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Prevalence of ESBLs and Biofilm Formation in *Escherichia coli* Isolated from Urinary Tract Infection in Isfahan, Iran

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ABSTRACT

Backgrounds: Uropathogenic *Escherichia coli* is a Gram-negative bacillus that is the most common cause of urinary tract infection. *E. coli* has the ability to produce biofilm as an important virulence factor. Due to the lack of sufficient information about ESBL resistance genes in this geographical area, this study aimed to investigate the prevalence of ESBLs in *E. coli* isolates to increase our knowledge about the role of these genes and biofilm formation in inducing resistance.

Materials & Methods: 139 *E. coli* strains were isolated from urine samples. Antibiotic susceptibility testing was performed for the isolates by disk diffusion method. ESBL production was confirmed using double-disk synergy test. Molecular detection of ESBL genes was performed using PCR. Biofilm formation assay was performed by microtiter plate method.

Findings: The most effective antibiotic against this bacterium was nitrofurantoin. Multidrug resistance was observed in 119 (85.6%) isolates. ESBL phenotype was detected in 93 (66.9%) isolates. The PCR test results showed that bla_{CTX}, bla_{VEB}, and bla_{TEM} were positive in 45 (32.4%), 87 (62.6%), and 10 (7.2%) isolates, respectively. The biofilm formation assay results revealed that 65 (46.8%), 58 (41.7%), 10 (7.2%), and six (4.3%) isolates were non-, weak, moderate, and strong biofilm producers, respectively.

Conclusions: The high prevalence of ESBL genes is a public health concern in this region because they could be transmitted to other susceptible bacteria and induce resistance. This study showed that biofilm production could increase antibiotic resistance.

Keywords: *Escherichia coli, ESBLs, Biofilm formation, Antibiotic resistant.*

CITATION LINKS

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Introduction

Urinary tract infection (UTI) is one of the most common infections usually caused by Gram-negative bacteria [1]. Various factors such as kidney stones, diabetes, and immune deficiency could increase the risk of this infection [2]. Approximately 150 million UTI cases are reported annually [3]. The most common cause of urinary tract infection is uropathogenic *Escherichia coli* (UPEC) [4]. *E. coli* strains are Gram-negative bacilli that live in the intestines of healthy individuals. *E. coli* has the ability to cause both community-acquired and hospital-acquired infections. These bacteria could cause diseases such as meningitis, bacteremia, urinary tract infections, and wound infections [5, 6]. In developing countries, some factors such as over-the-counter drug use and incorrect drug policies increase antibiotic resistance in bacteria [7]. On the other hand, factors such conjunct journey, horizontal gene transfer, and bacterial evolution increase the global burden of diseases caused by antibiotic-resistant pathogens. In addition, the acquisition of antibiotic resistance by pathogenic bacteria increases the virulence of these bacteria [8, 9]. The most important genetic factors involved in increasing resistance include: genetic mutations and mobile genetic elements such as plasmids, insertion sequences, and transposons. Currently, multidrug resistance to antimicrobial agents in nosocomial pathogens has become a major concern of the World Health Organization (WHO) [8]. Among the most important antibiotics used to treat these infections are beta-lactams. Unfortunately, resistance to these antibiotics has increased dramatically due to overuse of these antibiotics. ESBL (extended-spectrum beta-lactamase) production is one of the most common factors increasing resistance to this class of antibiotics. The most common type of ESBL is bla_{CTX-M}, and other important ESBL types are bla_{TEM} and bla_{SHV} [10]. These ESBL types belong to Ambler's class A / Bush's group 2be. This class induces resistance to penicillins, cephalosporins (except cephamycin), and monobactams [7]. Bla_{CTX-M} gene induces resistance to cefotaxime, and bla_{TEM} gene causes resistance to penicillin and first-generation cephalosporins [8, 11, 12]. Studies in Iran have shown that the prevalence of ESBL genes including bla_{TEM}, bla_{CTX-M}, and bla_{VEB} among clinical isolates of *E. coli* is about 51, 45, and 10%, respectively [8]. Several studies have demonstrated that the prevalence of ESBLs in developing countries such as East Africa (42%), Pakistan (40%), Israel (> 50%), and China (46%) is higher than in developed countries including German (4 to 12%) and US (4 to 12%) [14].

E. coli has the ability to produce biofilm, which is considered as an important virulence factor contributing to the stability and recurrence of the disease. Biofilm is a community of microbes that irreversibly adhere to a surface and secrete an extracellular matrix surrounding them [13]. *E. coli* biofilm is involved in several diseases caused by this organism and increases antibiotic resistance in this bacterium. Compared to planktonic bacteria, the bacteria in the biofilm exhibit a different behavior when exposed to antibiotics. Biofilms restrict antibiotic access to these bacteria, facilitate the transfer of resistance genes, and more efficiently pump efflux genes. As a result, the bacteria in the biofilm are 100 to 1000 times more resistant to antibiotics [14, 15].

Objectives: Considering the above mentioned points and the different causes of drug resistance in different geographical areas, this study aimed to investigate the prevalence of ESBLs in *E. coli* isolates. The results may contribute to our understanding of antibiotic resistance, ESBL genes involved in drug resistance in this geographical region, and also the role of biofilms in enhancing antibiotic resistance.

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Material and Methods

Patients and clinical specimens: The specimens used in this study were collected from Khorshid hospital in Isfahan during April 2018 to March 2019. After collection, accurate identification of organisms was done by routine culture and biochemical tests in the microbiology laboratory of the Faculty of Medical Sciences [16]. After accurate identification, the isolates were stored at -80 °C. This study was evaluated and approved by the Ethics Committee of Isfahan University of Medical Sciences.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was performed for the isolates against antibiotics discs (MAST, UK and Liofilchem, Italy) including tetracycline (30 µg), gentamicin (10 µg), ceftriaxone (10 µg), cefotaxime (10 µg), nitrofurantoin (10 µg), ciprofloxacin (5 µg), cefepime (30 µg), sulfamethoxazole (10 µg), amikacin (30 µg), and imipenem (10 µg) based on Clinical and Laboratory Standards Institute (CLSI) principles [17]. In this experiment, *E. coli* ATCC 25922 strain was used as the control.

ESBLs screening assay: Double disk synergy test was used for phenotypic identification of extended-spectrum beta-lactamases. Cefotaxime (10 µg) and cefotaxime-clavulanic acid antibiotic discs were placed at a distance of 24 mm on Mueller Hinton agar medium. After incubation at 35 °C for 18 hrs, the growth inhibition zone diameter was measured. If the difference in inhibition zone diameter between cefotaxime and cefotaxime-clavulanic acid antibiotic discs was more than 5 mm, it was considered as a broad-spectrum beta-lactamase producing strain [18].

Biofilm formation assay: Microtiter plate assay was carried out in triplicate to evaluate the biofilm formation ability of all assessed strains; the mean and standard deviation of all experiments were calculated. The isolates were incubated aerobically in a 96-well microtiter plate containing tryptic soy broth (TSB) and glucose at 37 °C for 24–18 hours. Then the supernatant was discarded, and the wells were washed with phosphate-buffered saline (PBS). Then the remaining attached bacteria were fixed with 300 µL of ethanol. The OD (optical density) values of the isolates coating the walls of the wells were measured using an ELISA reader after staining with crystal violet. At the end, the isolates were classified as non- (non-adherent), weak (weakly adherent), and strong (strongly adherent) biofilm producers according to the observed OD values [14].

DNA extraction and PCR: DNA of all samples was extracted by phenol chloroform method and used to evaluate resistance genes. PCR test was performed for *uspA*,

Table 1) List of primers, expected amplicon sizes, and annealing temperatures

Target gene	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)	References
uspA	*uspA-F* CCGATACGCTGCCAATCAGT *uspA-R* ACGCAGACCGTAGGCCAGAT	884	52	[19]
bla_{TEM}	*TEM*–F TTTCGTGTCGCCCTTATTCC *TEM*–R ATCGTTGTCAGAAGTAAGTTGG	403	58	[20]
bla_{CTX}	*CTX*-F CGCTGTTGTTAGGAAGTGTG *CTX*-R GGCTGGGTGAAGTAAGTGAC	569	55	[21]
bla_{VEB}	*Veb*-F CGACTTCCATTTCCCGATGC *Veb*-R GGACTCTGCAACAAATACGC	585	54	[22]

[DOI: 10.52547/iem.8.3.223]

Table 2) Demographic information and risk factors in hospitalized patients

Risk factor	Frequency(percent)
women	53(60.9)
men	34(39.1)
History of hospitalization	21(24.1)
The use of antibiotics in the last 3 months	32(36.8)
Use catheter now	12(13.8)
Use catheter in the past	14(16.2)
Surgical history	19(21.9)
History of urinary tract infection	31(35.6)
History of prostate inflammation	12(13.8)

Table 3) Antibiotic susceptibility of *E. coli* isolates (n = 139)

Antibiotics	Resistant (n %)	Intermediate (n %)	Susceptible (n %)
Tetracycline	111(79.9)	0(0)	28(20.1)
Gentamicin	50(36)	6(4.3)	83(59.7)
Ceftriaxone	95(68.3)	5(3.6)	39(28.01)
Cefotaxime	94(67.6)	0(0)	45(32.04)
Nitrofurantoin	15(10.8)	2(1.4)	122(87.8)
Ciprofloxacin	81(58.3)	1(0.7)	57(41.5)
Cefepime	54(38.8)	31(22.3)	54(38.8)
Sulfomethoxazole	117(84.2)	0(0)	22(15.8)
Amikacin	18(12.9)	11(7.9)	110(79.1)
Imipenem	42(30.2)	34(24.5)	63(45.3)

bla_{TEM}, bla_{CTX}, and bla_{VEB} genes. The names of the genes examined and the primers used for this purpose are listed in Table 1. PCR test was performed in a total volume of 25 µL using Master Mix Amplicon (Denmark). PCR amplification was performed in a thermocycler under the following thermal cycling conditions: an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of DNA denaturation at 94 °C for 1 min, specific annealing temperature for each primer for 1 min (Table 1), extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on 1% agarose gel.

Statistical Analysis: Statistical analysis was performed using IBM SPSS Statistics software Version 25.0 (IBM Corp., USA). The association between genes involved in biofilm formation and also the amount of biofilm formation by *E. coli* isolates with different antibiotic resistance phenotypes were evaluated by Chi-square and Fisher's exact tests. The analysis was performed with a confidence level of 95%. *P* values < .05 were considered statistically significant.

Findings

During the study period, 139 *E. coli* strains were isolated from urine samples (Figure 1). Among which, 97 (69.8%) cases were isolated from females, and 42 (30.2%) cases were isolated from males. Also, 87 (62.6%) strains were collected from inpatients, and

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52 (37.4%) isolates were collected from outpatients. The frequency distribution of demographic information about hospitalized patients is shown in Table 2. The antibiotic resistance test results are shown in Table 3. According to the results, the most effective antibiotic against this bacterium was nitrofurantoin, followed by amikacin and gentamicin, respectively. Sulfamethoxazole and tetracycline had the least effects on these strains. According to the results, 119 (85.6%) isolates were MDR (multidrug-resistant).

All the isolates were evaluated for the presence of ESBLs by phenotypic methods. According to the results, 93 (66.9%) isolates showed a positive phenotype using the DDST (double disk synergy test) method.

A total of 74 (53.2%) isolates were able to produce biofilms. Of these 74 isolates, 58 (53.2%) isolates were weak biofilm producers, 10 (13.5%) isolates were intermediate biofilm producers, and six (8.1%) isolates were strong biofilm producers.

The *uspA* gene was recognized in all the isolates. All 139 isolates were tested for ESBLs genotype by PCR (polymerase chain reaction) assay. According to the results, bla_{CTX}, bla_{VEB}, and bla_{TEM} were positive in 45 (32.4%), 87 (62.6%), and 10 (7.2%) isolates, respectively (Figure 2-4). All ESBL-producing isolates had ESBL genes. All three ESBL genes were observed in nine isolates, and one isolate harbored both bla_{TEM} and bla_{VEB} genes, and 30 isolates harbored both bla_{TEM} and bla_{CTX}. Also, eight isolates had extended-spectrum beta-lactamase genes that were not expressed in phenotypic experiments.

Statistical analysis indicated a significant correlation between biofilm production and resistance to tetracycline, ciprofloxacin, and imipenem ($p< .05$). There was also a significant correlation between the presence of extended-spectrum beta-lactamases and bla_{CTX} and bla_{TEM} ($p< .001$).

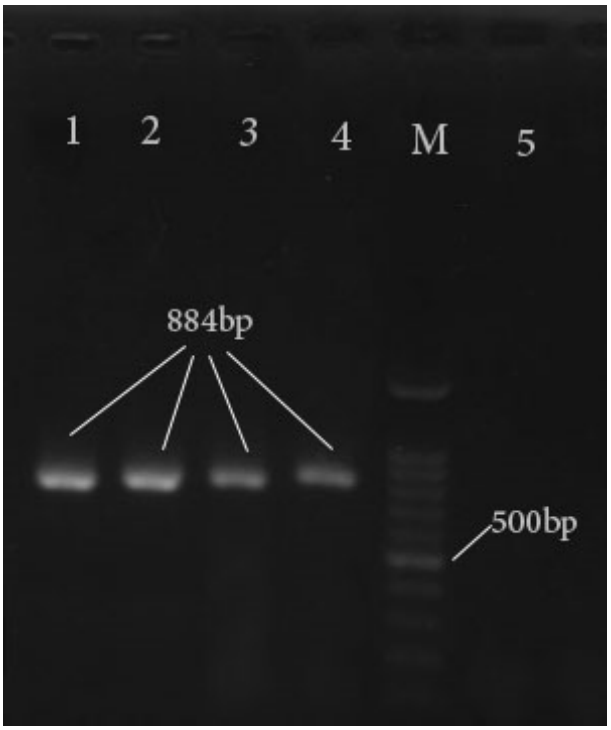

Figure 1) PCR product electrophoresis of *P. E. coli uspA* gene in agarose gel
Bands 1 - 3 of *E. coli* clinical specimens, Band M Ladder 100bp, band 4 positive control, band 5 negative control

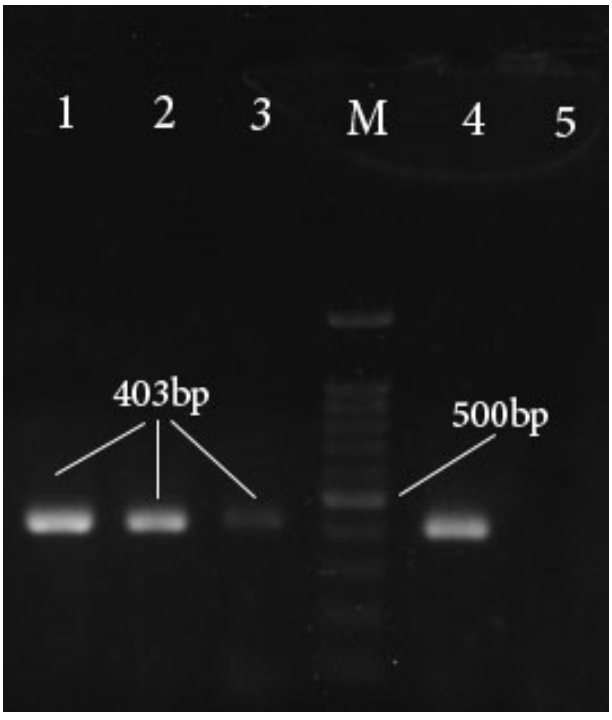

Figure 2) PCR product electrophoresis of *P. E. coli* bla_{TEM} gene in agarose gel
Bands 1 - 3 of *E. coli* clinical specimens, Band M Ladder 100bp, band 4 positive control, band 5 negative control

[DOI: 10.52547/iem.8.3.223]

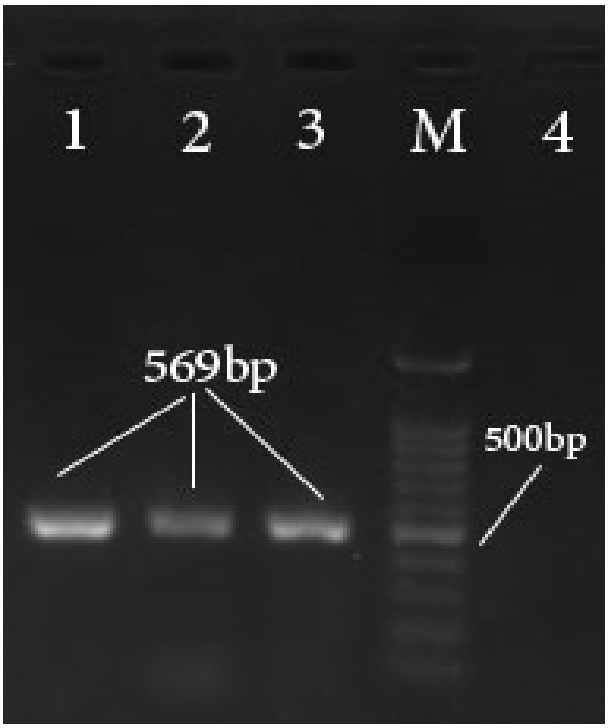

Figure 3) PCR product electrophoresis of *P. E. coli* bla_{CTX} gene in agarose gel
Bands 1 and 2 of *E. coli* clinical specimens, band 3 positive control, Band M Ladder 100bp, band 4 negative control

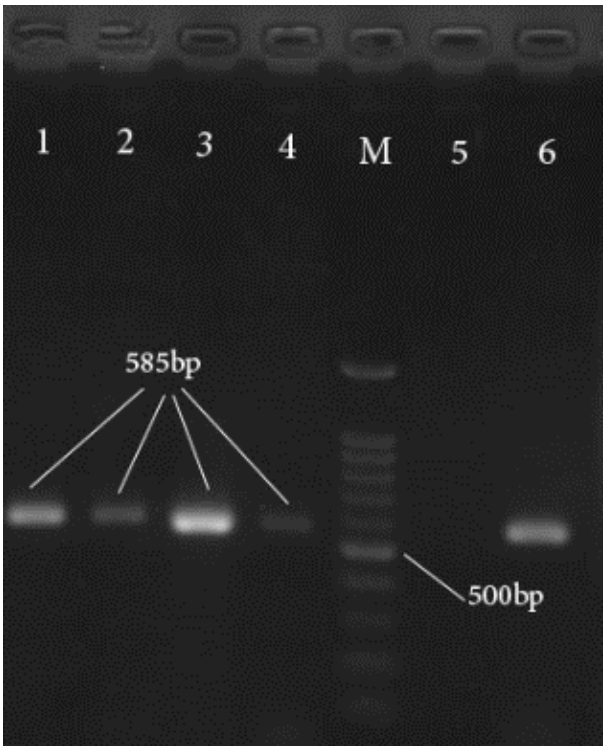

Figure 4) PCR product electrophoresis of *P. E. coli* bla_{VEB} gene in agarose gel
Bands 1 - 4 of *E. coli* clinical specimens, Band M Ladder 100bp, band 5 negative control, band 6 positive control

Discussion

Urinary tract infections are one of the major threats to global health due to widespread antibiotic resistance and high rates of recurrent infections [23]. *E. coli* is the most common cause of urinary tract infection in both inpatient and outpatient groups. If these types of infections are not treated, they infect higher areas and cause cysts, pyelonephritis, and finally kidney disease [24]. It is estimated that uropathogenic *E. coli* is responsible for 70 to 90% of urinary tract infections. Antibiotic resistance of these organisms is different in different geographical areas. In most cases, the long-term persistence of these organisms in different geographical conditions is due to the production of biofilm. On the other hand, biofilm production significantly increases antibiotic resistance in these bacteria [25]. The rate of resistance to common antibiotics is increasing, and this is due to the global spread of multidrug-resistant organisms [26]. This study results showed that 85.6% of the isolates were MDR, which is higher than the mean prevalence of MDR in Iran. This result is consistent with the results reported by Sharif and colleagues (2013) in Kashan and Fallah et al. (2012) in Tehran, showing high levels of MDR among the isolates [27, 28]. Ibrahim and colleagues (2012) in Saudi Arabia showed that the prevalence of MDR among UPEC isolates was 74%, which is similar to this study result [29]. One of the most important factors increasing resistance to beta-lactam antibiotics such as broad-spectrum cephalosporins is the presence of beta-lactamase genes. In recent years, ESBL-producing bacteria have caused many problems in the field of health, highlighting the need for new techniques to identify these bacteria in hospitals. A phenotypic method is a useful technique to differentiate between isolates producing ESBLs and isolates using other mechanisms for resistance to beta-lactams [30, 31]. In this study, the frequency of

[DOI: 10.52547/iem.8.3.223]

ESBLs based on phenotypic and genotypic methods was determined to be 66.9 and 73.4%, respectively. The higher prevalence rate obtained using the genotypic method could be due to the higher sensitivity of this method compared to the phenotypic method. Another reason is the presence of isolates which possess these genes but do not express them. In this study, the frequency of bla_{CTX}, bla_{VEB}, and bla_{TEM} was 32.4, 62.6, and 7.2%, respectively. In a study by Komijani and colleagues (2017), the prevalence of bla_{CTX} and bla_{TEM} was 38.4 and 22%, the prevalence of the bla_{CTX} gene is approximately similar to the present study result [5]. Farshad and colleagues (2008) conducted a study in Shiraz and reported a prevalence of 28, 8, and 49% for bla_{CTX}, bla_{VEB}, and bla_{TEM}, respectively, which are similar to this study results [32]. The frequency of bla_{TEM}*gene* is similar to our study result, while the frequency of bla_{CTX} is higher than that reported in a study by Rodríguez-Baño et al. (2009) in Spanish [33]. According to the results, it was found that the frequency of these genes is different in different regions of Iran and other parts of the world. Biofilm formation is an important virulence factor contributing to bacterial colonization and resistance to antibiotics. According to the present study results, 53.2% of the isolates were able to produce biofilm, and a significant relationship was found between biofilm formation and resistance to tetracycline, ciprofloxacin, and imipenem. Risal et al. (2018) in Nepal and Hassan et al. (2011) in Pakistan have also reported a prevalence of 64% for biofilm producing isolates, which is close to this study result [34, 35]. Risal et al. (2018) [34] showed that among biofilm-producing isolates, the highest resistance was observed to cefotaxime, ceftriaxone, and amoxicillin, which is inconsistent with the present study result. In another study conducted by Tajbakhsh et al. (2016) in Iran, the highest resistance in biofilm-producing isolates was observed against ampicillin and tetracycline, which is somewhat consistent with our study result [36].

In conclusion, urinary tract infections are one of the most important infections affecting both inpatients and outpatients. The present study evaluated the risk factors, antibiotic resistance, ESBL production, as well as biofilm production in *E. coli* strains in Isfahan. The high prevalence of ESBL genes in these isolates is considered as a public health concern in this region because they could be transmitted to other susceptible bacteria and induce resistance. Finally, this study results showed that biofilm production could increase antibiotic resistance.

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Ethical Permissions: Approval to conduct the study was obtained from the Research Ethics Committee of Isfahan University of Medical Sciences (ID- number: IR.MUI. RESEA RCH.REC.1397.114).

Conflict of interest: The authors declare that there is no conflict of interest.

Authors Contribution: Conceptualization: data curation: EH; formal analysis: EH, AR;funding acquisition: EH; investigation: EH AR; methodology: EH, AR; project administration: AR; resources:AR; software: EH; supervision: AR; writing of the original draft: EH, AR; writingreview and editing: EH, AR.

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