

Evaluating the Macrolide Resistance of Escherichia Coli Isolated from the Urinary Infection and Determining the Phylogeny Using the ERIC-PCR Method

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Abstract

Resistance to antibiotics among pathogen bacteria is a global concern nowadays. Considering that Escherichia coli (E. coli) is the most important urinary infection and nosocomial infection pathogen, the present study aimed to evaluate the macrolide resistance of E. coli isolated from urinary infection and determine the phylogeny using the ERIC-PCR method. The sampling was performed from patients admitted to Sanandaj Hospitals during 2017. Upon culturing urine samples, the E. coli strains were identified by biochemical tests. Then, the antibiotic sensitivity of the strains was performed using the disk diffusion test. Determining the erythromycin MIC using E-test was performed for species resistant to erythromycin. The bacterial resistance genes were identified using PCR, and ultimately, the gene relationships of the strains were determined using ERIC-PCR. Ninety-nine E. coli strains were isolated from urine samples. The strain resistance to ceftriaxone, piperacillin, erythromycin, azithromycin, carbenicillin, ciprofloxacin, cefotaxime, and amikacin was 51.51%, 84.84%, 84.84%, 30.30%, 75.75%, 41.41%, 84.4%, and 42.42%, respectively. Among the selected 19 strains, which were resistant to azithromycin and erythromycin, 15 mphA-positive isolates (78.9%), ten mphB-positive isolates (52.63%), and ten isolates (52.63%) simultaneously had mphA and mphB. Also, according to ERIC-PCR, it was shown that the strains are genetically related, and the same genetic source was not observed between the strains. Considering the prevalence of macrolide-resistant E. coli infections, it suggested that these strains be identified in urinary infection patients. Moreover, treating these patients with macrolide is not recommended. Moreover, the same genetic source was not observed between the strains; therefore, the antibiotic resistance gene spreads in strains with different genetic sources. Thus, there is a need to prevent the overuse of antibiotics.

Keywords: E. coli, macrolide resistance, urinary infection, ERIC-PCR

Introduction

Escherichia coli is a gram-negative bacillus from the Enterobacteriaceae, which is abundantly found in warm-blooded animals' intestines. The bacteria are transmitted from person to person through the fecal-oral route. E. coli is a facultative anaerobe motile gram-negative bacillus, which does not produce spores. The bacterium produces a mixture of acids in anaerobic conditions, such as lactate, succinate, ethanol, acetate, and carbon dioxide. The bacterium's optimal growth is at 37 C; however, it could tolerate 49 C and continue its growth. Moreover, it can grow in aerobic and anaerobic conditions (1).

Urinary tract infection is one of the most common bacterial infections, the second most common

infection, and the main reason for referring to hospitals. Each year, 150 million people in America are inflicted with UTI, and 6 billion dollars are spent on the treatment. *E. coli*, gram-negative bacteria, and the intestine normal flora are the most commonly isolated organisms in 75-90% of UTI infections among all urinary tract pathogens (2). Considering that the role of *E. coli* is well determined as the major and common reason for urinary tract infection in all ages, it is important to know its sensitivity pattern to various antibiotics. The antibiotic sensitivity of bacteria isolated from patients in various regions is different. The difference in the antibiotic sensitivity in various regions could lead to the differences in the dosage and antibiotics in those regions (3). Therefore, it is necessary to note that using antibiotics in treating the urinary tract and other infections in each region should be based on the antibiotic sensitivity of the region.

Initial antibiotic treatment in UTI is usually performed empirically; therefore, accurate and up-to-date information on the pattern of antibiotic sensitivity of regional strains is essential. Drug resistance is increasingly increasing among urinary tract pathogens worldwide. The type of antibiotic choice for empirical treatment of UTI is currently contradicted, as 20-50% of *Escherichia coli* strains are now resistant to first-line antibiotics, even in developed countries (4). Determining antibiotic sensitivity will play an important role in the correct treatment of these patients and preventing the overuse of broad-spectrum antibiotics and thus preventing the increase in microbial resistance and reducing patients' costs. Also, the pattern of antibiotic resistance in each region should be studied regularly so that based on the results, a more appropriate drug can be used in the treatment of patients, and in case of a change in the pattern of resistance, appropriate decisions should be made to prevent the increase of resistance (5).

Many antibiotics inhibit protein synthesis. Understanding how the bacteria become resistant to antibiotics is important for designing effective and new treatment regimens. Macrolides have been in the center of attention for decades as a drug with appropriate function and immunity in treating gram-positive coccus. Generally, macrolides are not competent against Enterobacteriaceae (6). Like most gram-negative organisms, the Enterobacteriaceae family is inherently resistant to low erythromycin A levels, probably due to the outlet pump mechanism. Macrolides contain a 14- to 16-membered lactone ring that combines with various sugars to form a key group that inhibits bacterial translation (7). The activities of macrolide inhibitors, such as erythromycin, depends on the binding to the site near the polypeptide exit tunnel of the large ribosome subunit (8). Considering that macrolides do not bind to a ribosome with a captured outlet tunnel and synthesize 2-10 amino acid peptides in protein translation tests in vitro, it is suggested that drug binding physically prevents transferable elongation proteins beyond this size (9). Some macrolide resistance mutations change the ribosomal target site and inhibit the binding (8). Nevertheless, macrolides still bind well to the mutated ribosomes (10). For example, the deletion of the M⁸²K⁸³R⁸⁴ sequence in *Escherichia coli* L22 ribosomal protein promotes growth in the presence of high levels of erythromycin and other macrolides. The same mutation leads to resistance in *Haemophilus influenzae* against macrolides. L22 different mutations lead to resistance against macrolides in other bacterial species (9). The resistance of Enterobacteriaceae against macrolides may occur through various mechanism: displacement of the target site by methylases encoded by *erm* genes, especially *ermA* and *ermB*; the inactivation of enzymes such as esterase encoded by *ere (A)* and *ere (B)* genes or phosphotransferases coded by *mphA* gene or producing output pumps encoded by the *mef (A)* gene (11).

Using advanced technology such as using molecular diagnostic tools and a molecular fingerprint may be an appropriate choice in molecular epidemiologic studies (12). Polymerase chain reaction-based techniques are accurate, rapid, repetitive, sensitive, and capable of a unique and reliable diagnosis used to determine various DNA fingerprints. ERIC is a simple, competent, and cost-efficient genotyping technology for identifying various species among PCR-based tools. ERIC sequences are identified in many bacterial genomes, such as the Enterobacteriaceae members, including *E. coli*. Incomplete palindromic sequences are generally identified in the translated regions. Moreover, numerous mixed versions of ERIC in bacterial species. Interestingly, there are many copies of the sequence in various bacterial species and even the strains of a single bacteria species, such as *E. coli* (13).

In a study by Mousavi et al. conducted in Isfahan and titled "Molecular Detection of Macrolide and

Lincosamide-Resistance Genes in Clinical Methicillin-Resistant *Staphylococcus aureus* Isolates,” the results showed that among 100 *Staphylococcus aureus* strains, 52 were MRSE. The frequency of iMLSB, MS and, cMLSB resistance phenotypes was 3.17, 4.13, and 0.48%, respectively. . The frequencies of *ermC*, *msrA*, and *ermA* resistance genes were 0.73, 5.11, and 7.5%, respectively (14). The study aimed to evaluate the macrolide resistance of *E. coli* isolated from urinary infection and determine the phylogeny using the ERIC-PCR method.

Methods

The present study was experimental. The sampling was conducted from patients referred to Sanandaj hospitals due to urinary tract infection. First, each sample was cultured on an EMB culture medium and blood agar in the laboratory; if the bacteria grew on both mediums, it indicated that the sample was gram-negative, and if it grew on only blood agar, it indicated that the sample is gram-positive. Finally, the colonies similar to *E. coli* (*Escherichia coli* strains have a metallic colony on the EMB medium) are identified, and citrate utilization, motility, and indole production, methyl red (MR), Voges Proskauer (VP), and TSI tests were performed.

The most common antimicrobial sensitivity test is the disk diffusion in agar medium, which is known as the Kirby-Boyer test. The method was proposed by Boyer and his colleagues in 1966 (15). In order to prepare a suitable microbial suspension to determine its antibiotic sensitivity, the number of bacteria in the inoculated sample must be correct and acceptable. The number of these bacteria for the antibiogram method is conventionally $10^8 \times 1.5$ bacterial cells per milliliter inoculation. For this purpose, the following was performed:

Half milliliter 0.048 mol/l BaCl₂ was added to 99.5 ml sulfuric acid, and the suspension was obtained by continuous stirring. The correct standard opacity density was then determined using the absorption measurement in a spectrophotometer with a 1cm light path. The absorbance at 625 nm should be between 0.08 and 0.13. An amount of 4-6 ml Barium sulfate suspension should be poured in in screw tubes of the same size as the bacterial suspension tubes. The lids of these tubes should be tightly closed and kept at room temperature and in the dark. The standard barium sulfate must be stirred vigorously (preferably with a mechanical vortex) before each use to create uniform turbidity. If large particles are observed, a new standard should be developed. The barium sulfate standard should be replaced monthly or have its uptake measured. After preparing the microbial suspension in accordance with a 0.5 McFarland Standard turbidity, impregnate a sterile swab with the microbial suspension. After extracting the excess liquid by pressing the swab into the inner wall of the tube, the wet swab was inoculated in all directions at a 60-degree angle evenly on the surface of the Mueller-Hinton agar medium, which was heated to room temperature to penetrate the agar. Then antibiotic discs (amikacin, piperacillin, ceftriaxone, cefotaxime, erythromycin, carbenicillin, azithromycin, gentamicin, and ciprofloxacin) were placed on the plate under sterile conditions. In the next step, the plates were inverted and housed for 18 to 24 hours at 35 to 37 ° C. After 18-24 hours of incubation, the diameter of the growth inhibition zone was measured and reported as resistant (R), sensitive (S), or intermediate sensitive (I) according to the Clinical and Laboratory Standard Institute (CLSI).

In order to determine the MIC of erythromycin using the E-test method, after preparing the bacterial suspension by half McFarland method, it was transferred to the Mueller-Hinton agar plate, and then the E-Test strips, which represent the antibiotic erythromycin, were put on Mueller-Hinton agar. After 24 hours of incubation in a 37-degree growth zone, a triangular shape growth inhibition zone was formed, and then, by referring to the table provided by the manufacturer of E-Test strips, the sensitivity of *Escherichia coli* bacteria to erythromycin was determined.

Then, for DNA extraction, a colony was removed from a simple agar medium such as a 24-hour (fresh) agar nutrient medium and inoculated into a liquid medium (nourish broth) and then incubated for 18 to 24 hours. The tubes were then centrifuged at 3000 g for 5 minutes, then the supernatant was discarded, and sediment was used in the extraction step. The primers used to amplify the studied genes are listed in Table 1.

Table 1. Primers used in PCR

Gene	Primer sequence (3'←'5')	PCR Product length	Source
<i>mphA</i> -F -	'-GTGAGGAGGAGCTTCGCGAG-3'5	403 bp	This study
<i>mphA</i> -R -	'-TGCCGCAGGACTCGGAGGTC-3'5		
<i>mphB</i> -F -	'-GATATTAACAAGTAATCAGAATAG-3'5	494 bp	This study
<i>mphB</i> -R -	'-GCTCTTACTGCATCCATACG-3'5		

Electrophoresis in the agarose gel medium and molecular typing was performed using ERIC-PCR. The regions which are placed between the duplicate parts are different in size and are reproduced using the ERIC-PCR technique; therefore, different patterns are created after PCR and electrophoresis, which could quickly help distinguish the strains. This is a suitable method because of its high sensitivity and speed. Primers used in ERIC-PCR are shown in Table 2.

Table 2. ERIC-PCR primers

Primer		Primer sequence (3'←'5')	Source
ERIC-PCR	F	5'-ATGTAAGCTCCTGGGGATTAC-3'	(Xia et al., 2012)
	R	5'-AAGTAAGTGACTGGGGTGAGCG-3'	

After the ERIC-PCR reaction, the product was loaded on 2% agarose gel. Then, 2000bp ladders with 50 bp resolution were used. Upon electrophoresis, the presence or the absence of bands were determined using 0 and 1 for the studied isolates, respectively. To analyze data, matrices 0 and 1 were drawn. Band detection was performed by manual detection. After creating matrices 0 and 1, the data were analyzed using NTSYS-pc ver. 2.02. After the formation of matrices 0 and 1, dendrograms of genetic relationships between isolates were drawn in NTSYS software.

Results

In this study, 99 strains of *Escherichia coli* were isolated from urine samples. Table 3 and Figure 1 show the biochemical tests used.

Table 3. The results of biochemical tests for *E. coli*

Urease	TSI	VP	MR	H ₂ S	Motion	Endol	Simmons Citrate	Catalase
-	A/A	-	+	-	+	+	-	+



Figure 1. An example of biochemical tests used.

In the phase of evaluating the antibiotic sensitivity of the strains, all the 99 isolated strains were evaluated using the disk diffusion. Table 4 shows the sensitivity of strains to different antibiotics. According to the data, the highest sensitivity belongs to piperacillin, erythromycin, and cefotaxime with 84.84%. After them, the highest sensitivity belongs to carbenicillin with 75.75%.

Antibiotic sensitivity of strains

Antibiotic	Sensitive (%)	Semi-sensitive (%)	Resistant (percentage)
CRO	30/3	18/18	51/51
PIP	7/7	8/8	84/84
E	15/15	0	84/84
AZM	22/22	47/47	30/3
GN	55/55	12/12	32/32
PY	7/7	17/17	75/75
CIP	46/46	12/12	84/84
CTX	3/3	12/12	84/84
AN	39/39	18/18	42/42

CRO: Ceftriaxone, PIP: piperacillin, E: erythromycin, AZM: azithromycin, GN: gentamicin, PY: carbenicillin, CIP: ciprofloxacin, CTX: cefotaxime and AN: amikacin

The least resistance of the strains was to azithromycin with 30.3%. On the other hand, the highest sensitivity of the strains was to gentamicin with 55.55%. High intermediate sensitivity was observed in the studied strains in relation to the antibiotic azithromycin (47.47%) (Figure 2).

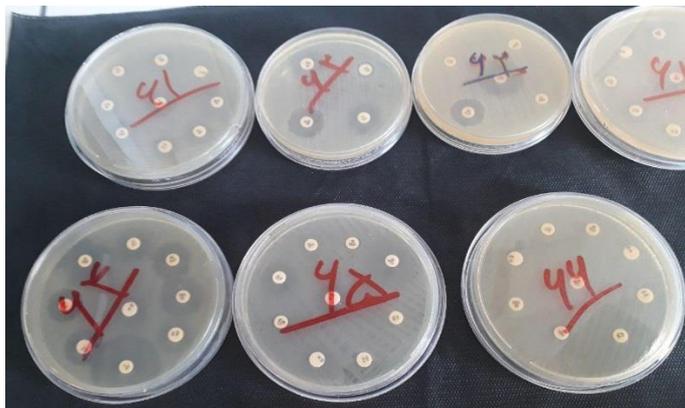


Figure 2. Disk diffusion samples

The results of this test for erythromycin-resistant samples were obtained in the range of 0.75-16 microgram/ml. Examples of this test are shown in Figure 3.



Figure 3. The creation of a triangular growth inhibition area due to increased antibiotic concentration around the E-test band

In the next step, the mpgA and mphB PCR gene results were evaluated. PCR test was performed for 17 strains, which were erythromycin and azithromycin resistant according to disk diffusion and for two semi-sensitive strains to these antibiotics. From the 19 samples, 10 were mphB positive isolates, and 10

isolates simultaneously *mphA* and *mphB* genes (Figure 4 and Table 1).

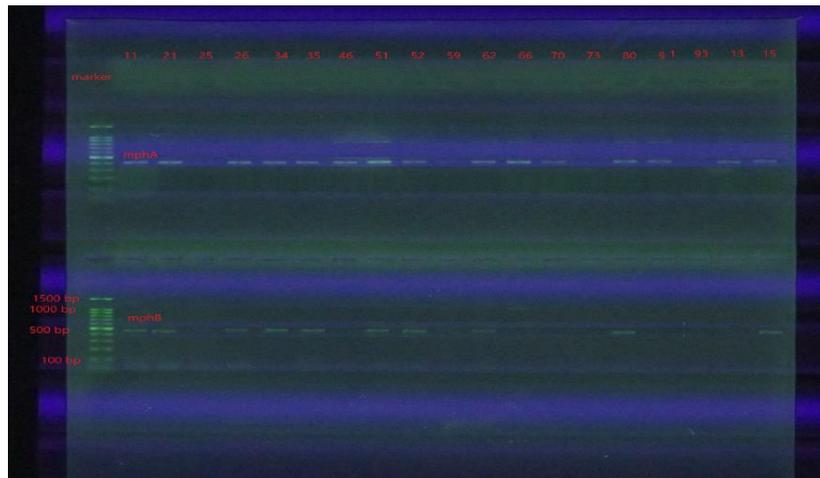


Figure 4. An example of the picture taken from PCR products gel; the above is *mphA* gene diagnosis, below is *mphB* gene diagnosis.

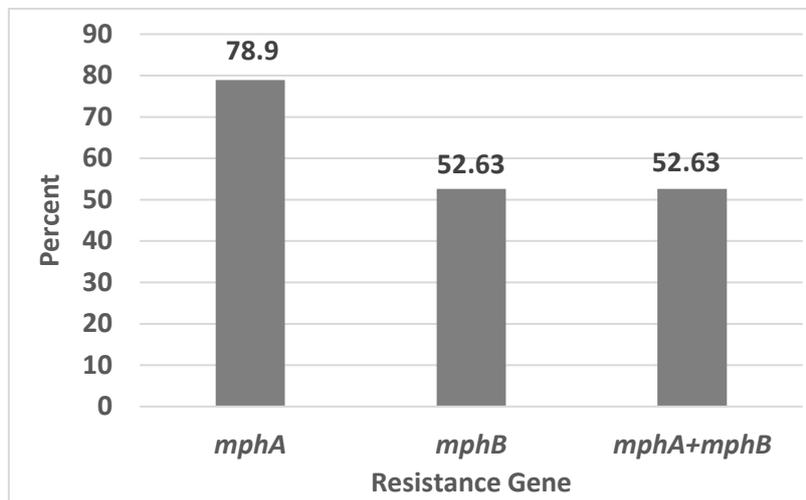


Table 1. The frequency of genes (%)

In the end, molecular typing was evaluated using the ERIC-PCR method. Then, using the bands observed on the gel, matrices 0 and 1 were formed (Figure 5).

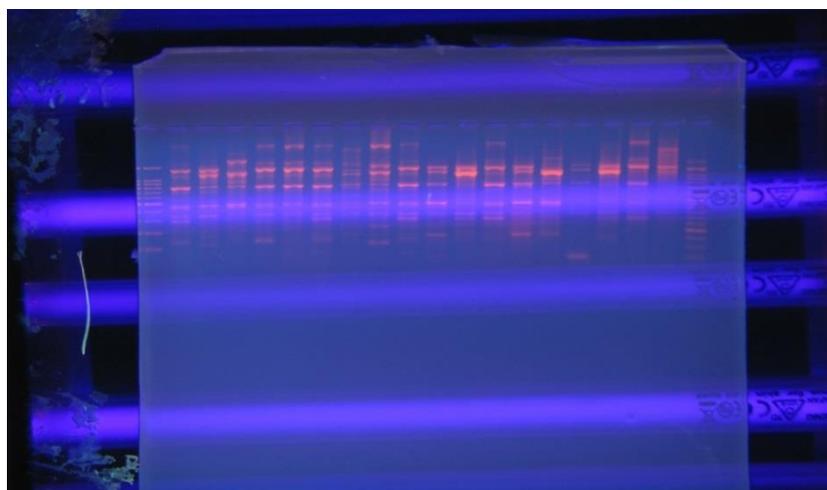


Figure 5. An example of ERIC-PCR product gel

Discussion

As mentioned, urinary infections are of the most common infections in humans and are the most common cause for referring to doctors in various age groups after respiratory infections. In most references, the most common urinary infection organism is perceived to be *E. coli*. The organism causes 75-90 % of urinary infections in both genders (16, 17). *E. coli* is one of the important pathogens which has shown resistance to most antibiotics. Resistant *E. coli* species are increasing day by day. The problems arise when patients do not complete their treatment course, and live bacteria become resistant, which has become a concern for physicians. In most cases, we witness numerous drug resistance in pathogens due to indiscriminate and arbitrary use of antibiotics, which has led to failure in the treatment and the emergence of many complications despite the hefty healthcare expenses. Drug resistance to antibiotics is different in various Iran regions due to genetic changes in the strains and the differences in antibiotics dosage and availability. Drug resistance is hereditary and acquired in bacteria. In hereditary resistance (chromosomal or plasmid), the cell's intrinsic and inherited traits inhibit the functions of antibiotics, and resistant strains emerge from the mass of susceptible bacteria after exposure to antibiotics (18).

E. coli causes 75-90% of urinary tract infections in the USA (19) and 85.9% in Russia (20). Therefore, according to the importance of urinary infections, this study was performed in Sanandaj. The studies show that *E. coli* isolated from humans is the most important pathogen that shows antimicrobial resistance to most antimicrobial drugs (21). On the other hand, this study shows that the most effective antibiotic for *E. coli* isolated from urinary infection in Sanandaj is gentamicin, ciprofloxacin, amikacin, ceftriaxone, respectively. Considering the present study results, it would be better to use piperacillin, erythromycin, cefotaxime, and carbenicillin less in the initial treatment of these infection because the results show the high resistance of *E. coli* to these antibiotics in Sanandaj.

In a study by Mohajeri et al. on the *E. coli* isolated from urinary infection in 2009 in Kermanshah, the sensitivity to ceftriaxone, amikacin, carbenicillin, ciprofloxacin, gentamicin, and cefotaxime were reported to be 71%, 97%, 17%, 71%, 85%, and 70%, respectively (22). These results are not consistent with that of the present study, which showed a sensitivity of 51.5%, 42.42%, 75.75%, 41.41%, 32.32%, and 84.84% to the antibiotics mentioned above. Kermanshah is a city close to Sanandaj, and it is expected that the antibiotic resistance pattern in the two close cities is similar. However, the apparent difference in the resistance pattern could be attributed to differences in the antibiotic regimens of the two provinces.

In the present study, 32.32% of *E. coli* strains were resistant to gentamycin, and 15 mphA positive isolates were identified, which was higher than that in Nakamura's study. The difference in mphA gene prevalence could be due to the geographical region and time difference because they conducted the study some 20 years ago, and the bacterial resistance gene is expanding daily. In a study in 2009 by Nguyen et al., 190 *E. coli* strains were studied. These strains were isolated from samples in five countries on four different continents. The countries were Ghana, Nigeria, Senegal, France, and Vietnam. Numerous macrolide genes were studied in this research, among which mphA, mphB, and ermB were detected in the studied strains. In all the 190 *E. coli* strains, six had ermB (3.15%), 48 had mphA (24.73%), and two had mphB (1.05%) (6). Comparing the two studies shows that the prevalence of mphA and mphB was higher in the present study. However, it should be noted that the gene frequency was determined among all the isolated strains, while in the present study, the frequency was determined among 19 azithromycin and erythromycin-resistant strains. If the frequency were determined among all the strains in the present study, the frequency of the mphA gene would probably be lower than that of theirs. However, if such happened for the mphB gene, its frequency would still be higher in our study because they reported a frequency of 1.05%. Another difference between the two studies is the width of the study area; they studied several countries while we studied a province of a country (Sanandaj, Iran). If their study was smaller, the results would probably be closer.

In a study by Soge et al. in 2009, 52 gram-negative bacteria isolates were isolated. They detected 11 tetracycline-resistant isolates. Then, they studied mphA and mphB genes in these 11 strains, which were

not detected in these strains (23). The present study results are not consistent with theirs because these two genes were detected in this study (15 mphA positive isolates (78.9), and ten mphB positive isolates (52.63%)). The difference is due to the different geographical properties of the study area, study timing, and antibiotic regimens in the regions. The results in the study of Jost et al. in 2016 showed that except for two atypical strains with codes 34396 and 36493 whose MIC for azithromycin was less than 256 mg/l, the MIC of azithromycin for other strains was between 0.25 and 16 mg/l. They also studied the plasmid capable of transmitting macrolide resistance. Two strains (0.393%) of isolates had the mphA gene. Strain 36493 of the E. coli clonal group 26: H11 has a plasmid containing mphA (11). The prevalence of the mphA gene in the present study was much higher than that of theirs (78.9%). The difference in antimicrobial resistance and resistance gene distribution in the study could be due to differences in the geographical area, studied strains, antibiotic application in different countries, the difference in personal and public health hygiene, the difference in meeting hospital health standards, meeting infectious waste disposal standards in hospitals, and the difference in prescribing antibiotics by physicians. The results obtained in a study whose objectives were in line with our study's objectives showed that 19 isolates (4.15%) have the mphA gene (24). Regarding the comparison of these two studies, it should be noted that this gene frequency was calculated from all isolated strains in that study, but in the present study, it was calculated among 19 azithromycin and erythromycin-resistant strains. If these genes were examined among all strains in the present study, the prevalence of the mphA gene was probably lower than in their study. The genus of the bacteria studied in the two studies is also different.

Typing techniques are highly important in understanding if the strains of patients in one or some areas are originated from an epidemic strain and distributed among patients, or there are different strains in the area. In antibiotic resistance studies, genetic typing could help study if antibiotic resistance genes are spreading among bacteria through gene transition or a single resistant strain is the cause. The present study showed that strains 42-22, 40-38, 28-8, and 3-43 have 100% genetic similarity in pairs. Therefore, these isolates are from the same genetic origin while others are not, and the hypothesis that resistance genes are spreading among strains through gene transition was confirmed in the study. Moreover, strains with genetic similarity did not have any of the two studied genes. Samples 21, 80, 34, 52, 66, 46, 11, 51, 62, 15, 35, 70, 13, 91 and 26 had the mphA gene. Also, samples 21, 80, 34, 11, 51, 62, 15, 35, 52 and 26 had mphB gene.

Conclusion

Considering the prevalence of macrolide resistance among E. coli strains in Sanandaj, detecting these strains in urinary tract infection patients is recommended. Moreover, it is not recommended to treat these patients with macrolides because these antibiotics would only lead to further resistance and development of macrolide-resistant strains in Sanandaj hospitals. Also, several factors, including the inhuman use of antibiotics in agriculture and veterinary medicine, cause the development of antibiotic resistance; in this regard, the present study recommended this study to meet more standards in different watersheds. Typing results showed that resistance genes are spreading among bacteria, and therefore, we should look for a solution to prevent further distribution of the genes. Hospitals are one of the main pillars for increasing and distributing strains and resistance genes, which have to implement health standards.

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