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ORIGINAL ARTICLE

Prevalence of main quinolones and carbapenems resistance genes in clinical and veterinary *Escherichia coli* strains

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ABSTRACT

Background and Objectives: Antibiotics-resistant *Escherichia coli* strains are considered one of the most important causes of human and animal infections worldwide. The aim of current study was to detect common resistance (carbapenems and quinolones) genes by PCR.
Materials and Methods: A total of 100 *E. coli* strains isolated from human urinary tract infection and 20 isolated strains of aborted sheep embryos were collected. PCR was performed using specific primers to detect the resistance genes.
Results: Overall, among the quinolones resistance genes, *qnrS* resistance gene had the highest frequency (48%) and among carbapenem resistance genes, *imp* resistance gene had the highest frequency (45%). The frequency of resistance genes, IMP (28.45%), KPC (9.5%), VIM (9.15%), NDM (7.20%) were observed in clinical and veterinary strains, respectively. According to the results, 38.6% of *E. coli* strains had at least one from five genes of resistance to quinolones. The lowest frequency of resistance gene was related to *qnrA*, which was observed in only 29 (24.2%) strains.
Conclusion: Monitoring of carbapenem and quinolone resistance in pathogenic *E. coli* to humans and animals has an important value in revising treatment guidelines and the national public health, and plays an important role in preventing the spread of resistant strains.

Keywords: *Escherichia coli*; Carbapenems; Drug resistance; Polymerase chain reaction; Quinolones

INTRODUCTION

Escherichia coli (E. coli) is a normal microbiota of various vertebrates that can cause gastrointestinal and extraintestinal infections. Extraintestinal pathogenic *E. coli* (ExPEC) strains are diverse microorganisms that can cause urinary tract, bloodstream, prostate and other non-gastrointestinal infections. In Iran, 80% of community urinary tract infections and 50% of hospitalized urinary tract infections are

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caused by *E. coli* (1). Every year, 150 million cases of urinary tract infections are diagnosed worldwide (2), and more than half of women have experienced a urinary tract infection at least once in their lifetime (1). Uropathogenic *E. coli* (UPEC) is one of the most common microbial causes of urinary tract infections in children and is estimated to be responsible for 70-90% of urinary tract infections in children (3, 4). Infections caused by avian pathogenic *E. coli* (APEC) cause colibacillosis, an acute and mostly systemic disease that causes significant economic losses to the poultry industry worldwide. Due to the indiscriminate use of antibiotics in medicine and veterinary medicine and the worldwide emergence of resistant strains, especially in developing countries, further studies are needed to detect the prevalence of antibiotic resistance in *E. coli*. By studying the antibiotic resistance patterns of human and animal strains of *E. coli* (5, 6) and estimating the frequency of resistant strains, clinicians can use better treatment strategies to combat infections caused by resistant strains. The inappropriate use of antibiotics in the treatment of urinary tract infections caused by *E. coli* has led to the emergence of resistant strains in recent decades (4). The main cause of bacterial antibiotic resistance is the excessive and indiscriminate use of antibiotics by humans and the misuse and overuse of antibiotics in food-producing animals for human consumption (5, 6). Today, antibiotic resistance is a major challenge in the prevention, treatment and control of infectious diseases such as urinary tract infections and a serious threat to public health (7). *E. coli* plays an important primary role in the acquisition and transfer of antimicrobial resistance genes and transfers antibiotic resistance genes to other gut bacterial strains via transposons, bacteriophages and plasmids through various pathways (8).

β-lactams were widely used to treat *E. coli* infections (9, 10). Over the last two decades, resistance to several β-lactams has increased rapidly, usually due to plasmids carrying extended-spectrum β-lactamase (ESBL) genes, and quinolone resistance genes are also located in the same plasmids (11).

Quinolones are widely available antibiotics used to treat many conditions, especially urinary tract infections. Resistance to quinolones arises mainly through chromosomal mutations and plasmids. Topoisomerase IV and DNA gyrase enzyme Qnr proteins include *qnrA, qnrB, qnrC, qnrD* and *qnrS* and belong to the family of penteptide proteins that lead to resistance to quinolones (12, 13).

Although the phenomenon of intrinsic and transferable resistance in *E. coli* has been recognised decades ago, this problem has not received significant attention and drug resistance remains a major threat to human and animal health (14-16). Investigation of the origin of the prevalence and epidemiological studies is one of the needs of the community in the control, treatment and prevention of drug resistance. The aim of this study was to investigate the frequency of resistance genes of *E. coli* strains isolated from human and animal infections to therapeutic antibiotics, in particular carbapenems and quinolones (*KPC, NDM, VIM, IMP, qnrA, qnrB, qnrC, qnrD* and *qnrS*).

MATERIALS AND METHODS

Ethics and consent to participate. Our experiments were conducted with the permission of Islamic Azad University Science and Research Branch. The license number is IR.IAU.SRB.REC.1400.266.

Sample collection. A total of 100 strains of *E. coli* isolated from urinary tract infections were collected from patients referred to the Hamadan Sina Hospital and 20 strains isolated from aborted sheep embryos were collected by the provincial veterinary organization. The samples were transferred to the Hamadan Medical University Reference Laboratory for culture and isolation of *E. coli*.

***Escherichia coli* isolation and identification.** To detect *E. coli* strains, samples were subjected to Gram staining, and after detection of Gram-negative bacteria, and if oxidase and catalase tests were negative for the isolated bacteria, SIM, TSI and urease differential cultures were used to detect the bacterial type. A streak culture was obtained on MacConkey agar medium and after incubation at 37°C for 18-24 hours, lactose fermenting bacteria were isolated. Pink colonies suspected to be *E. coli* were cultured on EMB agar medium and identified by a biochemical confirmation test (API 20E). Colonies with metallic green gloss were selected after cultivation on Nutrient Agar and stored at -20°C.

Antibiotic susceptibility testing. Susceptibility of *E. coli* isolates to 10 standard antibiotic discs (MAST,

London, United Kingdom), including tetracycline (30 µg), cefoxitin (30 µg), cefpodoxime (30 µg), ceftrioxime (30 µg), cephalothin (30 µg), ceftaxone (30 µg), amikacin (30 µg), amoxicillin (25 µg), colistin (10 µg) and piperacillin/tazobactam (100/10 µg), impenem (10 µg), meropenem (10 µg) and ertapenem (10 µg) were determined by the standardised disk diffusion method recommended by the CLSI (2020). *E. coli* ATCC 25922 strain was used for quality controlling.

DNA extraction. Genomic DNA was extracted from a pure colony of *E. coli* isolates on Nutrient Agar medium by the boiling method (17). In summary, samples were centrifuged at 15000 g for 15 min. The supernatant was removed and settled in sterile distilled water and centrifuged at 15000 g for 10 min. Subsequently, the supernatant was removed and the precipitate was suspended in 40 µl of sterile distillate, boiled at 100°C in a water bath for 10 min, cooled on ice and centrifuged at 15000 g for 10 s. The resulting pellet containing DNA extracted from the bacteria was poured into a separate microtube and placed in a -20°C freezer until used to PCR (18).

The amount of extracted DNA was estimated using a nanodrop spectrophotometer (Thermo Scientific™ 840274100) as ng/µl, and the adsorption rate was measured at two wavelengths 230 and 280 nm as control. To determine DNA quality, electrophoresis of DNA samples was also performed on a 1% agarose gel and the presence of intact DNA without contamination was examined.

Genetic confirmation of *Escherichia coli* strains. Genetic diagnosis of *E. coli* strains was performed by PCR using 16S rRNA gene primers (Table 1) in a BioRad T100 thermal seeder. All PCR reactions were performed in a final volume of 12.5 µl. The components used in the reaction contained 4.0 pmol/µL each primer, 25/6 µL Taq DNA polymerase master mix RED enzyme (Amplicon; Denmark), 50 ng/µL bacterial DNA and 75.4 µL distilled water. PCR was initiated by denaturation (95°C, 3 min). Each cycle included three steps (denaturation, annealing and extension). Each PCR reaction consisted of 30 replicate cycles (denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 30 s. The final extension cycle was performed at 72°C for 5 min.

Detection of quinolone resistant genes. PCR were performed using five pairs of specific primers (Table 1) in a BioRad T100 thermocycler. PCR thermal cycle for quinolone propagation was 5 min at 95°C and initial denaturation, 30 s at 95°C denaturation, 15 s at 54°C annealing of *qnrA* gene, 30 s at 64°C annealing of *qnrB* gene, 15 s at 56°C: *qnrC* annealing, 15 seconds at 64°C *qnrD* annealing, 30 seconds at 62°C *qnrS* annealing, 15 seconds at 72°C extension, 35 cycles, 5 minutes at 72°C final extension. After completion of the reaction, the amplified products were observed on a 1.5% agarose gel electrophoresis, and each strain was evaluated for the presence or absence of the expected amplification fragment for each primer pair.

Detection of carbapenem-resistant genes. PCR were performed using four pairs of specific primers (Table 1) in a BioRad T100 thermocycler. Each PCR reaction consisted of 30 amplification cycles (denaturation at 95°C for 30 s, annealing (58°C for 15 s for KPC, 64°C for 30 s for NDM, 60°C for 30 s for VIM, 66°C for 15 s for IMP) and extension at 72°C for 30 s. The final extension cycle was performed at 72°C for 5 min.

RESULTS

In this study, 100 *E. coli* isolates from urinary tract infections collected from Hamadan Sina Hospital were confirmed and tested. In addition, 20 *E. coli* isolates from samples isolated from aborted sheep embryos were confirmed and tested.

Demographic results. Of the 100 clinical samples validated in this study, 61 (61%) were from outpatients and 39 (39%) from inpatients. The demographic results of frequency of patients and age of patients are shown in Fig. 1. Out of 100 samples, 73 samples were taken from women and 27 from men.

PCR of 16S rRNA for genetic diagnosis of *Escherichia coli* strains. A total of 100 clinical bacterial samples and 20 isolated animal bacterial samples were tested using a 16S rRNA specific primer. After electrophoresis, all strains that amplified a 200 bp fragment were amplified (Fig. 2).

Antimicrobial susceptibility patterns. Among *E. coli* isolated from the clinic, the highest antibiotic sensitivity was to colistin with 90%, followed by pipera-

Table 1. Name, sequence, annealing temperature and length of the resulting piece in PCR with primers used

Primers	Sequneces (3-5)	Annealing Tempature (°C)	Length (bp)	References
16s rRNA-F	GCGGACGGGTGAGTAATGT	59	200	(14)
16s rRNA-R	TCATCCTCTCAGACCAGCTA			
qnrA-F	ATTTCTCACGCCAGGATTTG	54	516	(15)
qnrA-R	GATCGGCAAAGGTTAGGTCA			
qnrB-F	GATCGTGAAAGCCAGAAAGG	64	469	(15)
qnrB-R	ACGATGCCTGGTAGTTGTCC			
qnrC-F	GGGTTGTACATTTATTGAATC	56	447	(16)
qnrC-R	TCCACTTTACGAGGTTCT			
qnrD-F	GAGATCAATTTACGGGGAATA	64	582	(17)
qnrD-R	AACAAGCTGAAGCGCCTG			
qnrS-F	ACGACATTCGTCAACTGCAA	62	417	(15)
qnrS-R	TAAATTGGCACCCTGTAGGC			
IMP-F	GGGTGGGGCGTTGTTCCTA	66	182	(18)
IMP-R	TCTATTCCGCCCGTGCTGTC			
VIM-F	GAGCCGAGTGGTGAGTATCC	60	370	(19)
VIM-R	GAATCTCGTTCCCCTCTGCC			
KPC-F	ATCGCCGTCTAGTTCTGCTG	58	811	(19)
KPC-R	TCGCTGTGCTTGTCATCCTT			
NDM-F	AGGACAAGATGGGCGGTATG	64	281	(19)
NDM-R	CTTGGCCTTGCTGTCCTTGA			

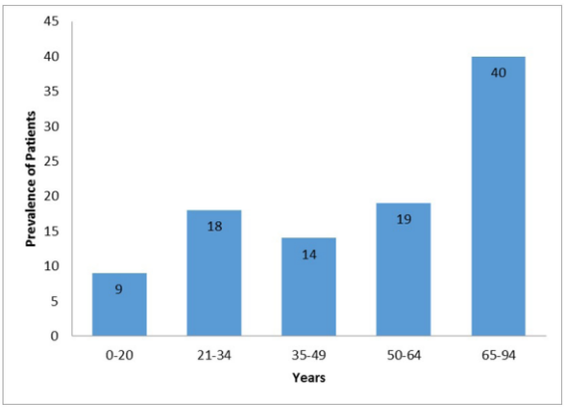

Fig. 1. Demographic results of frequency of patients and age of patients

cillin/tazobactam with 81% sensitivity, meropenem with 66% sensitivity, imipenem and amikacin with 62% sensitivity, and ertapenem with 60% sensitivity and the highest intermediate resistance in ertapenem was seen with 29%. In this study, the highest antibiotic resistance was found first for amoxicillin at 95% and then for cephalothin at 81%. Of the 100 *E. coli* isolates tested, 63% were resistant to cefoxitin, while 9 (9.5%) showed intermediate resistance. Of the 72% of cefoxitin-resistant isolates, 61%, 57% and 54.7% were resistant to ceftriaxone, cefpodoxime and ceftazoxime, respectively. The isolates were most sensitive to colistin 90%, piperacillin/tazobactam 81% and amikacin 62% (Fig. 3). Fig. 4 shows the frequency of resistance to different antibiotics in animals' isolates.

Presence of quinolones resistance genes. The detection of the expected amplification fragments of *qnrA* (516 bp), *qnrB* (469 bp), *qnrC* (447 bp), *qnrD* (582 bp) and *qnrS* (417 bp) in the PCR electrophoresis patterns of these genes indicated that clinical and animal strains carry resistance genes to these antibiotics (Figs. 5 and 6). The results of this study showed that these strains carry at least one resistance gene out of the five genes examined. In the clinical strains, the prevalence of the *qnrS* resistance gene was (48%), *qnrB* (47%), *qnrD* (39%), *qnrC* (30%) and *qnrA*: (29%) and in animal strains for the *qnrA* resistance gene (25%), *qnrD* (25%), *qnrC* (20%), *qnrS* (15%) and *qnrB* (5%).

Presence of main carbapenems resistance genes. In this study, resistance of *E. coli* strains isolated from

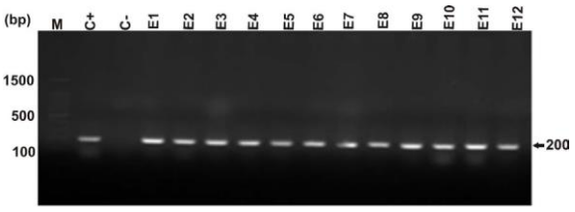

Fig. 2. Results of electrophoresis of PCR products in molecular confirmation of *E. coli* isolates using 16S rRNA specific primer. M: DNA molecular marker; C+: *E. coli* strain ATCC 25922 as positive control; C-: Sample without DNA template as negative control; E1 to E12: Clinical specimens of *E. coli.*

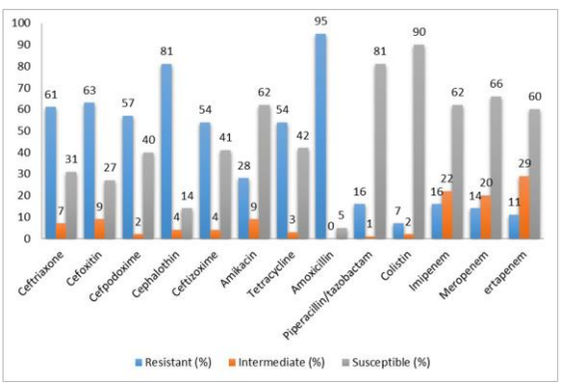

Fig. 3. The antimicrobial susceptibility profile of all clinical isolates (n=100)

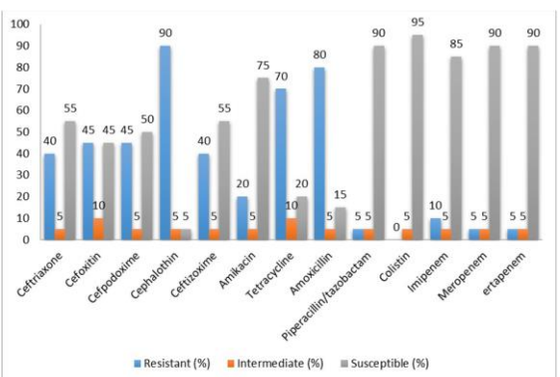

Fig. 4. The antimicrobial susceptibility profile of all animal isolates (n=20)

patient urinary tract infection samples and aborted sheep fetuses was investigated by PCR using specific primers for KPC, NDM, VIM and IMP resistance genes. The results showed that all resistance genes tested were present in clinical and animal strains (Fig.7), with a prevalence of 28% for the IMP resistance gene, 9% for the KPC gene, 9% for the VIM gene and 7% for the NDM gene in clinical strains and 45% for the IMP gene, 20% for the NDM gene, 15% for the VIM gene and 5% for the KPC gene in animal strains.

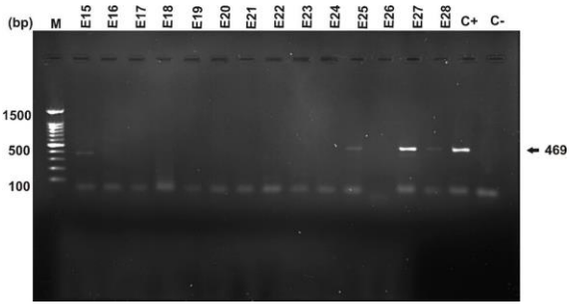

Fig. 5. Electrophoretic pattern of *qnrB* gene PCR. M: DNAbp100 marker; E15 to E28: clinical strains of *E. coli*; C-: negative control; C+: Positive control.

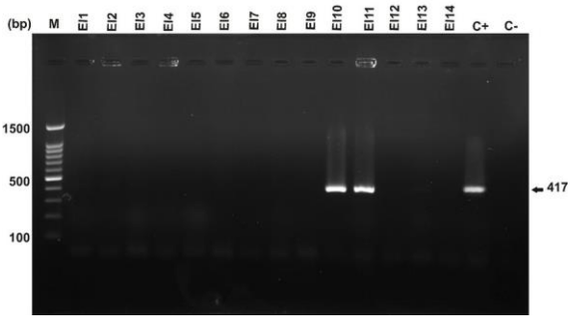

Fig. 6. Electrophoretic pattern of *qnrS* gene PCR. M: DNAbp100 marker; E15 to E28: clinical strains of *E. coli*; C-: negative control; C+: Positive control.

DISCUSSION

E. coli infection is one of the most common hospital-acquired infections in human and animal populations (18, 19). Today, the increasing mortality caused by these infections makes the detection of the causative agents and the development of antibiotic resistance very important. Some studies have shown that *E. coli* isolated from humans is the main pathogen in which an increase in antimicrobial resistance to most therapeutically used antibiotics has been observed (20).

ESBL-producing *E. coli* has become an emerging public health problem due to the clinical failure of empirical treatment protocols. Although the resistance mechanisms of ESBL-producing *E. coli* may

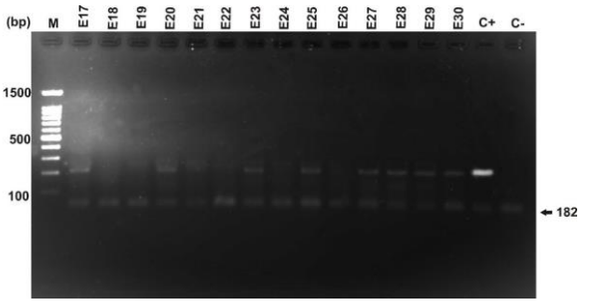

Fig. 7. Electrophoretic pattern of IMP gene PCR. M: DNAbp100 marker; E17 to E30: clinical strains of *E. coli*; C-: negative control; C+: Positive control.

vary, it has been shown that the most common resistance mechanism of *E. coli* isolates against β-lactams is the production of β-lactamases (21, 22). The proliferation of *E. coli* strains that produce a variety of ESBLs makes continuous monitoring necessary for appropriate antibiotic use and infection control. The number of resistance genes in ESBL-producing *E. coli* strains varies between different geographical areas and even between hospitals in the same region, depending on the infection control system and treatment programmed (23, 24). The excessive and inappropriate use of β-lactams is a major threat to the emergence of resistance genes and ESBL-producing strains. Infections should be managed through diagnosis of pathogenic microorganisms and precise antibiotic therapy. Therefore, the identification of antibiotic resistant strains is of utmost importance. Strains with reduced sensitivity to these antibiotics should also be screened for ESBL genes (25, 26).

Quinolones are widely used to treat infections caused by *E. coli*. The widespread use of quinolones has led to a high level of resistance in *E. coli* (22, 23, 26). Changes in antibiotic target site and permeability lead to antibiotic resistance. Plasmid facilitates quinolone resistance, in particular the translocation of the *qnrA, qnrB* and *qnrS* gene clusters, which are the main indicators of quinolone resistance (24, 27). The results of this study showed that 38.6% of clinical strains and 18% of animal strains were resistant to quinolones. The emergence of antibiotic resistance in human and animal *E. coli* strains showed that antibiotics are used indiscriminately, inappropriately and empirically in the study population. To date, there have been several studies worldwide on *E. coli* resistance to quinolones. Sedighi et al. (2014) studied the prevalence of β-lactamase genes and quinolone resistance in uropathogenic *E. coli* in Hamedan. The results of their study showed that 18.2% *qnrB* gene and 1.12% *qnrS* gene were present in quinolone resistant strains, and no *qnrA* gene was found in any strain (25). In 2016, Ali et al. investigated the multidrug resistance of *E. coli* strains isolated from urine. In this study, 59% of isolates were resistant to at least three antibiotics of the fluoroquinolone family, and *qnrA* genes were not detected in all strains, *qnrB* genes detected in 35.2% and *qnrS* genes in 53.3% of the population (25). Ramirez-Castillo et al. (2018) screened *E. coli* strains isolated from urinary tract infections in Mexico for multidrug resistance and *qnrA* had a frequency of 22.7%, *qnrB* 20.9%, *qnrS* 6.4%, *qnrD* 4.5% and *qnrC* 0.9% (29%) (26). In the results of a study by Hang et al. (2019) in Vietnam on antibiotic resistance genes of 144 *E. coli* strains isolated from stool samples of 14-day-old calves, 33 strains showed quinolone resistance phenotypes. 21% of all strains had a quinolone resistance gene, *qnrS*, and none of them detected other resistance genes to quinolones, *qnrA*, *qnrB* and aac(6)-Ib-Cr (27). Differences in these results may be related to antibiotic use patterns, geographical area and differences in resistance between regions, and overuse of antibiotics. The high proportion of resistant strains carrying multi-drug resistance genes in this study may be due to the concomitant use and combination of broad-spectrum antibiotics and antimicrobials in this region (28).

Qnr genes, which belong to the PMQR genes, are located in plasmids and cause bacteria to express proteins that protect against the action of quinolones. In addition, PMQR genes are always available with genes encoding ESBL production, allowing resistance to these antibiotics to spread globally, especially in hospitalized patients with long-term exposure (28, 29). In this study, 100 *E. coli* strains were collected from patients with urinary tract infections. The results showed that 38.6% of the strains examined had at least one of the five resistance genes (*qnrA, qnrB, qnrC, qnrD* and *qnrS*). Regarding the prevalence of PMQR genes in clinical strains of *E. coli*, this study showed that PMQR genes are the most prevalent genes in 38.6% of strains as single genes or as combinations of several genes. The clinical isolates with the lowest frequency were associated with *qnrA*, which was present in only 29 strains. Abdulsalam et al. (2018) investigated the quinolone resistance genes of *E. coli* strains isolated from urinary tract infections in Egypt and showed that 6.7%

of isolates had the *qnrA* gene, 23% had the *qnrB* gene and 36% had the *qnrS* gene. There was a significant association between the *qnrS* gene and quinolone resistance, whereas the association of *qnrA* and *qnrB* with quinolone resistance was not significant in this study (29). The results of this study were consistent with the results of another study by Farajzadeh et al. (2019), where the prevalence of PMQR gene in clinical *E. coli* isolates was 59.88%, with *qnrS* gene being the most prevalent (41%), followed by *qnrB* gene (21%) and finally *qnrA* gene (10%). The results also showed a significant association between the *qnrS* gene and quinolone resistance (30). The prevalence of PMQR genes in this study (38.6%) was lower than that reported by Farajzadeh et al. (33%) and higher than that reported by Sedighi et al. (26.1%) (11%). This difference may be due to the misuse of antibiotics in different regions and gene transconjugation mechanisms that occur in regions with high PMQR prevalence (25, 30).

Carbapenemase dissemination, often associated with extended-spectrum β-lactamase production, is one of the serious risks in the treatment of multi-drug resistant infections. The prevalence of ESBL in clinical species varies between countries and regions. In the present study, antibiotic resistance results of clinical and animal *E. coli* strains showed that all investigated β-lactam resistance genes are present in these strains. The level of resistance observed in this study is high considering that carbapenems are used as a last line of treatment for infections caused by Gram-negative bacteria. Many similar studies on drug resistance of *E. coli* strains against β-lactams have been conducted in Iran and other parts of the world. In France, results by Gauthier et al. (2018) on the frequency of OX-48, NDM, VIM and KPC3 genes in *E. coli* strains isolated from clinical samples showed that among the study strains (31, 32). Also, 74.3% of the carbapenemase gene carriers were OXA-48, 20% had the NDM gene, 3.6% had the VIM gene and 0.7% had the KPC3 gene (31). Nojoomi et al. (2017) studied *E. coli* isolated from urine, stool, blood and wound to determine IMP, VIM and NDM-1 carbapenem resistance genes, as well as oxacillinase (OXA-48) and quinolone resistance (*qnrA*) genes.

In this study, the prevalence of IMP genes in 16%, VIM in 0%, NDM-1 in 0%, *qnr* in 48% and OXA-48 in 8% of strains was determined which increases concerns in the management of infections (32). *E. coli* producing carbapenemase, which also produce ESBL, is very difficult to treat because, despite resistance to carbapenem, resistance to many cephalosporins exists and, on the other hand, many carbapenemase genes are carried in plasmids, which are larger and also carry resistance genes to other antimicrobials such as aminoglycosides, chloramphenicol, sulfonamides and tetracycline (33, 34). Thus, the transfer of resistance elements from one side to the other occurs and can simply cause the spread of resistance in hospitals and health centers. It is therefore recommended that, in addition to antibiotic tests to detect resistance, other tests should be performed to help physicians identify which types of effective antibiotics to prescribe and subsequently reduce the duration of illness and the cost of treatment (3, 5).

The high frequency of *E. coli* infections and the relative high level of antibiotic resistance make it necessary to select the right drug and to use it in a controlled manner on a large scale. To address this problem, it is necessary to study multiple strains with different human, animal and food sources from different geographical areas and to investigate the genotypic and phenotypic patterns of antibiotic resistance and susceptibility of the isolates. Given the presence of multiple antibiotic resistance genes in the isolates and the potential for these isolates to be transferred between bacterial strains and species in the community, this is considered a serious alarm about the presence of widespread and multiple resistances and its spread between humans and animals (10, 24). Therefore, to determine infection control strategies in the study area, it is necessary to conduct continuous surveillance, monitoring and screening programs and to determine the type of dominant strain or dominant genotype in the community in order to use the most appropriate orientation in the treatment of patients (26).

CONCLUSION

The overuse of carbapenems and quinolones in clinical and veterinary medicine has led to the emergence of resistant strains. As the related resistance genes are located in plasmids, they are easily transferred between Enterobacteriaceae, such as *E. coli*. In addition to clinical problems, they cause environmental problems and the emergence of quinolone-resistant *E. coli* strains in the environment, leading to high

economic costs to control and prevent the spread of this strain. In addition to quinolone resistance genes, *E. coli* has also acquired carbapenem resistance genes, which are highly resistant in strains isolated from Hamedan. To prevent the spread of plasmids, a way must be found to avoid indiscriminate use of antibiotics and to change the type of antibiotics prescribed periodically. The use of out-of-date antibiotics, such as carbapenems (imipenem) and quinolones (ciprofloxacin), in veterinary medicine as food supplements has led to the emergence of resistance to these antibiotics. We can screen and prevent the spread of *E. coli* bacteria resistant to these antibiotics through animal and human transmission.

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