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The presence of extended-spectrum β-lactamase as a risk factor for MDR in clinical isolation of *Escherichia coli*

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Abstract. Significant increases in antibiotic resistance have become a critical dilemma in healthcare systems around the world. *Enterobacteriaceae* acquired different mechanisms of antibiotic resistance such as ESBLs production and transposon attainment, which hold antibiotic resistance genes. This may create multidrug resistant (MDR) strains. Antibiotic resistance patterns vary in different geographical regions. The present estimated-cross sectional study is aimed to determine antibiotic resistance pattern of 182 *E. coli* strains to 20 antibiotics. Three different methods were applied to detect the ESBL-producing *E. coli*. Observations revealed that oxacillin, amoxicillin and ampicillin had the highest effect, while imipenem, gentamicin and nitrofurantoin had the highest impact on clinical *E. coli* strains in our region. Three different methods, including double disk synergy test (DDST) (30 mm), double disk synergy test (DDST) (20mm) and combined disk test were used to identify ESBL-producing *E. coli*. The prevalence of ESBL producers was at a 35.71% rate. Findings of this study indicate that there is no significant difference between these three methods in identifying ESBL-producing *E. coli*. There was a significant relation between ESBL production and resistance to three other classes of antibiotics, including protein synthesis inhibitor, Quinolones and Metabolite analogues. Moreover, antibiotic resistance rate in ESBL-producing *E. coli* was significantly higher than non ESBL- producing isolates. The MDR was at a 65.93% rate. Unfortunately, the rate of antibiotic resistance is globally increasing; this is due to several factors such as inappropriate antibiotic use, incomplete course of antibiotics use, protracted length of stay in hospitals and self-medication. Resistance mechanisms such as ESBL production and MDR cause treatment failure. Our findings suggest that ESBL production is a risk factor for MDR in clinical *E. coli*. Therefore, Physicians are recommended to stop excessive and long term administration of antibiotics.

INTRODUCTION

Antibiotic treatment plays a vital role in treatment of bacterial infections and postoperative care, as it saves lives in modern medicine (Nathan & Cars, 2014; Kapil, 2005). The increase of antibiotic resistance has turned into a critical dilemma in public health throughout the world (Spellberg *et al.*, 2013; Kapil, 2005; Levy, 1998).

There are different mechanisms that confer antibiotic resistance to micro-organisms. One of these mechanisms is attainment of transposons which hold several resistance genes and can cause different types of drug resistance in bacteria that are called Extensively Drug-Resistant (XDR), Pan Drug-Resistance (PDR) and Multidrug-resistant (MDR). These different patterns depend on resistance to a number of drugs that may be administered in medical treatments which are not the same for different types of bacteria. (CDC, 2013; Džidić *et al.*, 2008). MDR which is delineated by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) as microorganisms non-susceptible

to at least one drug in three or more antimicrobial classes, has been considered as an important defeat in the healing of infectious illnesses and led to increased morbidity, mortality and outlay of health systems (CDC, 2013; Brink *et al*., 2012; Ibrahim ME *et al*., 2012; Rowe-Magnus and Mazel, 2009). MDR, especially in *Enterobacteriaceae*, has been increasing in hospital settings during the current decade (Wajidi *et al*., 2011).

Another critical challenge to hospitalized patients can be nosocomial infections, and among pathogens, multi drug resistant Gram negative bacteria such as *Escherichia coli* are extremely capable of up regulating or getting genes that encode antibiotic resistance, especially under the pressure of antibiotic selection (Peleg & Hooper, 2010; Kapil, 2005).

Another mechanism is production of a group of enzymes in Gram negative bacteria. These enzymes called Extended-spectrum β-lactamases (ESBLs) are released into the periplasmic space of Gram negative bacteria. They enable bacteria to become resistant to third generation cephalosporins (i.e. cefotaxime, ceftriaxone, cefepime, ceftazidime and cefuroxime), monobactams and all penicillins, (i.e. aztreonam), but not to carbapenems and cephamycins (cefotetan and cefoxitin) (CDC, 2013; Yasmin, 2012; Džidić *et al*., 2008). β-lactam ring present in penicillins and cephalosporins is hydrolyzed by conventional hydrolytic amidases that are generated by ESBL-producing bacteria (CDC, 2013; Yasmin, 2012; Džidić *et al*., 2008). ESBLs are of a great variety since more than 180 different ESBLs have been recognized so far (Džidić *et al*., 2008). ESBL was initially isolated from *Klebsiella pneumoniae* in 1983 (Iroha IR *et al*., 2009). The majority of ESBLs are originated from *K. pneumoniae*, *K. oxytoca*, and *E. coli* (Nathan & Cars, 2014). Nowadays, the source of almost 26000 (19%) infections related to health-care is deemed to be ESBL-producing *Enterobacteriaceae* (CDC, 2013). Bloodstream infections caused by ESBL-producing *Enterobacteriaceae* result in mortality 57% more than probable bloodstream infections caused by non ESBL-producing strains (CDC, 2013).

Several studies in developing countries show the alarming tendency of enteric pathogens such as *Klebsiella*, *E. coli* and *Salmonella* spp. towards being resistant to almost all normally accessible antibiotics. There is no antibiotical resistance monitoring system in many developing realms, and infectious disorders are still empirically treated based on clinical diagnoses (Sosa *et al*., 2010). *E. coli*, one of the bacteria belonging to the family of *Enterobacteriaceae*, is the most significant causative agent of nosocomial and urinary tract infections. It has been estimated that about 70-80 percent of isolates are resistant to the normally administered antibiotics (Kapil, 2005).

Inappropriate antibiotic prescribing in different geographical regions leads to antimicrobial resistance among pathogens. Hence, area-based assessment of antibiotic susceptibility is required in order to improve antimicrobial medication (Ipek *et al*., 2011).

As ESBLs-production and MDR lead treatment of infectious caused by *E. coli* to fail, the aim of this study was to determine antibiotic resistance patterns, and whether ESBL production could be a risk factor for MDR in *E. coli* strains isolated from urinary tract infection in our region.

Experimental

Materials and methods

An estimated-cross sectional study was carried out in a six-month period.

A) Patients and Isolation of *E. coli*

During February to July 2014, 2000 patients with a chief complaint of urinary tract referred to five hospitals in Rasht (north of Iran). Urine samples were taken from all patients; then, being cultured on blood agar containing 5% defibrinated sheep blood and MacConkey agar, the plates were incubated at 37°C for 24 to 48 h. According to colony appearance, Gram staining, and standard microbiological tests, a total of 182 non-duplicated *E. coli* strains were isolated.

B) Antibiotic Susceptibility Test

Antibiogram was performed for every isolated *E. coli* using Kirby-Bauer method as claimed by the CLSI (Clinical Laboratory and Standards Institute) guideline. A suspension was prepared for each *E. coli* with a 0.5 Macfarland concentration, then cultured on Mueller Hinton agar (MHA) and 20 different antibiotic discs (Mast, UK) including Cotrimoxazole (25µg, TS25), Ciprofloxacin (5µg, CIP5), Ofloxacin (5µg, OFX5), Nalidixic acid (30µg, NA30), Nitrofurantoin (300µg, NI300), Gentamicin (120µg, GM120), Tetracycline (30µg, T30), Oxacillin (1µg, OX1), Amoxicillin (25µg, A25), Ampicillin (10µg, AP10), Imipenem (10µg, IMI10), Aztreonam (30µg, ATM30), Cephalothin (30µg, KF30), Cefoxitin (30µg, FOX30), Cefotaxime (30µg, CTX30), Cefixime (5µg, CFM5), Ceftriaxone (30µg, CRO30), Ceftazidime (30µg, CAZ30), Cefepime (30µg, CPM30) and Augmentin (30µg, AUG30) were put on MHA and incubated at 37°C for 24 h.

Wherever necessary, the quality control policy was carried out in sample processing.

C) Detection of ESBL Producing *E. coli*

All *E. coli* strains resistant to a number of beta-lactamase antibiotics in antibiogram test were subjected to the detection process. Using a single method may result in missing some ESBL producing strains, thus, in the present study, three different methods were adopted to identify the ESBL producing *E. coli*. Moreover, we aimed to compare different validity aspects of these approaches through the following methods.

1) Double Disk Synergy Test (DDST) (30 mm)

A suspension of resistant strains to β-lactams with a concentration of 1.5×10^8 cfu/ml were cultured on MHA and ceftriaxone (30µg), augmentin (30µg) and ceftazidime (30µg), ceftazidime/clavulanate (30µg/10µg), discs were put on MHA plate with a 30mm distance from center of each disc and incubated at 37°C for 24 h. The zone around a ceftriaxone disc was distorted and expanded on the side facing a disc that contained clavulanic acid, or a clear-cut expansion towards the augmentin disc was interpreted as synergy, also called "keyhole", "phantom image", "ellipsis" and "champagne-crok". These suggest the presence of an ESBL producing strain (Garrec *et al.*, 2011; Drieux *et al.*, 2008) (Figure 1).

2) Double Disk Synergy Test (DDST) (20mm)

Since DDST (30 mm) results turned ambiguous, it is recommended to decrease the distance between the third generation cephalosporin disc and clavulanate-containing disc down to 20 mm so as to enhance the test sensitivity. For example, in cases of lucid decrease in susceptibility to third-generation cephalosporins without explicit synergy, DDST was carried out again with decreased distance (Figure 2).

3) Combined Disk Test

A suspension of *E. coli* resistant strains with a concentration of 1.5×10^8 cfu/ml was provided and cultured on MHA, then discs containing the third generation of cephalosporins such as ceftazidime (30µg) and discs that have a combination of these antibiotics plus clavulanic acid (ceftazidime/clavulanate) (30µg/10µg) were put on MHA plate with a 30mm distance from center of each disc and incubated at 37°C for 24 h. In the ESBL producer strains, the zone diameter around ceftazidime/clavulanate was 5 mm larger than that of ceftazidime alone.

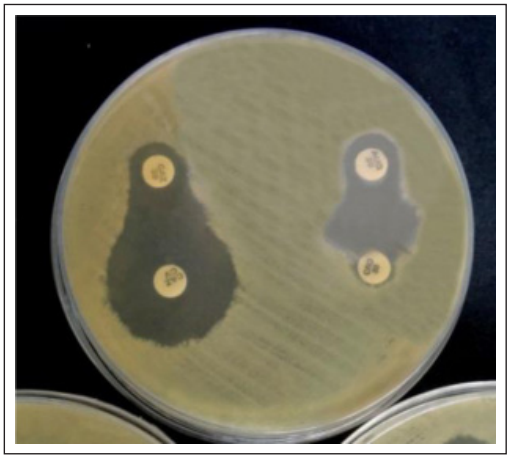

Figure 1. Double Disc Synergy Test (30mm).

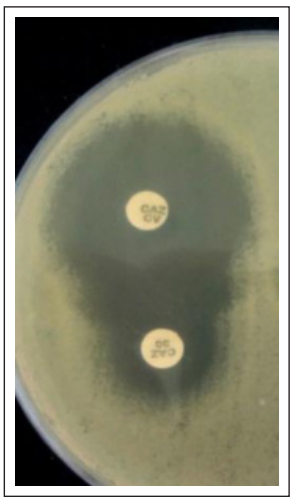

Figure 2. Double Disc Synergy Test (20mm).

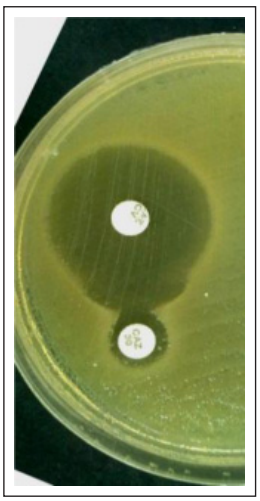

Figure 3. Combined Disc Test.

E. coli ATCC25922 was used as a controlling factor for antibiotic susceptibility determination and a negative control for ESBL tests; while *K. pneumoniae* ATCC 700603 was applied as a positive controlling factor for quality assessment of ESBL tests as recommended by CDC (Figure 3).

Statistics

Statistical analysis was performed by the Chi-square test using SPSS version 16 statistical software and a p value of either equal to or less than 0.05 was considered statistically significant. Comparisons were analyzed using 95% confidence intervals.

RESULTS

All 182 isolated *E. coli* were tested against 20 discrepant antibiotics (Table 1).

In general, the highest resistance rate belonged to oxacillin, amoxicillin and ampicillin (100%, 82.4% and 82.4% respectively); and the most effective antibiotics were imipenem, gentamicin and nitrofurantoin (susceptibility rate were 99.5%, 89% and 88.5% respectively).

DDST method and combined disc test revealed that 65 out of 182 *E. coli* strains (35.71%) were among ESBL producers.

No substantial difference was observed among these three methods; however, the results of the study revealed that, in some cases, Double Disk Synergy Test (DDST) (30 mm) led to loss of some certain ESBL strains, thus it is suggested to use Double Disk Synergy Test (DDST) (20mm) instead.

The results suggested there is a statistically significant relation between ESBL-producing *E. coli* and resistance to 3 classes of antibiotics, including protein synthesis inhibitors (P value=0.004), Quinolones (P value<0.0001) and metabolite analogues (P value<0.0001) (Table 2).

According to Table 3, resistance rate to 15 of 20 tested antibiotics among ESBL-producing *E. coli* was statistically more significant than non ESBL-producing strains, as percentage difference of antibiotic resistance in ESBL and non ESBL-producing *E. coli* in CRO30, CFM5, CTX30, ATM30, CAZ30, KF30, CPM30, OFX5, CIP5, NA30, AP10, A25, TS25, T30 and NI300 were 82.3, 81.2, 81.2, 67.8, 66.9, 57.3, 50.3, 45.8, 42.6, 28.5, 27.4, 27.4, 27.2, 18.1 and 15.1, respectively.

In this study, *E. coli* strains resistant to at least one antibiotic in three or more classes were considered as MDR. As shown in diagram 1, there was a significant relation between ESBL positive and negative strains and MDR (P<0.0001).

Table 1. Antimicrobial resistance pattern of the isolates to 20 antibiotics

Antibiotics \ Frequency		Resistant (%)	Intermediate (%)	Sensitive (%)
Ampicillin	AP10	82.4	2.7	14.8
Ciprofloxacin	CIP5	43.4	4.4	52.2
Cefoxitin	FOX30	11.0	8.2	80.8
Cefepime	CPM30	23.1	11.0	65.9
Aztreonam	ATM30	37.9	7.1	54.9
Amoxicillin	A25	82.4	1.6	15.9
Tetracycline	T30	63.7	0.5	35.7
Oxacillin	OX1	100.0	0.0	0.0
Cefotaxime	CTX30	47.8	2.7	49.5
Ofloxacin	OFX5	42.9	1.6	55.5
Cephalothin	KF30	63.2	12.6	24.2
Gentamicin	GM120	7.7	3.3	89.0
Nalidixic acid	NA30	70.9	6.6	22.5
Cotrimoxazole	TS25	61.0	0.0	39.0
Cefixime	CFM5	47.8	6.6	45.6
Nitrofurantoin	NI300	8.8	2.7	88.5
Imipenem	IMI10	0.0	0.5	99.5
Augmentin	AUG30	30.2	26.4	43.4
Ceftriaxone	CRO30	45.6	2.2	52.2
Ceftazidime	CAZ30	40.1	6.0	53.8

Table 2. The Relation between ESBL production and resistance to 3 different classes of antibiotics

Family	Protein synthesis inhibitors	Quinolones	Metabolite analogues
ESBL \ Frequency	Percent	Percent	Percent
Positive	81.5	89.2	78.5
Negative	60.7	61.5	51.3
P value	0.004	<0.0001	<0.0001

DISCUSSION

Ineligible use of antibiotics will cause bacterial resistance to ordinary antibiotics. These microorganisms, which are called "nightmare bacteria" have made serious health problems around the world, thus physicians should gain the required knowledge about the new details of regional rampancy of antimicrobial resistance (Naderi *et al.*, 2014; Villar *et al.*, 2014; CDC, 2013).

Through the present study, antimicrobial resistance pattern of *E. coli* isolates to 20 antibiotics was determined.

The highest resistance rates were observed to be against oxacillin, ampicillin and amoxicillin (100%, 82.4% and 82.4%, respectively).

The results of another study carried out through the same region revealed a 100% resistance rate to oxacillin, which was in line with our findings (Tavakoly *et al.*, 2014). It

Table 3. The Relation between resistance to 20 antibiotics and ESBL production

Antibiotics		ESBL	Positive (%)	Negative (%)	P value
Ampicillin	AP10	No Resistant	0.0	27.4	<0.0001
		Resistant	100.0	72.6	
Ciprofloxacin	CIP5	No Resistant	29.2	71.8	<0.0001
		Resistant	70.8	28.2	
Cefoxitin	FOX30	No Resistant	93.8	86.3	0.120
		Resistant	6.2	13.7	
Cefepime	CPM30	No Resistant	44.6	94.9	<0.0001
		Resistant	55.4	5.1	
Aztreonam	ATM30	No Resistant	18.5	86.3	<0.0001
		Resistant	81.5	13.7	
Amoxicillin	A25	No Resistant	0.0	27.4	<0.0001
		Resistant	100.0	72.6	
Tetracycline	T30	No Resistant	24.6	42.7	0.015
		Resistant	75.4	57.3	
Oxacillin	OX1	No Resistant	0.0	0.0	-
		Resistant	100.0	100.0	
Cefotaxime	CTX30	No Resistant	0.0	81.2	<0.0001
		Resistant	100.0	18.8	
Ofloxacin	OFX5	No Resistant	27.7	73.5	<0.0001
		Resistant	72.3	26.5	
Cephalothin	KF30	No Resistant	0.0	57.3	<0.0001
		Resistant	100.0	42.7	
Gentamicin	GM120	No Resistant	89.2	94.0	0.246
		Resistant	10.8	6.0	
Nalidixic acid	NA30	No Resistant	10.8	39.3	<0.0001
		Resistant	89.2	60.7	
Cotrimoxazole	TS25	No Resistant	21.5	48.7	<0.0001
		Resistant	78.5	51.3	
Cefixime	CFM5	No Resistant	0.0	81.2	<0.0001
		Resistant	100.0	18.8	
Nitrofurantoin	NI300	No Resistant	81.5	96.6	0.001
		Resistant	18.5	3.4	
Imipenem	IMI10	No Resistant	100.0	100.0	-
		Resistant	0.0	0.0	
Augmentin	AUG30	No Resistant	64.6	72.6	0.258
		Resistant	35.4	27.4	
Ceftriaxone	CRO30	No Resistant	1.5	83.8	<0.0001
		Resistant	98.5	16.2	
Ceftazidime	CAZ30	No Resistant	16.9	83.8	<0.0001
		Resistant	83.1	16.2	

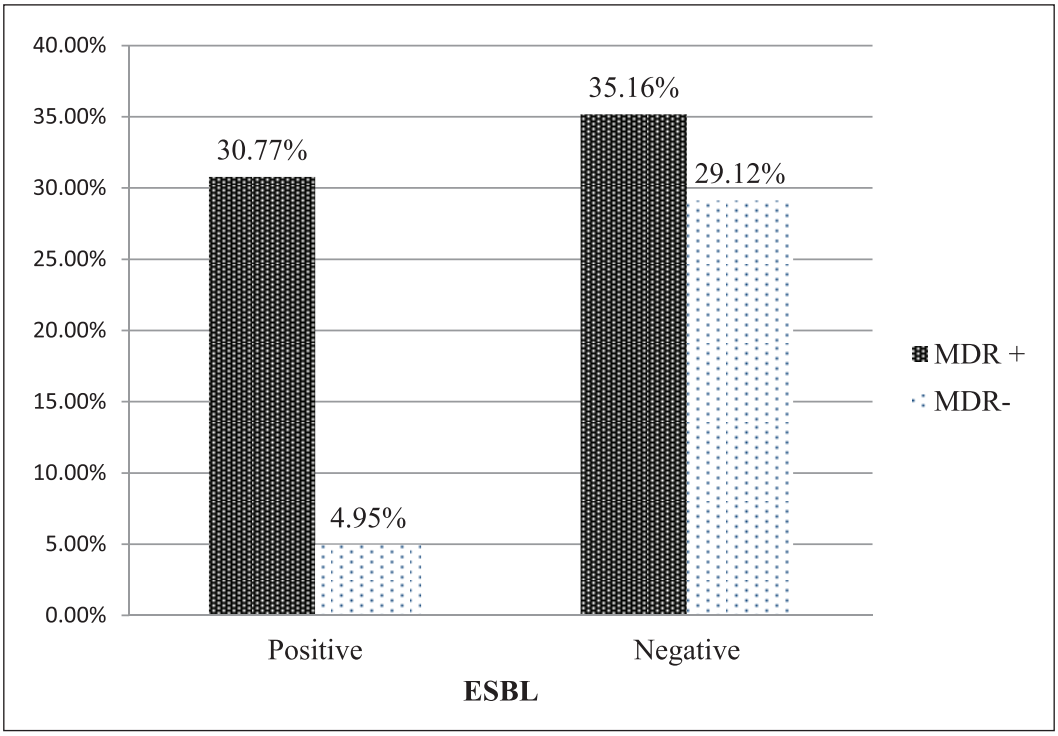

Diagram 1. The Relation between ESBL statue and MDR.

is a serious alarming about unauthorized use of this antibiotic.

The studies by Ipek *et al.* (Ipek *et al.*, 2011) and Charde *et al.* (Bhiwankar *et al.*, 2014) showed a 84.5% and 86% resistance rate to ampicillin, respectively, which were a little higher than the findings of this study; other studies conducted in Pakistan (Muhammad *et al.*, 2011) and Iran (Farshad *et al.*, 2008) reported 78.22% and 80.2% resistance rate to this antibiotic, which are less than what the results of the present study revealed.

In case of amoxicillin, it was observed that 82.4% of strains were resistant, but Kothari & Sagar (Kothari and Sagar, 2008) reported very low resistant rate to this antibiotic (14.7%).

High resistance rates reported in our study may be due to the inordinate use of such antibiotics in our region during the past decade.

The most effective antibiotics reported in this study include, imipenem, gentamicin, nitrofurantoin and cefoxitin with 0%, 7.7%, 8.8% and 11% resistance rate, respectively. Thus these antibiotics can be among potential candidates for treating UTI caused by *E. coli* in our region.

The results of several other studies showed zero or low resistance to imipenem (Bhiwankar *et al.*, 2014; Haghighatpanah *et al.*, 2014; Tavakoly *et al.*, 2014; Ipek *et al.*, 2011; Farshad *et al.*, 2008). Therefore, in cases of UTI caused by highly antibiotic resistant *E.coli*, imipenem can be used as a drug of choice for treating such infections.

In the study carried out by Ipek *et al.* (Ipek *et al.*, 2011), 12.7% of *E. coli* stains appeared resistant to gentamicin, which was similar to the findings of this study (8.7%); the results of studies conducted by Kwai-Lin Thong *et al.* (Lim *et al.*, 2009) and Charde *et al.* (Bhiwankar *et al.*, 2014) suggested resistance to gentamicin to be at 21% and 24% rates, respectively, which were higher than our findings.

In the case of nitrofurantoin, Habib *et al.* (Muhammad *et al.*, 2011) and Kothari & Sagar, 2008, have reported 78.22% and 75.6%

resistance rate, respectively, which were higher than the results of the present study. However, other studies reported as low resistance rates to this antibiotic as 16% and 4.9% that was almost similar to the results of this study (Bhiwankar *et al.*, 2014; Ipek *et al.*, 2011).

ESBLs are considered to be important defensive factors in Gram-negative bacteria against β-lactam antibiotics, therefore, they are clinically of a great significance because they may lead to alarming remedial dilemma worldwide, especially in developing realms, and they can be given as primitive line agents to wide range of severely ill patients (Iroha *et al.*, 2009; Wani *et al.*, 2009; Jacoby & Munoz-Price, 2005).

In this study, the prevalence of ESBL positive *E. coli* with urinary source was reported to be 35.71% that is similar to the results of other studies conducted in Iran (Moayednia *et al.*, 2014; Tavakoly *et al.*, 2014; Karimi *et al.*, 2012; Pourakbari *et al.*, 2012); while Wani *et al.*, reported 59.31% ESBL-producing *E. coli* with urinary source (Wani KA *et al.*, 2009), which is higher than our findings. Several studies carried out in Morocco (Iroha IR *et al.*, 2009), Saudi Arabia (Bourjilat *et al.*, 2011), Thailand (Somily *et al.*, 2014), Japan (Phongpaichit *et al.*, 2011), Nigeria (Harada *et al.*, 2013) and Iran (Ashrafian *et al.*, 2013) have reported lower rate of ESBL-producers *E. coli* comparing to our findings.

Winokur *et al.* in the SENTRY Antimicrobial Surveillance Program (1997–1999) have reported the rate of ESBL-producing *E. coli* to be at 4.2% in Canada, 5.3% in Europe, 8.5% in Latin America, 3.3% in United States and 7.9% in Western Pacific (Winokur *et al.*, 2001).

Kaya *et al.* claimed that previous use of fluoroquinolone as well as cephalosporin develops the likelihood of ESBL-producing *E. coli* bacteremia (Kaya *et al.*, 2013). On the other hand, our results showed that ESBL-producing isolates not only are resistant to beta-lactam antibiotics, but also have a significant impact in resistance to other antibiotics classes. Several studies have pointed to a part of the present study's finding. For example other studies have declared that ESBL-producing strains were resistant to different antibiotic classes including β-lactams, fluoroquinolones, aminoglycosides, trimethoprim/sulfa-methoxazole, and gentamicin (Bourjilat *et al.*, 2011; Nedjai *et al.*, 2013), as Wani *et al.* also declared that many ESBL producers are multi-resistant to non-β Lactam antibiotics such as quinolones and amino glycosides; and this reduces treatment options (Wani *et al.*, 2009). During their studies, Souwalak Phongpaichit *et al.* also, found a relation between the ESBL production and resistance to ciprofloxacin and norfloxacin (Phong-paichit *et al.*, 2011).

Unfortunately, 65.93% of isolated *E. coli* appeared to be MDR in our region. It is an alarming finding, but this rate is less than the findings of three other researches carried out in Iran reporting the presence of 77% (Farshad *et al.*, 2008), 87.1% (Eslami *et al.*, 1389) and 77% (Anvarinejad *et al.*, 2011) MDR in their studies, respectively. Although the results of a similar study showed a 100% MDR rate in our region, this cannot be completely reliable because of the low number of strains studied so far, which are as few as 33 strains (Hemmati *et al.*, 2015). These findings may be due to the excessive use of antibiotics in Iran.

Unreasonable use of antibiotics and animal source foods that have received antibiotics, self-medication, non-compliance with medication (Farshad *et al.*, 2008), and having no good hygiene habits (Bourjilat *et al.*, 2011) can lead to antibiotic resistance. In order to prevent antibiotic resistance, protracted hospitalized patients (Senbayrak Akcay *et al.*, 2014) must be trained about the consequence of poor hand-washing, avoiding redundant injections and transfusions and making sure that these processes are performed in aseptic conditions. Moreover, segregating patients with contagious diseases, safe management of waste products, and using antimicrobials appropriately could prevent the development of antibiotic resistance (Randrianirina *et al.*,

2010). Finally, physicians are recommended to stop redundant and longtime administration of antibiotics (Nedjai *et al*., 2013).

Using of probiotics as limited surrogate or add-on to antibiotic remedy may aid treating multi drug resistant UTIs (Naderi *et al*., 2014).

Accordingly, preventive use of antibiotics is not suggested, and exposure to antibiotics is considered to be a notable risk factor for colonization and infection with hospital acquired multidrug-resistant pathogens as noted by other authors (Joseph *et al*., 2010).

The deficiency of any new medicinal compounds in the close prospect implies that national, regional, and local scrutiny attempts are essential to provide clinicians with information for selecting appropriate experiential therapy. Akcay *et al*. claim that these empirical studies are a good support for programming more useful infection control policies and logical antibiotic therapies, and can diminish infection-related costs, morbidity, and mortality (Senbayrak Akcay *et al*., 2014).

Having sufficient information about the etiological agents of UTIs and their antimicrobial resistance patterns in particular geographical regions may help clinicians prescribe relevant antibiotics (Villar *et al*., 2014).

The findings of this study revealed that three different methods of detecting ESBL-producing *E. coli* did not come up with any considerable difference. According to the results of the present study, ESBL production appeared to be a risk factor for MDR in *E. coli* strains isolated from clinical samples.

More studies need to be performed for identification of the probable genes that encode co-resistance in ESBL-producing bacteria, because plasmids that code these enzymes often carry genes encoding co-resistance to other antibiotics such as aminoglycosides, tetracyclines, chloramphenicol, fluoroquinolones and sulfamethoxazole-trimethoprim.

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