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Prevalence of antibiotic resistance and genetic relatedness of *Escherichia coli* isolates in HIV and thalassemia patients in southeastern Iran

Alizade, H.[1,2], Aflatoonian, M.R.[2], Ghanbarpour, R.[3], Ranjbar, E.[4], Rezaei, A.K.[3] and Sharifi, H.[5*]
[1]Infectious and Tropical Disease Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[2]Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran
[3]Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[4]Behavioral Disease Counseling Center, Kerman University of Medical Sciences, Kerman, Iran
[5]HIV/STI Surveillance Research Center, and WHO Collaborating Center for HIV Surveillance, Institute for Futures Studies in Health, Kerman University of Medical Sciences, Kerman, Iran
[*]Corresponding author e-mail: hsharifi@kmu.ac.ir
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Abstract. The present study was carried out to evaluate the prevalence of antibiotic resistance genes in *Escherichia coli* isolates from HIV and thalassemia patients and to determine the phylogenetic group distribution and to genotype the isolates in southeastern Iran. This cross-sectional study was performed in *E. coli* isolates taken from fecal and urine samples of 43 HIV and 62 thalassemia patients. The *E. coli* isolates were examined for 13 antibiotic resistance genes and determine the phylogroups. The Rep-PCR DNA fingerprinting method was utilized to determine the genotype of the isolates. Among the 105 *E. coli* isolates, 66.7% isolates were positive for *qnrS*, 55.2% for *dhfrI*, 40.9% for *sulI*, 33.3% for *sulII* and 31.4% for bla_{TEM} genes. A $bla_{CTX\text{-}M\text{-}15}$ gene was detected in 20.9% isolates, *aac(3)-I* in 14.3% isolates and *aadA* in 12.4% isolates, whereas bla_{SHV} and *qnrB* genes were identified in 10.5% and 8.6% isolates, respectively. Out of the isolates, only 2.8% isolates possessed the $bla_{OXA\text{-}1}$ gene, and no *IMP* and *VIM* genes were detected. The significant phylogroup was A (37.2%), B2 (15.3%), B1 and unknown (each 14.3%), D (13.4%) and C, F and clade I (each 2%). Phylogroup A accounted for the highest antibiotic resistance. The results of Rep-PCR indicated that the isolates were closely related. These results showed a high prevalence of genes encoding antibiotic resistance in the *E. coli* isolates. The majority of *E. coli* isolates distributed among phylogroup A, whereas positive isolates for antibiotic resistance genes were disseminated among various phylogroups (A, B2, and D).

INTRODUCTION

Multiple-antibiotic resistance is a growing problem and a serious threat to public health, particularly in settings where few treatment options are available (Mwambete & Kamuhabwa, 2013). In European Union countries, it is estimated that 25,000 patients die annually from an infection with multidrug-resistant bacteria (ECDPC/EMA, 2009). In the recent estimates of global antibiotic resistance published by the WHO in 2014, *Escherichia coli* (*E. coli*) was named as the greatest concern and is associated with hospital and community acquired infections (WHO, 2014).

Bacterial infections are well-known to cause complication and the leading cause of death in patients with thalassemia and HIV. Since bacterial infections in these patients are very common, high consumption of antibiotics can result in antibiotic resistance

(Wanachiwanawin, 2000; Musiime *et al*., 2009; Alizade *et al*., 2015a; Yah *et al*., 2008). Antibiotic resistance to several groups of antibiotics in HIV-infected patients has been proven in many countries (Yah *et al*., 2008). The increased predisposition of HIV patients to invasive bacterial isolates has been reported but there are no detailed studies on antibiotics resistance with HIV and thalassemia.

Because of the scarcity of available data on antibiotic resistance genes in the *E. coli* isolates from special diseases infected patients (HIV and thalassemia) in Iran, this study was performed to: (i) determine the magnitude of *E. coli* resistance to β-lactam, carbapenem, quinolone, aminoglycoside, sulfonamide and trimethoprim (ii) identify the phylogenetic groups of *E. coli* based on the new Clermont method, (iii) characterize the genetic diversity of *E. coli* isolates using Rep-PCR, and (iv) examine the relationships between antibiotic resistance genes in *E. coli* isolates from HIV and thalassemia patients in southeastern Iran.

MATERIALS AND METHODS

Study population and bacterial isolates

The *E. coli* isolates were obtained from HIV-infected patients (43 isolates; 38 male and five female) over a 5-month period (September to November 2014) from the Voluntary Counseling and Testing Center in Kerman, Iran. Isolates from thalassemia patients (62 isolates; 39 male and 23 female) were obtained over a 4-month period (September to December 2014) from the Charity Foundation for Special Diseases in Kerman, Iran. The *E. coli* isolates of HIV patients were recovered from fecal samples (n=40) followed by isolates from urine (n=3). Among the 62 *E. coli* isolates from thalassemia patients, 57 isolates were recovered from fecal samples and five isolates from urine samples. This study has an ethics approval certificate from the research council at the Kerman University of Medical Sciences, Kerman, Iran (Reference code K.93.668).

Molecular characterization of antimicrobial resistance genes

Template DNA was extracted using the lysis method. Several genes conferring resistance to different groups of antibiotics were examined using PCR: (i) β-lactam: $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, bla_{SHV} and bla_{TEM} genes; (ii) carbapenem: *VIM* and *IMP* genes; (iii) quinolone: *qnrB* and *qnrS* genes; (iv) aminoglycoside: *aadA* and *aac(3)-I* genes; (v) sulfonamide: *sulI* and *sulII* genes; and (vi) trimethoprim: *dhfrI* gene (Sharma *et al*., 2010; Garza-Ramos *et al*., 2008; Messai *et al*., 2006; Colom *et al*., 2003; Cattoir *et al*., 2007; Van *et al*., 2008).

Phylogenetic analysis

Bacterial genotypes were determined by analyzing the genomic DNA with PCR-based methods (Bhattacharya *et al*., 2003). Phylogenetic groups were determined as recently described by Clermont *et al*. (2013) using quadruplex PCR. Regarding the presence/absence of *arpA*, *chuA*, *yjaA*, and TspE4.C2 genes, an *E. coli* strain could be classified into one of the main phylogroups, A, B1, B2, C, D, E, F and clade I.

Rep-PCR

Among a variety of existing genotyping techniques, repetitive extragenic palindromic elements PCR (Rep-PCR) were used successfully to generate DNA fingerprints to distinguish between genetically unrelated and closely related bacterial isolates (Bhattacharya *et al*., 2003). Rep-PCR fingerprints were obtained by using a primer $(GTG)_5$ (52-GTG GTG GTG GTG-32) (Versalovic *et al*., 1994). The amplification reaction was carried out in MJ Mini thermal cycler (Bio-Rad) applying for the following amplification program: the initial denaturation at 95°C for 2 minutes and the next 30 cycles consisting of a denaturation step 94°C for 3 seconds and 92°C for 30 seconds; annealing at 40°C for 1 minute; and a single final extension step at 65°C for 8 minutes. Gel images were analyzed using CLIQS 1D Pro software (Total Lab, Newcastle upon Tyne, UK). Amplicon sizes were determined by comparison with a 1kb

DNA ladder (Fermentas) as an external reference standard. The dendrogram was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm.

Statistical analysis

Data were analyzed using descriptive statistics and 95% Confidence Interval (95% CI). Differences in antimicrobial resistance between HIV and thalassemia patients were compared using the Chi-square test, with a p-value<0.05 indicating statistical significance. Antimicrobial resistance genes data was managed using the Stata version 13.0 software.

RESULTS

Distribution of antimicrobial resistance genes

All the *E. coli* isolates were screened for the presence of antimicrobial resistance genes. In totally, 58 out of 62 (93.5%; 95% CI: 84.3–98.2) *E. coli* isolates obtained from thalassemia patients harbored at least one of the 13 resistance genes chosen for analysis. The most prevalent resistance gene in these patients was *dhfrI* (61.3%; 38/62), followed by *qnrS* (59.7%; 37/62). The aminoglycoside encoding genes *aadA*, and *aac(3)-I* were present in 17.8% (11/62) and 19.4% (12/62) of isolates, respectively. The extended spectrum beta-lactamases (ESBLs) encoding genes bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$ and bla_{SHV} were observed in 32.3% (20/62), 19.4% (12/62) and 12.9% (8/62) of isolates, respectively. The most common genotypes of sulfonamide were *sul1*, with the highest prevalence 37.1% (23/62), followed by *sulII* 30.6% (19/62). Only three isolates were positive for the *qnrB* and two for the $bla_{OXA\text{-}1}$ gene. None of the *E. coli* isolates were positive for *IMP* and *VIM* genes (Table 1).

Overall, 42 of 43 (97.7%; 95%CI: 87.7–99.9) HIV *E. coli* isolates were positive for at least one of the examined antimicrobial resistance genes. The presence of *qnrS* in 76.8% (33/43) of the *E. coli* isolates examined from HIV patients was the most prevalent represented. The *E. coli* isolates studied frequently exhibited resistance to antimicrobial agents, including *sulI* 46.5% (20/43), *dhfrI* 46.5% (20/43), *sulII* 37.2% (16/43), bla_{TEM} 30.2% (13/43), $bla_{CTX\text{-}M\text{-}15}$ 23.2% (10/43), *qnrB* 13.9% (6/43), bla_{SHV} 7% (3/43), *aac(3)-I* 7% (3/43). The resistance genes $bla_{OXA\text{-}1}$ and *aadA* were the least prevalent in one and two isolates, respectively. Neither *IMP* nor *VIM* was detected among the isolates of HIV-infected patients (Table 1).

Table 1. Distribution of antibiotic resistance genes among *E. coli* isolates in HIV and thalassemia patients

Gene	Thalassemia no (%)	HIV no (%)	Total no (%)
$bla_{CTX\text{-}M\text{-}15}$	12 (19.4)	10 (23.3)	22 (20.9)
$bla_{OXA\text{-}1}$	2 (3.3)	1 (2.4)	3 (2.9)
bla_{TEM}	20 (32.3)	13 (30.3)	33 (31.5)
bla_{SHV}	8 (12.9)	3 (6.9)	11 (10.5)
IMP	–	–	–
VIM	–	–	–
qnrS	37 (59.7)	33 (76.8)	70 (66.7)
qnrB	3 (4.9)	6 (13.9)	9 (8.6)
aadA	11 (17.8)	2 (4.7)	13 (12.4)
aac(3)-I	12 (19.4)	3 (6.9)	15 (14.3)
sulI	23 (37.1)	20 (46.6)	43 (40.9)
sulII	19 (30.7)	16 (37.5)	35 (33.4)
dhfrI	38 (61.3)	20 (46.6)	58 (55.3)

Distribution of phylogenetic groups

According to the results, the predominant phylogenetic group was A (37.2%; 39/105) followed by B2 (15.3%; 16/105), B1 and unknown (each 14.3%; 15/105), D (13.4%; 14/105) and C, F, and clade I (each 2%; 2/105). It is worth noting that strains from groups A (32.3%) and B1 (19.4%) were predominant among thalassemia patients, whereas strains from groups A (44.2%), B2 (18.7%) and D (14%) were found in HIV patients. As shown in Table 2, 62 *E. coli* isolates isolated from thalassemia patients and 43 isolates from HIV patients were allocated into seven phylogenetic groups.

Prevalence of antimicrobial resistance genes in phylogenetic groups

Phylogenetic analysis revealed that the most prevalent antibiotic resistance genes belonged to phylogroup A. The phylogenetic groups A and D were the predominant groups of the isolates possessed antibiotic resistance genes of thalassemia patients. In contrast, the number of HIV *E. coli* isolates belonging to phylo-genetic groups A and B2 were significant (Table 3).

Rep-PCR

The genomic diversity analysis of *E. coli* isolates was carried out with the use of the Rep-PCR fingerprinting method with a primer $(GTG)_5$. In general, the band patterns of isolates from HIV and thalassemia patients were very similar, and the data indicated that the isolates were closely related. Eighty-two isolates with identical patterns (100% similarity) were observed. The greatest genetic similarity was found among isolates belonging to A (27 isolates) in comparison with those in the B1 and B2 (15 isolates each) phylogenetic groups (Figure 1).

Correlation between antimicrobial resistance genes in HIV and thalassemia patients

Of the 62 isolates from thalassemia patients, 16 isolates possessed aminoglycoside genes. However, only four of the 43 isolates from HIV patients were positive for these genes (Table 2). The bivariate analysis confirmed that this difference between the number of isolates was significant ($p = 0.034$). Among *E. coli* isolates, the rate of positive isolates was more significant in thalassemia isolates than in HIV isolates for sulfonamide genes ($p = 0.283$). Of the thalassemia patient isolates, 33 were positive for ESBL genes; however, only 17 of the HIV patient isolates were positive to these genes ($p = 0.167$). There was a significant difference in resistance rate between thalassemia and HIV isolates to quinolone ($p = 0.013$). The positive rate to the trimethoprim gene in thalassemia *E. coli* isolates was similar to that in HIV isolates ($p = 0.134$).

Table 2. Distribution of HIV and thalassemia *E. coli* isolates in phylogenetic groups

Phylo-groups	HIV no. (%)	Thalassemia no. (%)	Total
A	19 (44.2)	20 (32.3)	39 (37.2)
B1	3 (7)	12 (19.4)	15 (14.3)
B2	8 (8.7)	8 (13)	16 (15.3)
C	1 (2.4)	1(1.7)	2 (2)
D	6 (14)	8 (13)	14 (13.4)
E	–	–	–
F	1 (2.4)	1 (1.7)	2 (2)
Clade I	–	2 (3.3)	2 (2)
Unknown	5 (11.7)	10 (16.2)	15 (14.3)
Total	43	62	105

Table 3. Details of positive *E. coli* isolates for antibiotic genes according to phylogenetic background

Gene	Isolates from HIV patients								Isolates from thalassemia patients							
	Phylotype															
	A	B1	B2	C	D	F	clade I	unkonwn	A	B1	B2	C	D	F	clade I	unknown
$bla_{CTX\text{-}M\text{-}15}$	3	1	3	–	2	–	–	1	4	4	2	–	–	–	–	2
$bla_{OXA\text{-}1}$	1	–	–	–	–	–	–	–	1	–	1	–	–	–	–	–
bla_{TEM}	5	2	4	-	2	–	–	–	8	4	4	1	2	–	–	–
bla_{SHV}	–	–	–	–	–	–	–	3	1	2	–	–	–	–	–	5
IMP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
VIM	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
qnrS	15	1	9	1	4	1	-	2	16	2	8	1	4	1	1	4
qnrB	4	1	–	–	–	–	–	1	1	–	1	–	–	–	–	1
aadA	1	–	1	–	–	–	–	–	2	2	1	–	4	–	–	2
aac(3)-I	–	1	2	–	–	–	–	–	6	2	–	–	3	–	–	1
sulI	11	1	1	–	3	1	–	3	8	1	2	–	5	1	2	4
sulII	8	–	2	–	4	–	–	2	6	1	3	–	5	–	1	3
dhfrI	8	2	3	1	4	1	–	1	11	10	6	1	6	–	2	2
Total	56	9	25	2	19	3	–	13	64	28	28	3	29	2	6	24

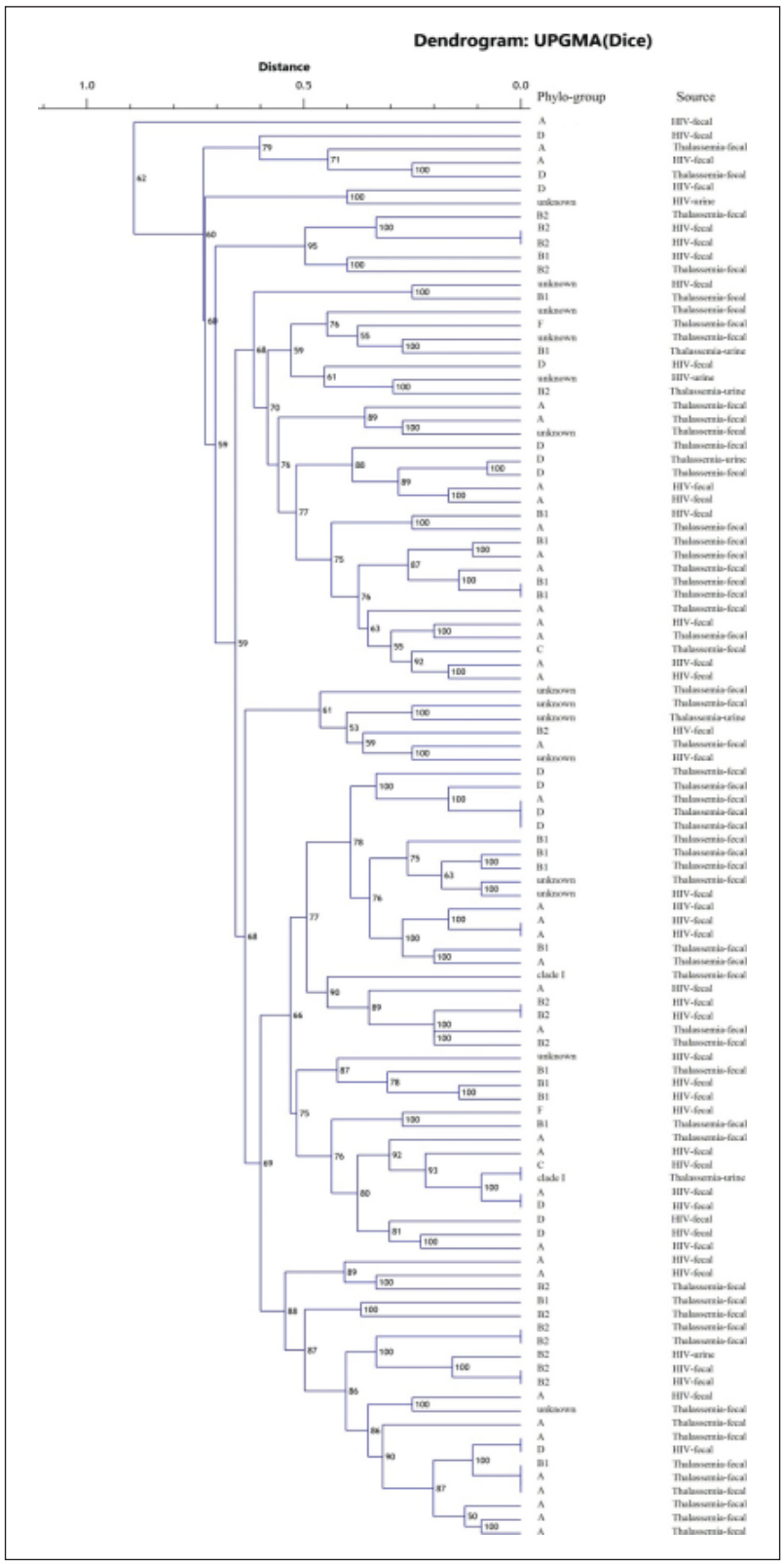

Figure 1. Cluster analysis of $(GTG)_5$-PCR fingerprints of HIV and thalassemia *E. coli* isolates.

DISCUSSION

The present study confirms a high carriage of antibiotic resistant *E. coli* from fecal and urine samples in HIV and thalassemia patients. The quinolone encoding gene *qnrS* was identified as one of the most common resistance genes among the studied isolates. The increase resistance to quinolone is threatening the clinical utility for treatment of various infections (Kim & Hooper, 2014). Fluoroquinolones resistance in *E. coli* isolates from clinical samples is very high in many European countries (25% to 50%) (Wiedemann *et al.*, 2014). The results of the present study shows high (88.4%) resistance rate to trimethoprim among the HIV *E. coli* isolates. Use of trimethoprim and sulfonamides (co-trimoxazole) is thought to have a synergistic effect (Huovinen, 2001). Co-trimoxazole has been used as a prophylactic agent against HIV patient's infections (Marwa *et al.*, 2015). The findings by Sibanda *et al.* (2011) showed that co-trimoxazole prophylaxis for opportunistic infections in HIV patients protects against resistance to other classes of antibiotics. The continuous use of co-trimoxazole among HIV patients is expected to increase resistance due to selective pressure (Marwa *et al.*, 2015). A rapid increase in the use of the prophylactic co-trimoxazole has resulted in an increase in the levels of resistance to this drug among all clinically significant bacterial species, especially *E. coli*, in the HIV units of San Francisco General Hospital (Martin *et al.*, 1999). The existence of ESBL-producing *E. coli* in the community has been one of the significant epidemiologic changes in infectious diseases worldwide (Ahmed *et al.*, 2013). Ahmed *et al.* (2013) suggests that the intestinal tract acts as a reservoir for ESBL-producing bacteria, and a trafficker of antibiotic resistance genes. The results of this study show that 47.7% of *E. coli* isolates were positive for ESBL genes among thalassemia and HIV patients. Cotton *et al.* (2008) in Cape Town, South Africa, evaluated the presence of antimicrobial resistance patterns of potentially pathogenic bacteria in nasopharyngeal swabs from HIV-infected children and found that 50% of *Enterobacteriaceae* produced ESBL (resistant to third generation cephalosporins). Another study showed the presence of gram-negative ESBL-producing bacteria infecting hematologic patients at the Amazon Hematology and Hemotherapy Foundation in the Amazon region in Brazil. These strains carried the bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{OXA} genes and were resistant to tetracycline, ciprofloxacin, cefepime and amikacin (Ferreira *et al.*, 2011).

Schouten *et al.* (1999) demonstrated that high-level resistance to gentamicin had been detected with prevalence ranging from 1% to 48% in all investigated European countries, noting that, there were no geographic relationships among the studied countries. According to the results of the current study, aminoglycoside genes were detected in less than one-fifth of the isolates, which is significantly lower than previously reported (Li *et al.*, 2015). Houghton *et al.* (2010), however, showed that novel aminoglycosides have a potential to overcome resistance as well as to be utilized in the treatment of HIV-1 and even human genetic disorders.

In Iran, like other developing countries, drug utilization is an important aspect of primary healthcare as governments have insufficient control of the drug supply system. Most developed countries have sufficient control of over-the-counter sales on many drugs including antibiotics. Unfortunately, in Iran almost any drug can be acquired in pharmacies without a prescription (Sharifi *et al.*, 2013).

Extended phylogenetic analyses in the current study showed that *E. coli* isolates fall into seven main phylogenetic groups (A, B1, B2, C, D, F and clade I), with most *E. coli* isolates belonging to the A group. This result is in accordance with other studies in Iran and worldwide (Alizade *et al.*, 2015b; Alizade *et al.*, 2014a; Alizade *et al.*, 2014b; Derakhshandeh *et al.*, 2013; Dhanji *et al.*, 2011). Several studies indicated that most commensal *E. coli* strains belong to groups A and B1 and extra-intestinal strains usually belong to group B2 and a lesser extent to group D (Dhanji *et al.*, 2011; Moreno *et al.*,

2006). In Germany the majority of the uropathogenic *E. coli* isolates, which were isolated from hospital inpatients and outpatients with a suspected urinary tract infection, fell into *E. coli* phylogenetic group B2 (49.4%), A (16.2%), B1 (15.5%), C (6.8%) and D (6.4%) (Toval *et al.*, 2014). Also, the prevalence of antibiotic resistance of *E. coli* strains was shown to be greater in non-B2 phylogenetic group shift towards group A (Giufre *et al.*, 2012). In this study, about 6% of *E. coli* isolates belonged to C, F, and clade I phylogenetic groups. We cannot find any study on utilizing a new quadruplex PCR method for phylotyping *E. coli* isolates in fecal and urine samples in HIV and thalassemia patients.

In general, the band patterns of *E. coli* isolates indicated that the isolates were closely related.

Approximately three-quarters of the bands had 100% similarity, and a few bands shared a more than 70% similarity. According to the Rep-PCR results, the greatest genetic similarity was found among isolates belonging to the A phylogenetic group. Also, phylotype analysis revealed that the most prevalent antibiotic resistance genes fell into phylogroup A.

To the best of our knowledge, this study is the first study of antibiotic resistance genes among *E. coli* isolates in HIV and thalassemia infected patients in Iran. In summary, our study shows a high variability of genes encoding antibiotic resistance in the *E. coli* isolates in HIV and thalassemia patients. The frequency of *qnrS*, *dhfr* and *sulI* genes was higher than the other genes in analyzed *E. coli* isolates. The majority of *E. coli* isolates belonged to phylogroup A, whereas positive isolates for antibiotic resistance genes were found among various phylogenetic groups (A, B2 and D). Finally, our findings demonstrate that the spread of *E. coli* possessing antibiotic resistance genes in HIV and thalassemia patients is complex and diverse, complicating the challenge of control. Future studies are needed to understand better the antibiotic resistance and phylogeny in *E. coli* isolates.

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