use molecular methods [40].

Conclusion:

The prevalence of AmpC *β*-lactamase production in the clinical isolates is critical as part of the surveillance and monitoring activity of antibiotic resistance for infection control. The results of this study suggested that physicians should pay attention while prescribing antibiotic and send the sample to laboratory for the antibiogram tests so that the best medical choice is given to the patients. The emergence of plasmid-mediated AmpC and ESBL *β*-lactamase producing *E. coli* and *K. pneumonia* may attitude possible risk to the spread of antibiotic resistance in the clinical situations. As plasmid-mediated genes may serve as the reservoir for the emergence of antibiotic resistance in a clinical setting, surveillance and infection control measures are essential to limit the spread of these organisms in the hospital.

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References

1. Brook I, Frazier EH. Clinical features and aerobic and anaerobic microbiological characteristics of cellulitis. *Arch Surg* 1995; 130 (7): 786-92.
2. Alikhani MY, Hashemi SH, Aslani MM, Farajnia S. Prevalence and antibiotic resistance patterns of diarrheagenic Escherichia coli isolated from adolescents and adults in Hamedan, Western Iran. *Iran J Microbiol* 2013; 5(1): 42-47.
3. Haghighatpanah M, Mozaffari Nejad AS, Mojtahedi A, Mozafari NA, Zeighami H. Detection of extendedspectrum β-lactamase (ESBL) and plasmid-borne blaCTX-M and blaTEM genes among clinical strains of Escherichia coli isolated from patients in the north of Iran. *J Glob Antimicrob Resist* 2016; 7:110-13.

4. Zamani A, Yousefi Mashouf R, Ebrahimzadeh Namvar A, Alikhani MY. Detection of magAGene in Klebsiella spp. Isolated from Clinical Samples. *Iran J Basic Med Sci* 2013; 16(2): 173-176.
5. Aslani MM, Alikhani MY. Serotypes of enteropathogenic Escherichia coli isolated from children under 5 years of age. *Iranian J Publ Health* 2009; 38(3): 70-77.
6. Sedighi I, Mozaffari Nejad AS, Amanati A, Nakhaei S, Alikhani MY. Virulence Factors and Antibiotic Resistance in Uropathogenic and Commensal Escherichia coli Isolates. *JKIMSU* 2016; 5(4): 50-57.
7. Dever LA, Dermody TS.Mechanisms of bacterial resistance to antibiotics. *Arch Intern Med* 1991;151(5):886-95.
8. Fisher JF, Meroueh SO, Mobashery S. Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity. *Chem Rev* 2005;105(2):395-424.
9. Sandanayaka VP, Prashad AS. Resistance to β-lactam antibiotics: structure and mechanism based design of β-lactamase inhibitors. *Curr Med Chem* 2002;9(12):1145-65.
10. Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. *ClinMicrobiol Rev* 2007;20(3):440-58.
11. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type β-lactamases. *Antimicrob Agents Chemother* 2002;46(1):1-11.
12. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis* 2006;6(10):629-40.
13. Walther-Rasmussen J, Høiby N. Plasmid-borne AmpC β-lactamases. *Canadian J Microbiol* 2002;48(6):479-93.
14. Jacoby GA. AmpC β-lactamases. *Clin Microbiol Rev* 2009;22(1):161-82.
15. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *JClin Microbiol* 2002;40(6):2153-62.
16. Stürenburg E, Mack D. Extended-spectrum β-lactamases: implications for the clinical microbiology laboratory, therapy, and infection control. *J Infect* 2003;47(4):273-95.
17. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement, M100-S23. Wayne, Pa: Clinical and Laboratory Standards Institute; 2013.
18. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 2001;14(4):933-51.
19. Lehman DC. Biochemical Identification of Gram-Negative Bacteria. Textbook of Diagnostic Microbiology. 2007;3:226-32.
20. Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods. Manual of Clinical Microbiology, Eleventh Edition: American Society of Microbiology; 2015. p. 1253-73.
21. Safari M, Mozaffari Nejad AS, Bahador A, Jafari R, Alikhani MY. Prevalence of ESBL and MBLencoding genes in Acinetobacter baumannii strains isolated from patients of intensive care units (ICU). *Saudi J Biol Sci*2015; 22(4): 424–29.
22. Dudley MN, Ambrose PG, Bhavnani SM, Craig WA, Ferraro MJ, Jones RN. Background and rationale for revised Clinical and Laboratory Standards Institute interpretive criteria (breakpoints) for Enterobacteriaceae and Pseudomonas aeruginosa: I. Cephalosporins and aztreonam. *Clin Infect Dis* 2013;56(9):1301-9.
23. Davis L. Basic methods in molecular biology: Elsevier; 2012.
24. Manchanda V, Singh N, Goyal R, Kumar A, Thukral S. Phenotypic characteristics of clinical isolates of Klebsiella pneumoniae & evaluation of available phenotypic techniques for detection of extended spectrum beta-lactamases. *Indian J Med Res* 2005;122(4):330-7.
25. Hiva S, Asgari Niaraki N, Ghahroudi Tali A, Baseri Z, Abdollahi A, Yalfani R. Research Article Prevalence of AmpC β-lactamase in Clinical Isolates of Escherichia coli, Klebsiella spp., and Proteus mirabilisin a Tertiary Hospital in Tehran, Iran . *Jundishapur J Microbiol* 2016; 9(12):e39121.
26. Xu Y, Gu B, Huang M, Liu H, Xu T, Xia W, *et al.* Epidemiology of carbapenem resistant Enterobacteriaceae (CRE) during 2000-2012 in Asia. *J Thorac Dis*2015;7(3):376-85.
27. Turnidge J. The pharmacodynamics of β-lactams. *Clinical Infect Dis* 1998;27(1):10-22.
28. Nikaido H, Pagès J-M. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev* 2012;36(2):340-63.
29. Drawz SM, Bonomo RA. Three decades of β-lactamase inhibitors. *Clin Microbiol Rev* 2010;23(1):160-201.

30. Vila J, Sáez-López E, Johnson J, Römling U, Dobrindt U, Cantón R, et al. Escherichia coli: an old friend with new tidings. *FEMS Microbiol Rev*2016; 40 (4): 437-63.
31. Espy M, Uhl J, Sloan L, Buckwalter S, Jones M, Vetter E, *et al.* Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiol Rev* 2006;19(1):165-256.
32. Niakan M, Chitsaz M, Metwaei A. Prevalence of AmpC type extended spectrum beta lactamases genes in clinical isolates of Klebsiella pneumoniae. *Iranian J Med Microbiol* 2008;2(2):1-8.
33. Mansouri S, Chitsaz M, Haji Hr, Mirzaei M, Gheyni M. Determination of resistance pattern of plasmid-mediated ampc β–lactamases producing isolate of Escherichia coli. *Daneshvar Medicine*2009; 16: 61-70.
34. Kalantar-Neyestanaki D, Mirsalehian A, Jabalameli F, Fatahi-Bafghi M, Mansouri S. The Importance of Extended-Spectrum β-lactamases in Gram-Negative Enteric Bacilli and the Phenotypic Methods of detection. *J Kerman Uni Med Sci* 2016;23(3):380-405.
35. Shanthi J, Balagurunathan R. Characterisation of heteroresistant subcolonies for MBL, AmpC genes in Klebsiella pneumoniae and Acinetobacter baumannii. *Indian J Med Microbiol* 2014;32(2):210-1.
36. Fam N, Gamal D, El Said M, Aboul-Fadl L, El Dabei E, El Attar S, *et al.* Detection of plasmid-mediated AmpC beta-lactamases in clinically significant bacterial isolates in a research institute hospital in Egypt. *Life Sci J* 2013;10(2):2294-304.
37. Amyes S. Antibacterial Chemotherapy: Theory, Problems, and Practice: Oxford University Press; 2010.
38. Tzouvelekis L, Markogiannakis A, Psichogiou M, Tassios P, Daikos G. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. *Clinical Microbiol Rev*2012;25(4):682-707.
39. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, *et al.* Antibiotic resistance—the need for global solutions. *Lancet Infect Dis* 2013;13(12):1057-98.
40. Yang S, Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. *Lancet Infect Dis* 2004;4(6):337-48.

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