DOI: 10.7860/JCDR/2016/17722.7739

Original Article

Microbiology Section

Prevalence of Class D Carbapenemases among Extended-Spectrum β-Lactamases Producing *Escherichia coli* Isolates from Educational Hospitals in Shahrekord

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ABSTRACT

Introduction: Extended-spectrum β-lactamases (ESBLs) are a set of plasmid-borne, various and quickly evolving enzymes that are a main therapeutic issue now-a-days for inpatient and outpatient treatment.

Aim: The aim of this study was to determine multi-drug resistance (MDR) and ESBLs producing *E. coli* strains, prevalence of class D Carbapenemases among ESBLs producing *Escherichia coli* isolates from educational hospitals in Shahrekord, Iran.

Materials and Methods: Uropathogenic *Escherichia coli* strains were isolated from patients with Urinary Tract Infections (UTIs). The agar disc diffusion test was used to characterize the antimicrobial sensitivity of the *E. coli* isolates. The ESBL positive strains were identified by phenotypic double-disk synergy test, by third-generation cephalosporin in combination with or without clavulanic acid. Multiplex PCR was carried out for detection of the three families of OXA-type carbapenamases including OXA-23, OXA-24, and OXA-48 in *E. coli* strains.

Results: All bacterial isolates were susceptible to meropenem. Ninety isolates produced ESBL, 55 *E. coli* isolates from inpatients, and 35 isolates from outpatients, with a significant association ($p< 0.05$). The prevalence of OXA-23, OXA-24, and OXA-48 in the ESBLs producing isolates was respectively 21%, 18%, and 11% for inpatients, and 10%, 8%, and 6% for outpatients.

Conclusion: ESBL-producing *E. coli* isolates are also a major threat in the clinical setting. The findings of this study indicated the high occurrence of ESBLs and multiple antibiotic resistance in *E. coli* isolates.

Keywords: Bacterial resistance, Multiplex polymerase chain reaction, Urinary tract infections

INTRODUCTION

Escherichia coli (*E. coli*) is an opportunist pathogen in the human intestinal tract. When this bacterium enters into unnatural sites, it can cause a variety of infections, for example UTI, sepsis, pyelonephritis, etc. Serotypes that lead to UTIs are designated as uropathogenic *E. coli*. Due to the high rate of morbidity and mortality of UTIs, the topic of uropathogenic *E.coli* is attracting lots of attention [1]. *E. coli* is the most common cause of UTIs, accounting for about 85% of community-acquired and 50% of hospital-acquired infections. One of the most common infectious diseases, out of community and hospital-acquired infections, is UTI, resulting in high rates of mortality and morbidity, and consequently high economic costs are associated with its treatment [2-4].

β-lactam antimicrobial agents, especially extended-spectrum cephalosporins, carbapenems and fluoroquinolones are the principal therapeutic choices for the treatment of Enterobacteriaceae infections. The rate of bacterial resistance to these antimicrobial agents is to rise worldwide [5]. Among the bacterial resistance mechanisms, production of Extended-Spectrum β-Lactamases (ESBLs) is the most common mechanism of bacterial resistance. These enzymes are multitudinous and mutate incessantly in response to the high use of antibiotic drugs, resulting in ESBLs [6]. Due to ESBL producers, therapeutic choices for infections have also become increasingly limited [7].

E. coli and *Klebsiella pneumonia* are among the most common Enterobacteriaceae, involving in both community and hospital-acquired infections. These bacteria become increasingly resistant to expanded-spectrum cephalosporins via production of various ESBLs [8,9]. Among all β-lactam antibiotics, carbapenems are still the drug of choice for serious infections with ESBL-producing Enterobacteriaceae [10].

Nevertheless, resistance to carbapenem can be caused by several mechanisms including production of enzymes such as carbapenemases Class A KPCs, Class B metallo β-lactamases IMP, VIM, NDM, or Class D OXA-type enzymes such as OXA-23, OXA-24, and OXA-48 [11,12]. Class D β-lactamases or oxacillinases are widely distributed among clinically relevant gram-negative species. OXA-48 shows a hydrolysis profile that includes penicillins, carbapenems and cephalosporins [13]. OXA-24 and OXA-23 are a class D β-lactamase with carbapenem-hydrolyzing activity that commonly isolated from a multi-drug resistant *Acinetobacterbaumannii* [14,15]. Carbapenemases class D such as OXA-23, OXA-24, and OXA-48 are always associated with low levels of resistance to carbapenems such as meropenem, imipenem and doripenem [16].

AIM

The aim of this study was to determine the multi-drug resistance (MDR) and ESBLs producing *E. coli* strains, prevalence of class D carbapenemases among ESBLs producing *Escherichia coli* isolates from educational hospitals in Shahrekord, Iran.

MATERIALS AND METHODS

Clinical Isolates

As E. coli is the most important causative agent of UTIs in both inpatients and outpatients [17]. In this study, *E. coli* strains isolated from UTI samples, including inpatients and outpatient, were examined. The isolates were collected from September 2013 to July 2014 in educational hospitals in Shahrekord, Iran.

Bacterial Identification and Susceptibility Testing

Uropathogenic *E. coli* isolates were characterized through culture on respective mediums such as MacConkey agar, Blood agar

and Eosin Methylene Blue agar (Hi Media, India), and biochemical tests. After incubation of plates at 35°C for 24 h, the pure isolates were identified by biochemical tests such as oxidative, catalase, citrate utilization, indole production, triple sugar iron agar, Voges-Proskauer and methyl red, as described in standard bacteriological methods. Antimicrobial susceptibility testing was performed by the Kirby–Bauer method in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations. The antibiotic discs used in this study were amikacin 30 µg, ciprofloxacin 5 µg, nitrofurantoin 300 µg, norfloxacin10µg, Trimethoprim-sulfamethoxazole (1.25/23.75 µg), nalidixic acid 30 µg, gentamicin 10µg, ceftazidime 30µg, and meropenem 10 µg (MAST Co., England). The protocol for antibiotic susceptibility test has been explained in the CLSI. The diameters of the inhibition zone were assessed and the isolates were classified as susceptible, intermediate, and resistant categories according to CLSI criteria [18]. The MDR bacteria were resistant to three or more antimicrobial classes [19].

Phenotypic ESBL Detection

ESBLs production was examined for the isolates that were resistant to one or more third generation cephalosporins (3GCs) by DDST using ceftazidime (30) and ceftazidime/clavulanic acid (30/10) (MAST Co. UK). A higher than or equal to 5 mm increase in diameter of the inhibition zone of the cephalosporin-plus-clavulanate disc, when compared to the cephalosporin only disc, was interpreted as phenotypic evidence of ESBLs production. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively.

Preparation of DNA Template for PCR

Since most ESBL genes are plasmid borne, plasmid DNA was extracted and purified using the GeNet bio Plasmid kit (Plasmid DNA Isolation Kit, Korea). Plasmid DNA from these microorganisms was extracted from overnight bacterial growth in 5 ml Luria Bertani medium at 35±2°C. The GeNet bio Plasmid kit protocol was used for bacterial plasmid isolation. Plasmid DNA concentrations were determined by the Nano Drop Spectrophotometer (Thermo Scientific, USA).

Detection of OXA-type Carbapenamases Encoding Genes

All ESBL-producing *E. coli* isolates were screened by Multiplex PCR for the following β-lactamase encoding genes. Multiplex PCR was conducted for detection of the three families of OXA-type carbapenamases found in *E.coli*. Sequences of primers for the detection of gene encodings bla_{OXA-23}, bla_{OXA-24}, and bla_{OXA-48} genes are given in [Table/Fig-1].

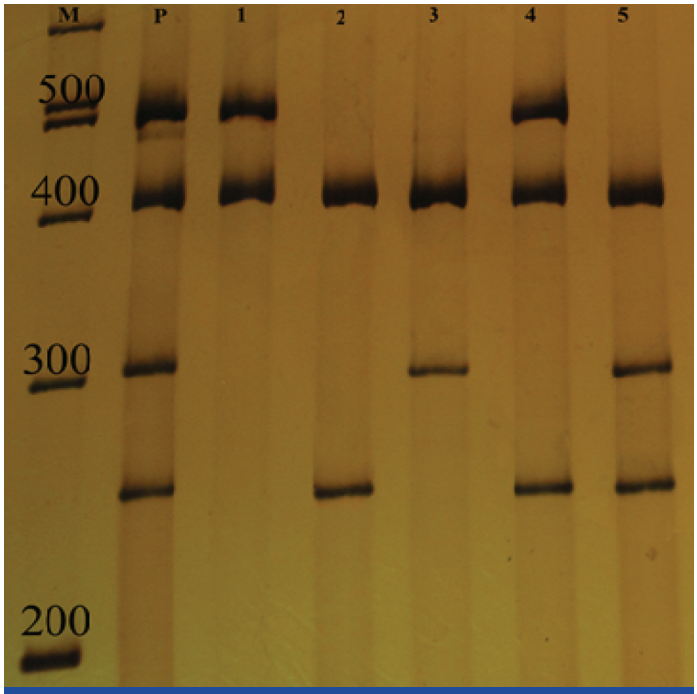
We also utilized the 16S rRNA gene as an internal control. The Multiplex PCR conditions are as follows: Initial denaturation at 95°C for 5 min, 35 cycles of 94 °C for 30 s, 56°C for 40 s and 72°C for 40 s, followed by a final extension step at 72°C for 5 min.

Ethical Consideration

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

STATISTICAL ANALYSIS

We compared the differences in susceptibility or resistance between the outpatient and inpatient with use of correlations between nominal variables. The data were assessed for significance by the Pearson Chi-square test or Fisher's-exact test. The differences between groups were considered significantly different with p-values smaller than 0.05. We analysed the data with Statistical Package for the Social Sciences software for Windows Version 18.0 (SPSS, Inc., Chicago, IL, USA).



[Table/Fig-1]: Multiplex PCR for detection of the class D carbapenemase; blaOXA-23, blaOXA-24 and blaOXA-48. Lane M, DNA size marker (bp); lane P, positive control (*KlebsiellaPneumoniae* ATCC 700603); Lane numbers 1 and 4 are showed a 501bp fragment of OXA-23 gene; Lane numbers 2,4 and 5 are showed a 249bp fragment of OXA-24 gene. Lane numbers 3 and 5 are shown a 307bp fragment of OXA-48 gene. All lane numbers are shown a 420bp fragment of 16SrRNA gene

RESULTS

Antimicrobial Drug Susceptibility

During a 10-month period, 200 *E. coli* strains were isolated from UTIs of 100 from inpatients and 100 from outpatients. Although the resistance to antibiotic in isolated *E. coli* in outpatient urinary infection was remarkably lower than inpatient samples, this relationship was only significant for Ceftazidime ($p<0.05$) [Table/Fig-2]. Isolated MDR of the outpatients was 39% and of the inpatients was 60%. The results of statistical analysis by Chi-Square test showed that there was a significant relationship between the number of isolates that were resistant to multiple-drug in two groups of inpatients and outpatients isolates ($p=0.005$). Nine antimicrobial agents are tested for all *E. coli* isolates and the obtained data are summarized in [Table/Fig-3].

Prevalence of ESBL-producing *E. coli* Isolates

Out of 100 *E. coli* isolates from inpatients UTI, 60 isolates were multiple-drug resistant and 55 isolates were ESBL positive. Moreover, out of 100 *E. coli* isolates from outpatient UTI, 39 isolates were multiple-drug resistance and 35 were ESBL positive. DDST was applied to detect ESBL in *E. coli* isolates that were resistant to Ceftazidime using ceftazidime alone or with clavulanic acid. Of these, 55 (55%) isolates of inpatient and 35(35%) of outpatient were positive for production of ESBL. Statistical analysis suggested that there was a significant relationship between ESBL-borne bacteria in outpatient and inpatient isolates ($p=0.007$). In this study, the prevalence of bacteria producing ESBLs in inpatient and outpatient isolates was 55% and 35%, respectively.

Carbapenemases Genes Detected

Multiplex PCR was applied to detect the presence of Class D carbapenemase genes, including bla_{OXA-23}, bla_{OXA-24}, and bla_{OXA-48} in *E. coli* isolates from inpatients and outpatient UTIs. The PCR products of 501 bp bla_{OXA-23}, 249 bp bla_{OXA-24}, and 307 bp bla_{OXA-48}

Primer name Sequence (5' to 3')	Size(bp)	References
OXA-23 F GATCGGATTGGAGAACCAGA OXA-23 R ATTTCTGACCGCATTTCCAT	501	[20]
OXA-24 F GGTTAGTTGGCCCCCTTAAA OXA-24 R AGTTGAGCGAAAAGGGGATT	249	[20]
OXA-48 F GCGTGGTTAAGGATGAACAC OXA-48 R CGCTCCGATACGTGTAACTT	307	This study
16S rRNA F AGGCCTTCGGGTTGTAAAGT 16S rRNA R ACCTCCAAGTCGACATCGTT	420	This study

[Table/Fig-2]: Primers used for detection of ESBL-producing *E.coli* isolates by Multiplex-PCR.

Antibiotic	Inpatients		Outpatients		p-value
	Resistance (%)	Sensitive (%)	Resistance (%)	Sensitive (%)	
AK (30µg)[a]	30	70	25	75	0.42
CIP (5µg)[b]	48	52	40	60	0.25
GEN (10µg)[c]	35	65	28	72	0.24
FM (300µg)[d]	10	90	7	93	0.42
NOR (10µg)[e]	45	55	40	60	0.4
SXT (1.25/23.75µg)[f]	75	25	72	28	0.47
NA (30µg)[g]	62	38	55	45	0.24
MER (10µg)[h]	0	100	0	100	-
CTZ (30µg)[i]	55	45	35	65	0.004

[Table/Fig-3]: Antibiotic susceptibility pattern of *E. coli* in both in patients and outpatients. [a] AK, Amikacin; [b] CIP, Ciprofloxacin; [c] GEN, Gentamicin; [d] FM, Nitrofurantoin; [e] NOR, Norfloxacin; [f] SXT, Trimethoprim-Sulfamethoxazole; [g] NA, Nalidixic acid; [h] MER, Meropenem; [i] CTZ, Ceftazidime.

Antimicrobial agent (%)	Susceptible No	Resistant No (%)	Drug Resistant rates % in groups		
			In-patient/ outpatient (p-value)	MDR/non-MDR (p-value)	ESBLs/non-ESBLs (p-value)
AK (30µg)[a]	145(72.5)	55(27.5)	30/25(0.42)	42/13(0.001)	48/8(0.001)
CIP (5µg)[b]	112(56)	88(44)	48/40(0.25)	53/35(0.007)	58/30(0.001)
GEN (10µg)[c]	137(68.5)	63(31.5)	35/28(0.24)	38/25(0.038)	42/21(0.001)
FM (300µg)[d]	183(91.5)	17(8.5)	10/7(0.42)	13/4(0.02)	15/2(0.001)
NOR (10µg)[e]	115(57.5)	85(42.5)	45/40(0.4)	49/36(0.048)	52/33(0.001)
SXT (1.25/23.75µg)[f]	53(26.5)	147(73.5)	75/72(0.47)	82/65(0.003)	67/80(0.78)
NA (30µg)[g]	83(41.5)	117(58.5)	62/55(0.24)	75/42(0.001)	64/53(0.001)
MER (10µg)[h]	200(100)	0(0)	0/0(0)	0/0(-)	0/0(-)
CTZ (30µg)[i]	110(55)	90(45)	55/35(0.004)	81/9(0.001)	95/4(0.001)

[Table/Fig-4]: Antimicrobial susceptibility results of 200 *E. coli* strains and their resistant rates in different groups.
[a] AK, Amikacin; [b] CIP, Ciprofloxacin; [c] GEN, Gentamicin; [d] FM, Nitrofurantoin; [e] NOR, Norfloxacin; [f] SXT, Trimethoprim-Sulfamethoxazole; [g] NA, Nalidixic acid; [h] MER, Meropenem; [i] CTZ, Ceftazidime.

OXA	No. of isolates	No. (%) of isolates resistant to: type genes OXA type/total						
		CIP[a]	AK[b]	CAZ[c]	GEN[d]	FM[e]	SXT[f]	MEM[g]
Inpatient								
OXA-23	21/(100)	11(52.3)	8(38)	14(66.6)	6(28.5)	3(14.2)	16(76.1)	0(0)
OXA-24	18/(100)	8(44.4)	7(38.8)	10(55.5)	4(22.2)	3(16.6)	13(72.2)	0(0)
OXA-48	11/(100)	4(36.3)	3(27.2)	8(72.7)	5(45.4)	5(45.4)	9(81.8)	0(0)
Outpatient								
OXA-23	10/(100)	4(40)	3(30)	7(70)	4(40)	1(10)	7(70)	(0)
OXA-24	8/(100)	2(25)	3(37.5)	4(50)	3(37.5)	1(12.5)	6(75)	(0)
OXA-48	6/(100)	2(33.3)	2(33.3)	3(50)	2(33.3)	1(16.6)	4(66.6)	(0)

[Table/Fig-5]: Antimicrobial susceptibility data of *E. coli* isolates producing OXA-type carbapenamases.
[a] CIP, Ciprofloxacin; [b] AK, Amikacin; [c] CAZ, Ceftazidime; [d] GEN, Gentamicin; [e] FM, Nitrofurantoin; [f] SXT, Trimethoprim-Sulfamethoxazole; [g] MEM, Meropenem

were visualized by 8% polyacrylamide gel electrophoresis [Table/Fig-4].

In inpatients *E. coli* isolates, the bla_{OXA-23} gene was detected in 21 (21%). Only 18 isolates (18%) carried bla_{OXA-24}, and only 11 isolates (11%) carried bla_{OXA-48}. In addition, in outpatients *E. coli* isolates, 10 isolates (10%) were positive for the bla_{OXA-23} gene, 8 (8%) were positive for the bla_{OXA-24}, and 6 (6%) were positive for the bla_{OXA-48} gene. The 16S rRNA gene was observed in all clinical strains. Antimicrobial susceptibility data of *E. coli* isolates producing OXA-type ESBLs are summarized in [Table/Fig-5].

DISCUSSION

The issue of elevated bacterial resistance to antimicrobial agents and treatment of infectious diseases caused by these bacteria is a public health problem. In addition, the rate of bacterial resistance varies worldwide, nationwide and even among various geographical areas of a country [20]. In our study, all bacterial isolates were susceptible to meropenem. In a study by Gorgec et al., in Turkey, all the extended-spectrum beta-lactamase producing nosocomial *E. coli* isolates was susceptible to meropenem [21]. Besides, similar sensitive pattern were found in China and Nigeria [22,23]. Because of stability issues encountered with some imipenem samples in gram negative bacteria, imipenem was replaced by meropenem in 2006 [24]. In the obtained results by disc diffusion method, *E.coli* isolated from urinary infections in inpatients and outpatients have shown the highest resistance to trimethoprim-sulfametoxazole (73.5) antibiotics and nalidixic acid (58.5%). Other studies also showed the highest resistance to trimethoprim-sulfametoxazole and nalidixic acid [25,26] in *E. coli* samples collected from patients with UTIs.

In the obtained results by disc diffusion method, *E.coli* isolated from urinary infections in inpatients and outpatients have shown the highest resistance to Trimethoprim-Sulfamethoxazole antibiotics and nalidixic acid. In a study in Tehran, Iran Soltan Dallal et al., revealed a similar anti-bio gram pattern for trimethoprim-sulfamethoxazole (80.5%), nalidixic acid (74%), ceftazidime (55.5%), and ciprofloxacin (55.5%) for 200 clinical isolates of *E. coli*. They also found that 70% of isolates were multi-drug resistant phenotype [25].

Other studies also showed the highest resistance to trimethoprim-sulfametoxazole and nalidixic acid [26,27] in *E. coli* samples collected from patients with UTIs [28,29]. But this pattern of resistance have been different in studies investigating drug resistance in strains of ESBL-producing *E. coli* compared to those study working upon the third generation of cephalosporins. For example, in a study by Gholiour et al., the level of antibiotic resistence for most studied antibiotics was lower than that of the present study. for example in a study that was performed by Gholipour et al., in Isfahan, Iran, the level of antibiotic resistance for most studied antibiotics was lower than that of the present study [26].

During treatment of UTIs, whenever resistance to routinely used antibacterial happens, specific cephalosporins can be used for UTIs as alternatives [27]. But ESBL production rate by Enterobacteriaceae has increased considerably, which is the main problem with use of these cephalosporins [28]. Moreover, during recrudescent UTI, the carbapenem-resistant strains appear after long-term treatment with meropenem and imipenem. Since carbapenem antibiotics are often the drugs of choice for therapy of severe infections resulting from ESBL-producing *E. coli*, the incidence of carbapenem resistant *E. coli* is disturbing [29]. In the present study, the rate of antibiotic resistance in inpatient isolates was higher than outpatient, that is consistent with other studies [30].

It can be inferred that further resistance of strains in hospital was due to greater consumption of antibiotics and antiseptics in hospital environments, resulting in higher levels of antibiotic resistant strains of hospital origin in outpatient's comparing to inpatients.

Even some debilitating illnesses such as severe sepsis can also cause antibiotic resistant gene transfer of exogenous pathogens resistant to susceptible endogenous pathogen during treatment with antibiotics that it demands multi-drug treatment against this pathogen [31,32].

In this study, the prevalence of ESBLs producing *E. coli* in out patients and inpatients UTI was 35% and 55%, respectively. Based on the finding of this study, the prevalence of ESBL producing *E. coli* was higher in inpatient isolates than in outpatient isolates. Similarly, researches from other countries demonstrated that the prevalence of ESBL-positive bacteria are more common in patients who, having a history of hospitalization, than in outpatients [31].

In Isfahan, Iran, a study by Mobasherizadeh et al., revealed 44.1% and 21.2% frequency for ESBL positive in *E. coli* strains isolated from inpatients and outpatients isolates [32]. Thus, our results were consistent with Mobasherizadeh et al., study and the frequency of ESBLs in bacteria isolated from inpatients was higher than that of outpatients. This suggests that infection control in hospitals could decrease the emergence of ESBL-producing bacteria and subsequently reduce the spread into societies. Various frequencies of ESBLs producing *E. coli* have been reported by some previous studies in Iran. In a study in Tehran, Soltan Dallal et al., showed that 115 (89.8%) *E. coli* isolates were identified as ESBLs producers [25]. In a study in Iran, Zaniani et al., revealed that 43.9% of *E. coli* was ESBL producers [33].

In other countries, several studies have revealed that the distribution of ESBLs varies. For instance, in a study in Turkey by Bali et al., [34], 42 of 50 *E. coli* strains (84%) isolated from different clinical samples were ESBL positive. The prevalence of the organisms in Poland was reported 49.3% in *E. coli* isolates [35]. The prevalence of ESBL-producing bacteria has been on the rise, mainly in Asia compared with other regions. Thus, the geographical distribution of ESBL-producing bacteria could vary among countries and settings [36].

The prevalence of the OXA-23, OXA-24, and OXA-48 in our study was 21%, 18% and 11%, respectively for *E. coli* isolates from inpatient and 10%, 8% and 6%, respectively for *E. coli* isolates from outpatient. This difference is significant because the p-value of the Chi-Square is $P < 0.001$ and as mentioned above for ESBLs, the frequency of OXA type carbapenemase in bacteria isolated from inpatients was higher than that from outpatients.

In addition, the present study showed that OXA-23 and OXA-24 and OXA-48 were found more frequently in inpatients isolates, and that ESBL-positive bacteria was also more frequent in inpatients. In a study in India, the prevalence of OXA-48 gene was 32.6 in *E. coli* isolates, but OXA-23 and OXA-24 genes were not identified in the *E. coli* isolates [37]. These results are comparable to the results of other studies that have emphasized the spread of OXA-48-producing Enterobacteriaceae worldwide, especially in Europe, Africa and East-Central Asia [38,39].

In Iran, OXA-type carbapenemases was found more frequently in *A.baumannii*, *P.aeruginosa* and Enterobacteriaceae especially *K. pneumonia*, but no study has been yet investigated *E. coli* OXA producing isolates and to our knowledge, this is the first report of the frequency of OXA-type carbapenemases genes in *E. coli* isolates in Iran. In a study by Sohrabi et al., in Northwest of Iran, 88.7% of *A. baumannii* isolates carried blaOXA-23 [40]. In another study by Kooti et al., in Iran, 40% of *A. baumannii* isolates had positive results for blaOXA-23-like, 7% for blaOXA-24-like and 0.5% for blaOXA-58-like [41].

CONCLUSION

It is quite alarming to note that many of the isolates included in this study were found as multiple drugs resistant. Antibiotic resistance has been appearing as a main issue in the management of inpatients and outpatients and increases the costs imposed on patients in health care systems. Briefly, the prevalence of ESBL-positive *E. coli* isolates found in this study is of enormous concern that needs revision of infection control guidelines such as management of antibiotic prescription and traditional laboratory identification of ESBL-positive isolates to prevent ESBLs spread.

ACKNOWLEDGMENTS

The result described in this paper are part of Msc thesis of microbiology with grant no.1882. We thanks deputy of research and technology for thier technical and financial support.

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FINANCIAL OR OTHER COMPETING INTERESTS: As declared above.

Date of Submission: **Nov 06, 2015**
Date of Peer Review: **Dec 13, 2015**
Date of Acceptance: **Jan 06, 2016**
Date of Publishing: **May 01, 2016**