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Detecting of beta-lactamase genes in clinical isolates of *Escherichia coli* isolated from Baqiyatallah Hospital of Bashagard and evaluating their antibiotic resistance and biofilm formation

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Abstract---Biofilm formation has been observed in different species of bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. The

formation of biofilms in the airways of pneumonia patients and chronic lung patients is one of the important factors in prolonging the treatment period, exacerbating clinical symptoms and even death of patients. The present study aimed to investigate beta-lactamase genes in clinical isolates of *Escherichia coli* isolated from Baqiyatallah Bashagard Hospital and to evaluate their antibiotic resistance and biofilm formation. In the present study, 60 isolates from fecal samples of patients with symptoms of diarrhea and suspected *Escherichia coli* infection were collected and selected from Baqiyatallah Hospital of Bashagard. After confirmation of *Escherichia coli* strains using microbial culture and biochemical tests, the presence of three beta-lactamase genes in these isolates was examined. The ability of isolates to form biofilms and their resistance to two different antibiotics were also measured. A phenotypic study of isolates based on optical absorbance measurements showed that among the clinical *Escherichia coli* isolated from Baqiyatallah Hospital of Bashagard, only 10 isolates could form a strong biofilm in the microtiter plate. Most of the samples were resistant to the antibiotic penicillin, and unlike ciprofloxacin, penicillin could not remove the biofilm. Out of 60 samples collected, 41 samples had CTX beta-lactamase (68.3%) and 28 samples had TEM beta-lactamase (46.6%) and SHV beta-lactamase of no sample was positive. The results of present study emphasize the use of adequate doses of antibiotics in the treatment of biofilm infections. The ability to form biofilms plays a major role in the virulence of this bacterium. Thus, rapid detection of isolates that can form biofilm is essential for treatment and management decisions.

Keywords---*Escherichia coli*, biofilm, antibiotic resistance, gastrointestinal infection.

Introduction

Escherichia coli are the most common gram-negative bacillus isolated from patients with sepsis. It is the cause of a significant percentage of urinary tract infections and nosocomial infections, and the leading cause of gastroenteritis in developing countries. Most infections caused by *Escherichia coli* have an internal origin, except for neonatal meningitis and gastroenteritis. It means that *Escherichia coli*, which are a part of the natural microbial flora of the human body, can cause disease when the immune system is weakened. *Escherichia coli* opportunistically cause various diseases and complications such as sepsis, wound infection, urinary tract infection, pneumonia, meningitis, gastroenteritis and sepsis in humans and animals (Knight et al., 2017).

Uropathogenic *Escherichia coli* strains account for 80% of these urinary tract infections (Schuppe et al., 2017). Some strains, especially acute strains, cause urinary tract infections (Talaro & Chess, 2018). Among all bacterial and pathogenic agents in outpatients and hospitalized patients, *Escherichia coli*, gram-negative bacilli, and normal intestinal flora are the most common organisms, isolated in about 75 to 90% of cases of urinary tract infections

(Katongole et al., 2020). Also, an increase in drug resistance in recent years has caused many problems in the treatment of infections such as genitourinary tract infections. Due to importance of timely diagnosis and effective antibiotic treatment, the use of a sensitive and accurate method to identify the resistance pattern of this bacterium in any geographical area can be a valuable guide for experimental treatment (Bryce et al., 2016).

Since the role of *Escherichia coli* as a major and common cause of many infections at all ages is well known, knowing its pattern of susceptibility to various antibiotics is crucial. The susceptibility level of bacteria isolated from patients to antibiotics varies in different areas. Differences in the pattern of antibiotic susceptibility in different parts of the world might be due to differences in the amount and type of antibiotics used in each area (Ahmed, 2019). Thus, it should be noted that the use of antibiotics in each area should be based on the pattern of antibiotic susceptibility in that area, since 20-50% of *Escherichia coli* strains in developed countries are currently resistant to first-line antibiotics (Petty et al., 2014). Treatment of urinary tract infections is performed experimentally before obtaining laboratory results. Excessive and uncontrolled use of antibiotics has caused resistance to bacterial infections, so that antibiotic resistance in uropathogenic *Escherichia coli* strains in the world is increasing and has caused many problems. To control the increase in the prevalence of resistance to antimicrobial agents, the selection of an appropriate antibiotic based on accurate data seems to be necessary (Drobniewski et al., 2015).

Beta-lactamase enzymes are the most important factor in resistance to Beta-lactam family antibiotics among gram-negative bacteria. Beta-lactamase genes in bacteria, especially extended-spectrum β -lactamases (ESBLs), are one of the most effective factors in increasing resistance to beta-lactam antibiotics, including extended-spectrum cephalosporins. Organisms that carry these genes increase morbidity and mortality among people and the continued growing trend in the development of such resistance may cause a serious threat to society. Nowadays, the number of infections caused by them is increasing around the world and it is one of the emerging health problems at the global level (Abdallah et al., 2015). Identification of *Escherichia coli* isolates requires the isolation of these organisms from non-pathogenic isolates (normal intestinal flora). It is done by molecular detection, especially PCR. Considering the phylogenetic background, most studies show isolates causing extra-gastrointestinal infections such as urinary tract infections, neonatal septicemia and meningitis, mostly in phylogenetic group B2 and to a lesser extent in group D (Massot et al., 2016).

Bacteria are also found in nature in both planktonic and biofilm forms. Bacterial biofilms are complex accumulations of bacteria enclosed in a glycocalyx coating that adhere to mucosal surfaces. Biofilm formation has been observed in various bacterial species, including *Escherichia coli* and *Pseudomonas aeruginosa*. Biofilm infections rarely recover, even in healthy people with adequate immune systems, and the surrounding tissue of the biofilm is damaged by the immune response, and the infection will persist until surgery or removal by an external device. Also, biofilms are observed in infections of foreign bodies such as prosthetic heart valves, teeth, prosthetic joints, and urinary catheters. The most important distinguishing characteristic of biofilms is the difference in their

growth, which results in drug resistance and the need for different treatments and different methods of biofilm identification (Madigan et al., 2017). In a study conducted by Alirezai et al. the ability of biofilm formation by *Escherichia coli* and *Staphylococcus aureus* and the effect of some antibiotics and industrial biocides used in Iran to control the biofilm of these bacteria were examined. The results revealed an increase in the resistance of double-species biofilm compared to single-species biofilm caused by each of these two bacteria, which was probably due to better cooperation of these two bacteria in the biofilm against stress caused by antimicrobial agents (Alirezai et al. 2015).

Due to many health problems and the extensive economic losses caused by *Escherichia coli* in Iran due to the failure of human resources, it seems that research in this regard can be useful in controlling and preventing the disease. Antibiotic resistances are formed during treatment and most of them performed traditionally yield negative result and are not isolated using conventional methods. It also shows a high prevalence of genes that produce antibiotic resistance and relatively high phenotypic expression of the genes that produce the genes, and they should be detected using specific methods to control the disease and infections. Molecular epidemiological analysis of drug-resistant strains is crucial. Rapid detection of isolates with antibiotic resistance capability is essential to make treatment and management decisions.

Materials and Methods

In the present study, 60 isolates of fecal samples of patients with symptoms of diarrhea and suspected *Escherichia coli* infection were collected and selected from selected from Baqiyatallah Hospital of Bashagard. The samples were transferred to a microbiology laboratory in a sterile container with a special lid at 4 ° C. In the laboratory, to prepare the samples, fecal samples were homogenized with Phosphate-buffered saline. The solid particles in the feces were then precipitated using a centrifuge at 3000 rpm for 5 minutes and the upper liquid was stored in another sterile container at 4 ° C for the next experiment. Samples were cultured on MacConkey agar, Xylose Lysine Deoxycholate agar (XLD), *Salmonella shigella* agar (SSA), Thiosulfate Citrate Bile Salts Sucrose (TCBS) and *Escherichia coli* were incubated for 24 hours at 37 ° C for isolation. Red colonies on MacConkey were identified as *Escherichia coli*. After one day of incubation at 37 ° C, colonies suspected of *Escherichia coli* were transferred to agarose medium. If negative, it was transferred to biochemical media such as TSI, Simon Citrate, MR-VP and SIM medium for final approval, and finally, DNA extraction and PCR were performed for 60 samples for molecular identification.

Samples were cultured on Blood Agar, MacConkey Agar, EMB, ECC and *E.coli* Chrome Agar media to confirm the presence of *Escherichia coli* and bacterial isolation. Detection of *Escherichia coli* by microbiological methods including catalase and oxidase tests and the use of differential media such as TSI, SIM, MRVP, simon citrate, urea agar, nitrate agar as well as additional biochemical tests such as fermentation of sugars and the use of amino acids were performed according to the microbiological table. Accordingly, lactose-positive colonies were studied on MacConkey culture medium, lactose-positive colonies on EMB medium with metal polish and blue colonies on ECC medium and *E.coli* chrome Agar

medium. Then, the isolated strains were stored in TSA medium at 4 ° C until the experiment. To extract DNA, the kit of the National Center for Genetic and Biological Resources of Iran was used. The PCR program for this research was determined and performed according to similar articles after optimization. The total PCR time was also determined according to the number of cycles. DNA fragments extracted from clinical specimens were used as template DNA. The DNA used in PCR should be as pure as possible and its RNA and protein should be removed. In this study, the amount of template DNA for each sample tube is 3 µl. In primer design, SINCE 3 genes were examined in this study, 6 primers were added in each of the sample tubes and 0.5 µl was added from each primer. Several and valid articles were used to select and obtain the required specific primers. The sequences of specific primer pairs used are listed in Table 1.

Table 1
Sequence of primers used

Primer name	Sequence	TM in °C	Amplicon size in bp
bla-SHV.SE	ATGCGTTATATTCGCCTGTG	45	747
bla-SHV.AS	TGCTTTGTTATTCGGGCCAA	45	
TEM-164.SE	TCGCCGCATACACTATTCTCAGAATGA	53	445
TEM-165.AS	ACGCTCACCGGCTCCAGATTAT	52	
CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	54	593
CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG	58	

High concentrations of agarose gel are used to separate short-length DNAs and low concentrations of agarose are used to separate long-length DNAs. In this study, 1% gel was used. After the gel was removed from the electrophoresis tank, it was transferred to the ethidium bromide tank for a few minutes.

Biofilm assay method in optical absorbance changes

This method is performed using plates with polystyrene wells. Then, the ability of *Escherichia coli* isolates in forming the biofilms was evaluated in the laboratory using the method reported by Peters et al. in 2008 with slight changes. The ability to form optical absorbance was recorded in 4 hours.

OD = Optical absorbance rate

OCD = Negative control optical absorbance rate

OD < OCD = lack of binding

ODC × 4 < OD < ODC × 2 = moderate

ODC × 2 < OD < ODC = weak

ODC < OD × 4 = strong

Finally, the percentage of biofilm reduction due to antibiotics was obtained through optical absorbance of the treated well, blank and control according to Formula 1.

$$\text{Percentage reduction} = [(C-B)-(T-B)] \times 100$$

Formula 1

C = Mean optical absorbance of control wells

B = Mean optical absorbance of blank wells

T = Mean optical absorbance of treated wells

Results

Phenotypic study of isolates based on optical absorbance measurement showed that among the clinical *Escherichia coli* isolated from Baqiyatallah Hospital of Bashagard, only 10 isolates could form a strong biofilm in plate microtiter. The results of the effect of the antibiotic penicillin on biofilm formation are presented in Table 2 and the effect of ciprofloxacin is presented in Table 3.

Table 2
Results of the effect of penicillin antibiotic on the optical absorbance of *Escherichia coli* biofilm isolated and obtained by plate microtiter and ELISA

Mean optical absorbance (OD)	Formation of biofilm in microtiter plate				Penicillin mean MICml / gı biofilm 0.25 ≤ susceptible Semi-susceptible 0.05 0.25 ≥ resistant	n	Genus and species	source
	strong	moderate	weak	negative				
32.0	10	6	4	-	63.0	20	<i>Escherichia coli</i> <i>Escherichia coli</i>	clinical
29.0	1	-	-	-	-	1	Positive control without antibiotic effect	ATCC 25923

Table 3
Results of beta-lactamase gene detection

Mean optical absorbance (OD)	Formation of biofilm in microtiter plate				Ciprofloxacin mean MICml/gı biofilm 1 ≤ susceptible(μg/mg)	n	Genus and species	source
	strong	moderate	weak	negative				

						Semi-susceptible 2 4 $\geq$ susceptible		
26.0	3	6	11	-	9.1	20	Escherichi a coli	clinical
29.0	1	-	-	-	-	1	Escherichi a coli Positive control without antibiotic effect	ATCC 25923

The presence of three beta-lactamase genes CTX, TEM and SHV was examined by PCR in isolates isolated from Baqiyatallah Hospital of Bashagard, the results of which are shown in Table 4 and Figures 1 to 5.

Table 4
PCR results of CTX and TEM beta-lactamases

beta-lactamase gene	Positive number	%
Bla CTX	41	68.3
Bla TEM	28	46.6
Bla SHV	0	0

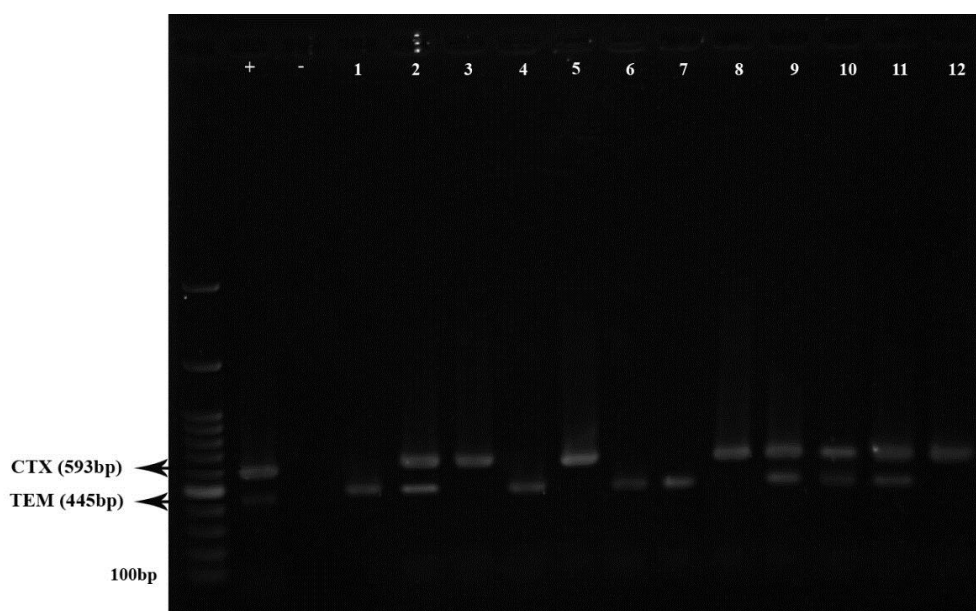


Figure 1. M-PCR test results from left to right: Ladder 100 bp - Positive control - Negative control - positive samples of bla CTX12, 11, 10, 9, 8, 5, 3 and 2; positive samples of bla TEM 11, 10, 9, 7, 6, 4, 2 and 1

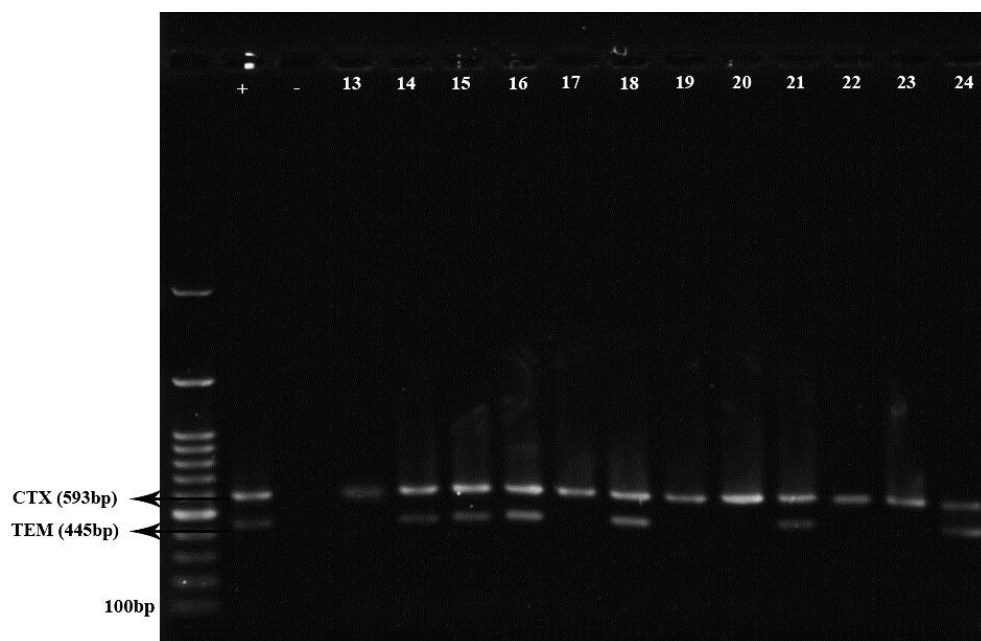


Figure 2. M-PCR test results from left to light: Ladder 100 bp - positive control - negative control - positive bla CTX24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14 and 13 samples; positive samples of TEM 24, 21, 18, 16, 15 and 14

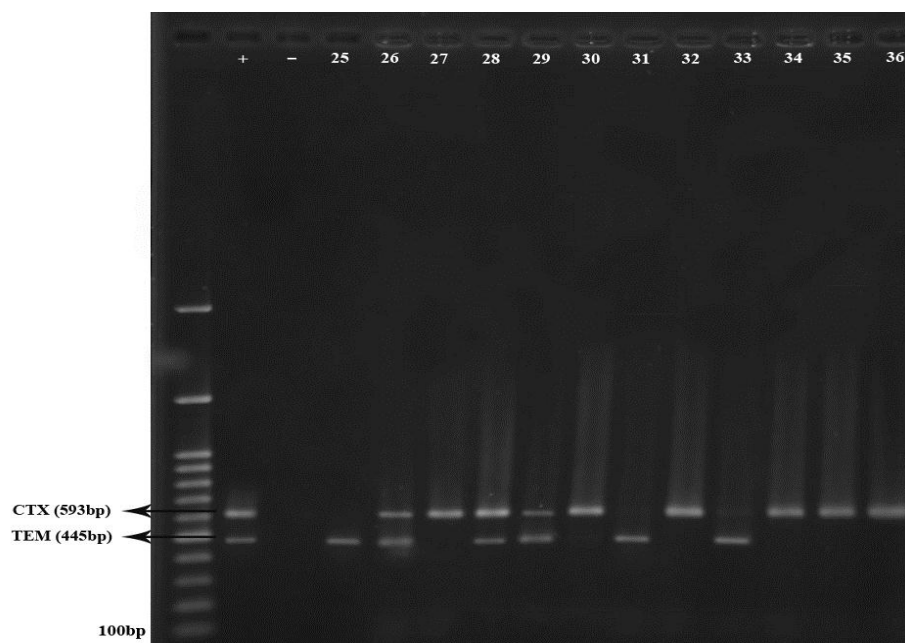


Figure 3. M-PCR test results from left to right: Ladder 100 bp - positive control - negative control - positive samples of bla CTX36, 35, 34, 32, 30, 29, 28, 27 and 26; positive samples of bla TEM33, 31, 29, 28, 26 and 25

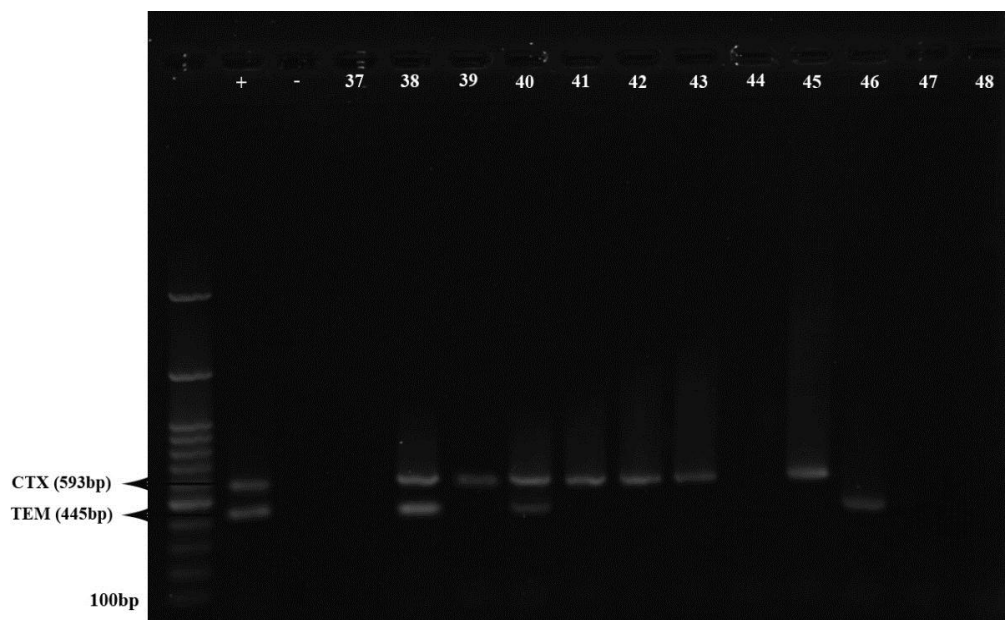


Figure 4. M-PCR test results from left to right: ladder bp 100 - positive control - negative control - samples of bla CTX 45, 43, 42, 41, 40, 39 and 38 and positive Samples of TEM 46, 40 and 38

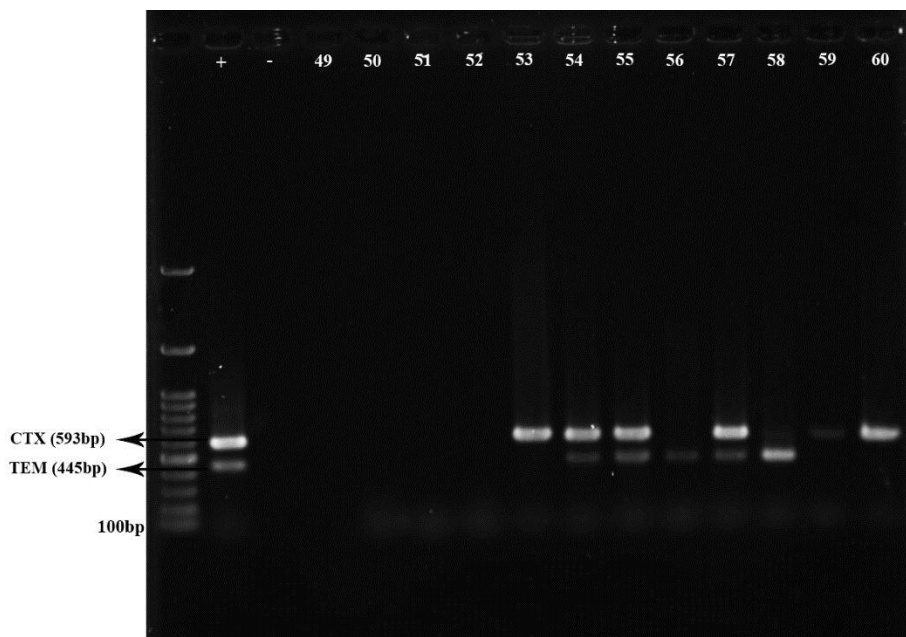


Figure 5. M-PCR test results from left to right: ladder 100 bp - positive control - negative control - positive samples of CT blax 60, 57, 55, 54 and 53, positive samples of TEM 58, 57, 56, 55 and 54

Discussion

In the present study, 60 fecal samples with symptoms of diarrhea and suspected colibacillosis were selected and transferred to a microbiology laboratory in a sterile container with a special lid at 4 ° C. After culture and biochemical tests by specific primers, Multiplex PCR was examined. Multiplex PCR method can amplify and identify several genes simultaneously due to the use of multiple primers. Saving on required cases of PCR and specificity and susceptibility by reducing the time of genetic testing and required templates with a few pairs of primers in general and specific and with high reliability and speed are used for molecular identification of *Escherichia coli* pathogens and beta-lactamase genes. As a result, out of 60 samples collected, 41 samples had CTX beta-lactamase (68.3%) and 28 samples had TEM beta-lactamase (46.6%) and SHV beta-lactamase was not positive in any of the samples. In one study, 100 *Escherichia coli* bacteria were isolated from patients with colibacillosis in Ilam province and confirmed using biochemical characteristics. Isolates were analyzed for the presence of beta-lactamase genes (blaTEM and blaSHV) by PCR. Out of 100 isolates, ten isolates (10%) had blaTEM gene, which is much lower than 68% of the results obtained in our study, and two isolates (2%) had blaSHV gene, which was not detected in the present study, and 2 isolates (2%) were positive for both blaTEM and blaSHV genes.

Various in vitro and in vivo studies have shown that *Escherichia coli* can adhere to and penetrate epithelial cells. Biofilms are structures made up of bacterial cells inside a polymer matrix that are made by the bacteria themselves and cause them to adhere to living or non-living surfaces. Biofilm formation has significant health and economic consequences. It is estimated that 65% of nosocomial infections in the United States are associated with biofilm formation, and the economic loss caused by biofilm is more than \$ 1 billion annually. Many bacteria grow in the body in a biofilm phase. According to the histopathological signs and extra-structural appearance of the bacteria inside the tissue, it is estimated that the biofilm is present in important diseases such as pneumonia, liver abscess, enteritis and ulcerative infections. The role of biofilm in most chronic and resistant infections has been proven.

Escherichia coli infections are known as long-term and resistant infections among other infections. Biofilm formation consists of two stages. The first stage is adhering of cells to a surface that is facilitated by cell wall binding factors. The second stage involves the proliferation of cells and the formation of an adult structure composed of a large number of different cellular layers that are adhered by polysaccharide intercellular adhesins (PIA). The ica locus is composed of the icaA, icaB, icaC, and icaD genes, which synthesize PIA-mediated proteins. Among the icaA and icaD locus genes, it plays a greater role in biofilm formation. icaA encodes N-acetylglucosaminyl transferase. IcaD has been reported to play a critical role in maximal expression of N-acetylglucosaminyl transferase and leads to phenotypic expression of capsular polysaccharides. The relationship between biofilm formation and related virulence factors varies widely and depends on the ability of binding factors and the origin of the isolates. Since virulence factors have a proven role in biofilm formation, they also play a major role in non-

treatment of disease by antibiotics and the inconsistency of antibiotic test results and treatment results.

Bacterial virulence is related to the ability to produce toxins and extracellular factors such as biofilm, which give the bacterium its ability to adhere and resist phagocytosis. The ability to form biofilms helps bacteria survive in the host environment. Nowadays, biofilms have been suggested as one of the causes of chronic *Escherichia coli* infections. *Escherichia coli* are known among other bacteria for producing a glazed layer. In a research conducted by Hussain et al., the effects of different environmental conditions, including the density of oxygen gas (air) and carbon dioxide and the composition of the culture on biofilm formation were examined.

The results revealed that the amount of glaze (external polysaccharide) isolated from a bacterium cultured in a synthetic medium with a specific chemical composition (HHW) in the presence of air relative to the amount of glaze produced in both HHW medium and synthetic dialysis fluid medium in the presence of air with CO₂ 5% was negligible. The presence of a physiological level of CO₂ during culture in TSB medium prevented the formation of glaze. Biofilm formation was preferred in SDF and HHW but was weakened in TSB and peritoneal dialysis fluid when air was used with CO₂ (Hussain et al., 2014). The majority of cells in a biofilm are not necessarily resistant to planktonic cells, and when treated with a lethal antibiotic that can kill slow-growing cells, they die rapidly and resistant cells survive, and they remain by the presence of an antibiotic that inhibits their growth. Another research revealed that the resistance of *Staphylococcus aureus* bacteria in ruptured biofilms was equal to their resistance in healthy biofilms at MIC concentrations of antibiotics.

At higher concentrations, the bacteria in ruptured biofilms showed significantly less resistance than those in healthy biofilms ($P < 0.001$), but more resistant to planktonic cells. Quinupristin / dalfopristin is the best activity against ruptured biofilm cells at high concentrations of MICS and vancomycin at 500 and 1000 ug / ml was significantly more active against methicillin-resistant and methicillin-susceptible biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis*. By examining the effects of different polymers on the CNS, Pasquel and Lopezia concluded that these bacteria could metabolize biomaterials in the absence of nutrients and remain on their surface. A research conducted by Pasquel in November 2002 on bacterial adhesion and biofilm formation on the surface of PVC, latex and Teflon silicone catheters concluded that the results of this study were consistent with those of a study conducted by Pasquel. In a study conducted by Toosi et al., they examined the ability of biofilm formation in *Escherichia coli* isolated from livestock around Tehran.

A total of 90 *Escherichia coli* isolates were prepared from raw milk of 5 livestock around Tehran. All isolates were detected according to standard NMC methods and *Escherichia coli* isolates were confirmed by polymerase chain reaction (PCR). Phenotypic examination of biofilm formation was performed using microplate titration method. All isolates were evaluated in terms of presence of *icaA* and *icaD* genes by polymerase chain reaction. Phenotypic examination of biofilm formation using titration microplate method showed that 4.4, 3, 43.40% of the isolates,

respectively, could form strong, medium or weak biofilm and only 12.2% of the isolates could not form biofilms in the laboratory. Also, 87.7% (79.90) of isolates had *icaA* and *icaD* genes separately and 85.5% (77.90) had both genes simultaneously. The results of the research show a high prevalence of biofilm-producing genes, a relatively high phenotypic expression of biofilm-producing genes and a relatively high phenotypic expression in livestock around Tehran.

In the present study, out of total number of clinical samples exposed to the antibiotic penicillin for the formation of antibiotic biofilm with a mean MIC of 0.32 µg / µl, 10 samples of strong biofilm, 6 moderate strains and 4 weak strains were obtained. In contrast, in the biofilm formed with ciprofloxacin antibiotic, out of total of clinical samples exposed to antibiotics for the formation of antibiotic biofilm with a mean MIC of 0.26 µg / µl, 3 samples of strong biofilm and 6 moderate strains and 11 weak strains were obtained. It suggests that most of the samples were resistant to the antibiotic penicillin and penicillin could not eliminate the biofilm against the antibiotic ciprofloxacin, so a stronger biofilm related to antibiotic penicillin is created.

Conclusion

The results of present study are in line with indigenous strains and the results of similar studies on other strains and emphasize the use of adequate doses of antibiotics in the treatment of biofilm infections. The ability to form biofilms plays a major role in bacterial virulence. Thus, rapid detection of isolates that can form biofilm is essential for treatment and management decisions.

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