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ORIGINAL ARTICLE

Characterization of integrons, extended spectrum beta lactamases and genetic diversity among uropathogenic *Escherichia coli* isolates from Kerman, south east of Iran

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ABSTRACT

Background and Objectives: The study aimed to investigate the distribution of genes encoding integrons, extended spectrum beta-lactamase (ESBL) in *E. coli* isolated from UTIs, as well as the genetic diversity among the isolates.

Materials and Methods: *E. coli* isolates were recovered from the patients with UTI in Kerman Iran. Antibiotic susceptibility was done according to CLSI guidelines. The presence of ESBL genes and integrons was evaluated using PCR. PCR and sequencing were applied for the evaluation of cassette content of integrons. Genotyping of the isolates was performed by multiple-locus variable-number tandem repeat analysis (MLVA).

Results: Imipenem was the most effective antibiotic, while the highest resistance was observed to streptomycin. In total 40.2% of isolates were ESBL producers. Of 69 integron-positive isolates, 59 only had class I integrons, 4 only had class II integrons and 6 had both types. The most common gene cassette found within class I integrons was *dfrA17-aadA5* (n=27). The *E. coli* isolates were divided into 16 MLVA clusters.

Conclusion: The current study demonstrated the simultaneous presence of class I integrons and ESBLs involved in the resistance of UPEC isolates to antibacterial agents. Our finding also revealed that the *E. coli* isolates belonged to diverse clones.

Keywords: Uropathogenic *Escherichia coli*; Drug resistance; Integrons; Extended spectrum beta-lactamase; Tandem repeat sequences

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INTRODUCTION

Urinary tract infections (UTIs) are the second most prevalent community-acquired infection and the main cause of nosocomial infection (1). UTIs are caused by a range of bacteria, but *Escherichia coli* is the most frequent urinary tract pathogen, accounting for 75% to 95% of UTI cases (2). Treatment of UTI has become problematic in recent years due to the increased resistance to the first-line antimicrobial agents used for UTI empirical therapy, including beta-lactams, trimethoprim-sulfamethoxazole (SXT), and fluoroquinolones (3, 4). Dissemination of antibiotic resistance genes by horizontal transfer has led to alarming rates of antibiotic resistance among pathogenic bacteria (5). Resistance genes are distributed by plasmids or transposons and also can be integrated by using a site-specific recombination mechanism into DNA elements designated integrons (6).

Integrons play a major role in the acquisition, expression, and dissemination of antibiotic resistance genes among bacterial populations (5). Integrons are composed of three different elements: an integrase gene (intI) that mediates gene cassette integration and excision through site-specific RecA-independent recombination; an attI recombination site where cassettes are inserted; and a Pc promoter responsible for gene cassette expression (5). To date, four classes of integrons have been identified based on their integrase (intI) gene (5). Class I (intI I) and class II (intI II) integrons are commonly detected in Gram-negative bacteria, including *E. coli,* and are responsible for conferring resistance to multiple antibiotics (7).

Extended-spectrum β-lactamases (ESBLs) are a group of diverse and rapidly evolving enzymes that can cause resistance to various types of β-lactam antibiotics, including third-generation cephalosporins and monobactams (1). ESBL-producing *E. coli* is a serious problem in public health since these ESBL genes can be spread by plasmid-mediated integrons, insertion sequence (IS) elements, and transposons among different bacteria species causing outbreaks as well as sporadic infections (8). Most ESBLs have evolved by genetic mutation from narrow spectrum β-lactamase, especially $bla_{\text{TEM-1}}$, $bla_{\text{TEM-2}}$, and $bla_{\text{SHV-1}}$. In the late 1980s, a new ESBL, $bla_{\text{CTX-M}}$ enzyme was discovered, which is associated with community-acquired urinary tract infections. The $bla_{\text{CTX-M}}$β-lactamases play an important role in resistance to third-generation cephalosporin, especially cefotaxime (9).

Molecular typing methods are frequently utilized to establish the clonal relationships between isolates for the purpose of epidemiological investigation. Multilocus variable-number tandem-repeat analysis (MLVA) is a PCR-based method that has been successfully used for the genotyping of numerous bacterial pathogens including *E. coli* (10-12).

Understanding the molecular mechanism of resistance is important to develop new techniques for preventing the spread of resistance determinants among pathogens. In this study, the aim was to determine the antibiotic susceptibility pattern, prevalence of ESBL genes, and class I and II integrons among *E. coli* isolated from patients with UTI in Kerman, Southeast of Iran, as well as the genetic relationship among these isolates.

MATERIALS AND METHODS

Bacterial isolates. The study was conducted from March 2020 to September 2021 in Afzali-Pour hospital in Kerman, Iran. A total of 102 non-repetitive *E. coli* isolates were obtained from inpatients who presented with symptomatic UTI. The isolation and identification of *E. coli* strains were performed using standard microbiological and biochemical tests (11). Confirmed *E. coli* isolates were kept frozen in tryptic soy broth (Merck Co., Germany) containing 20% glycerol (Merck KGaA, Germany) at - 70°C until further experiments.

Antibiotic susceptibility testing. Antibiotic susceptibility testing was performed by Kirby-Bauer disk diffusion technique according to the CLSI guidelines (13). The following antibiotics (Mast, United Kingdom) were used: ampicillin (10 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), streptomycin (10 µg), imipenem (10 µg), trimethoprim-sulfamethoxazole (25 µg), ciprofloxacin (5 µg), and gentamicin (10 µg). *E. coli* ATCC 25922 was used as the quality control.

Detection of ESBL producers by combination disc method. The *E. coli* isolates with resistance to any of the tested third-generation cephalosporins were further screened for the production of ESBL by combined disc method using cefotaxime (30 mg) and ceftazidime (30 mg) alone and in combination

with clavulanic acid (10mg). ESBL production was interpreted if the zones produced by the disks with clavulanic acid were ≥5 mm larger than those without inhibitors (10). *Klebsiella pneumoniae* ATCC 700603 was used as an ESBL positive and *E. coli* ATCC 25922 as a negative control.

Molecular detection of β-lactamase and integrin genes and sequencing of resistance-encoding gene cassettes. Genomic DNA templates were extracted using the boiling method (14). PCR was performed to detect β-lactamase genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}) and *intI* I and *intI* II. Integron-positive isolates were further subjected to PCR and sequencing using specific primers for the analysis of gene cassette arrays. For sequencing, PCR products were purified and direct sequencing of internal variable regions (gene cassettes) of class I and II integrons was done using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea). Nucleotide sequences were analyzed and compared with all available sequences in the GenBank database. The primer sequences used in this study are shown in Table 1.

Multi-locus variable number tandem repeat analysis (MLVA). Seven VNTR loci (ms06, ms07, ms09, ms11, ms21, ms23, and ms32) were selected and amplified using the primers indicated in Table 2. PCR was carried out according to a method previously described (15). After performing PCR, the size of each amplicon was assessed on a 1.5% agarose gel and the number of repeats was calculated using the criteria of Gorge et al. (16). Data were entered into Microsoft Excel and analysed with Bionumerics software (Applied Maths, Saint-MartensLatem, Belgium). In our study, any difference in one or more VNTR loci was regarded as a distinct type, and genetic similarity of 50% was considered as the cut-off value for clustering.

Statistical analysis. The study utilized SPSS version 20 to analyze data. Qualitative data were presented as frequency and percentage, and the chi-square test was used to determine the statistical significance of qualitative variables. A P-value of less than 0.05 was considered significant.

RESULTS

Antibiotic susceptibility testing. Among the antibiotics tested in this study, streptomycin had the least antimicrobial effect as 72.5% of the isolates were resistant to this antibiotic. The rates of resistance to other antibiotics were as follows: ampicillin (71.6%), trimethoprim/sulfamethoxazole (67.6%), ceftriaxone (42.2%), ceftazidime (39.2%), ciprofloxacin (28.4%) and gentamicin (14.7%). All *E. coli* isolates were sensitive to imipenem. Altogether, 30 (29.4%) isolates were characterized as MDR.

ESBL screening. The ESBL phenotypic screening by double disc synergy test indicated that 41 (40.2%) of isolates were ESBL producers. Overall, ESBL-pro-

Table 1. Nucleotide sequences of the primers used for PCR

Genes	Oligonucleotide sequence (5'→3′)	Size of amplicon (bp)
bla_{SHV}	F: 5'- TCAGCGAAAAACACCTTG-3'	471
	R: 5'- TCCCGCAGATAAATCACC-3'	
bla_{TEM}	F: 5'- GAGTATTCAACATTTCCGTGTC-3'	861
	R: 5'- TAATCAGTGAGGCACCTATCTC-3'	
bla_{CTXM}	F: 5'- CGCTTTGCGATGTGCAG R-3'	550
	R: 5'- ACCGCGATATCGTTGGT-3'	
intI	F: 5'-GCCTTGCTGTTCTTCTACGG-3'	558
	R: 5'-GATGCCTGCTTGTTCTACGG-3'	
intII	F: 5′-CACGGATATGCGACAAAAAGGT-3′	789
	R: 5′-GTAGCAAACGAGTGACGAAATG-3′	
CS	F: 5′-GGCATCCAAGCAGCAAGC-3′	Variable
	R: 5′-AAGCAGACTTGACCTGAT-3′	
hep	F: 5′-CGGGATCCCGGACGGCATGCACGATTTGT-3′	Variable
	R: 5′-GATGCCATCGCAAGTACGAG-3′	

Table 2. List of locus-specifc PCR primers selected for MLVA assay

Genes	Oligonucleotide sequence (5'→3′)	Repeat Sizes at Each Locus, bp
ms06	F: 5'-AAACGGGAGAGCCGGTTATT-3′	39
	R: 5'-TGTTGGTACAACGGCTCCTG-3′	
ms07	F: 5'-GTCAGTTCGCCCAGACACAG-3′	39
	R: 5'-CGGTGTCAGCAAATCCAGAG-3	
ms09	F: 5'-GTGCCATCGGGCAAAATTAG-3′	179
	R: 5'-CCGATAAGGGAGCAGGCTAGT-3′	
ms11	F: 5'-GAAACAGGCCCAGGCTACAC-3′	96
	R: 5'-CTGGCGCTGGTTATGGGTAT-3′	
ms21	F: 5'-GCTGATGGCGAAGGAGAAGA-3′	141
	R:5'-GGGAGTATGCGGTCAAAAGC-3′	
ms23	F: 5'-GCTCCGCTGATTGACTCCTT-3′	375
	R: 5'CGGTTGCTCGACCACTAACA-3′	
ms32	F: 5'-GAGATTGCCGAAGTGTTGC-3′	101
	R:5'AACTGGCGGCGTTTATCAAG-3′	

ducing isolates showed significantly higher rates of resistance towards ciprofloxacin (56.1%), gentamicin (36.6%), and trimethoprim-sulfamethoxazole (95.1%) compared to non-ESBL producers ($p < 0.05$). The rate of MDR was significantly higher (P-value < 0.05) among the ESBL-producers (65.9%) than non-ESBL-producing (4.9%) isolates.

Molecular investigation of β-lactamase-encoding genes revealed that a high number of ESBL-producing *E. coli* isolates carried bla_{CTX-M} (n=36, 87.8) and bla_{TEM} (n=35, 85.3). bla_{SHV} was found in only one (2.4%) isolate. Three isolates that were positive for the bla_{TEM} were not phenotypically ESBL producers.

Detection of integrons. Of the 102 *E. coli* isolates, 69 (67.6%) carried integrons. Out of 69 integron-positive isolates, 59 isolates contained only class I integrons, four isolates contained only class II integrons, and six isolates harbored both class I and II integrons. The results showed a significant association between ESBL and class I integrons (P-value < 0.05). Also, the carriage of class I integrons was found to be significantly higher in MDR isolates (P-value < 0.05).

We amplified cassette regions of class I and II integrons by primers 5'CS/3'CS and hepF/hepR, respectively. Gene cassettes of class I integrons were detected in 47 (72.3%) of the 65 isolates harboring class I integrons. Four different amplicons were obtained in class I integrons with the following size, 650 bp (2 isolates), 750 bp (10 isolates), 1500 bp (34 isolates) and 2900 bp (1 isolate). Six different cassette arrays were detected within the class I integrons. The most common gene cassette found within class I integrons was *dfrA17-aadA5* (n=27), followed by *aadA1- dfrA1* (n=7), *dfrA17* (n=6), *dfrA7* (n=4), *dfrA5* (n=2) and *dfrA32-ereA-aadA2* (n=1). Only two isolates carried a class II integrase gene along with a 2100 bp variable region containing *dfrA1-sat1-aadA1* gene cassette.

MLVA assay. UPGMA cluster analysis revealed high genetic diversity among UPEC isolates. The isolates were divided into 94 unique MLVA types. The analysis revealed also, Sixteen MLVA clusters (clusters A to P), each containing 2 or more than 2 isolates, and five singletons. The dominant cluster, cluster F, included 18 isolates (Fig. 1). Most of the isolates in the same cluster were different in the combination of resistance genes. MLVA assay set-up for the ms32 locus is shown in Fig. 2. The image represents how the repeat copy numbers can be easily deduced by manual reading.

DISCUSSION

Antimicrobial resistance is a serious problem in the treatment of urinary tract infections (5). UTIs are generally treated empirically by physicians, therefore understanding the local epidemiological data for efficient therapy is necessary and useful (4). UPEC isolates in this study were highly resistant to streptomycin (72.5%), ampicillin (71.6%), and trimethoprim-sulfamethoxazole (67.6%). These findings are comparable to those of Oliveira-Pinto et al. and

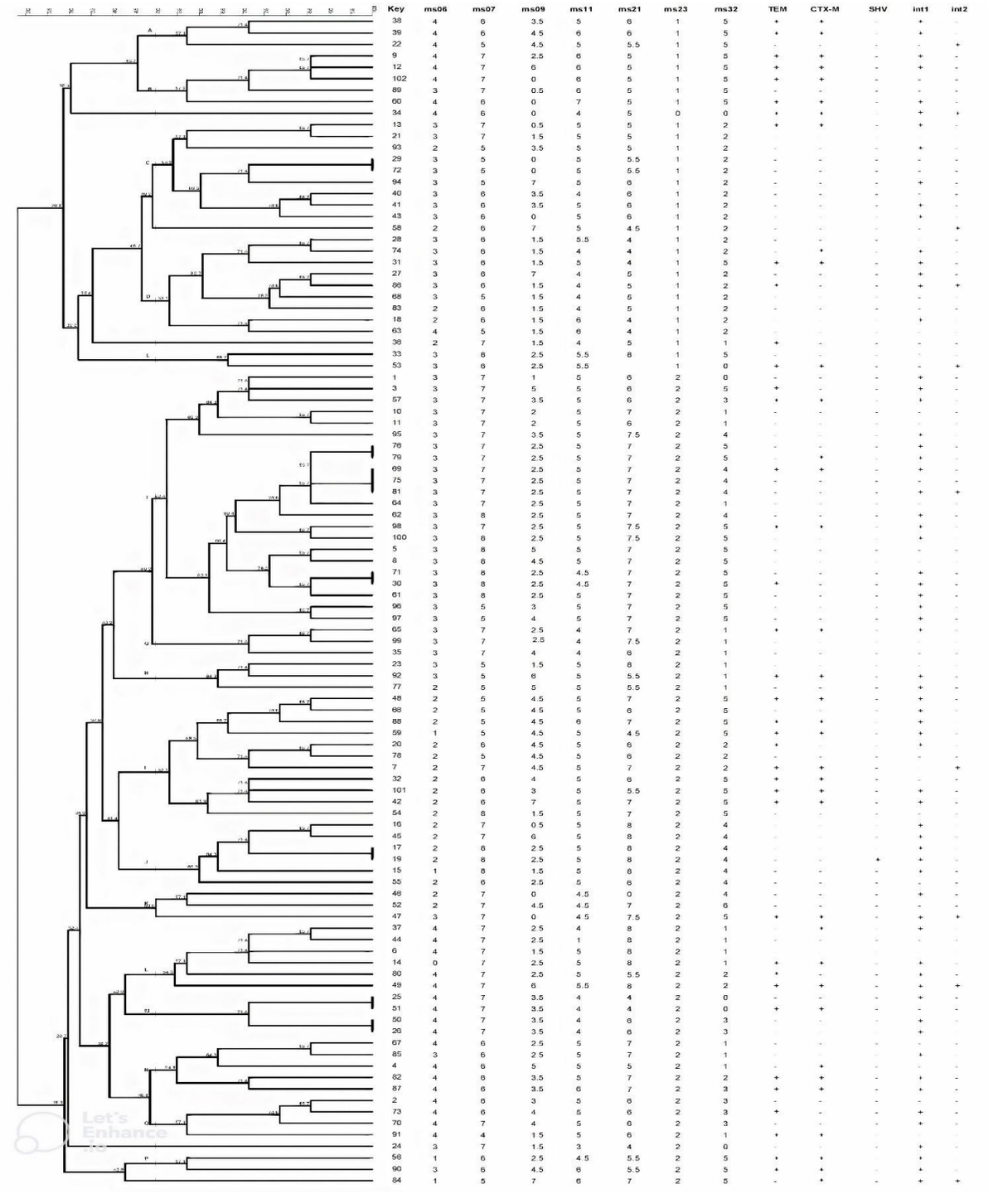

Fig. 1. UPGMA dendrogram for the 102 UPEC isolates based on seven VNTR loci. Clusters were defined using a similarity cut-off value of 50%, as indicated by the vertical line. The assigned clusters are represented by letters. Strain numbers, integron, and ESBL genes of these isolates are also indicated (+, positive and -, negative).

Kumar et al. who have reported high resistance of UPEC isolates to ampicillin and trimethoprim-sulfamethoxazole (17, 18). This high resistance may be due to the inappropriate and uncontrollable use of these antibiotics (18). In the present survey, imipenem was the most effective antibiotic, as all the investigated isolates were susceptible to it. A high level of susceptibility of UPEC strains to imipenem has been

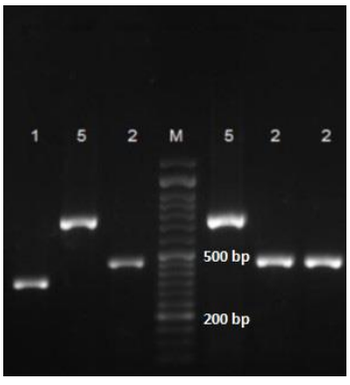

Fig. 2. An Example of Gel Electrophoresis of VNTR Products. The image shows how the number of repeats can be directly deduced by manual reading. The numbers above the PCR amplicons represent the repeat numbers of ms32 locus. Lanes M, DNA markers

also reported in Iran and other countries (4, 19-21). Despite the good activity of imipenem against UPEC isolates, this antibiotic is not suggested for routine use in the treatment of UTIs due to the concern of emerging resistance. However, imipenem is recommended as the drug of choice for only ESBL-producers (3, 4).

In the present study, 34.3% of isolates were identified as MDR. Although the percentage of MDR isolates was lower than the pooled percentage of MDR uropathogenic *E. coli* with 49.4% in Iran, it has been determined that the frequency of MDR isolates varied from 10.5% to 79.2% in different regions (20).

Over the past two decades, extended broad-spectrum antibiotics have been widely used to counter the increasing rates of ESBL-producing bacteria in patients with UTIs worldwide (22). The percentage of ESBL-producing *E. coli* ranges from 2.16% in Switzerland (23) to 83% in India (24). Our prevalence rate of ESBL producing *E. coli* (40.2%) is close to the findings reported by previous studies in different parts of Asia, including 38.9 % from Nepal (25), 37% from Iran (26), and 41.4% from Turkey (27). This prevalence of ESBL-producing UPEC isolates is consistent with the median values reported from Iran varied from 24% to 72.9% among UPEC (20, 28). Extremely higher rates of ESBL-producing *E. coli* (79%) have also been reported by Hawser et al. from India (29). The global differences in the prevalence of ESBL-producing UPEC strains could be partly due to variations in geographical regions, infection control policies, antibiotic usage patterns, and sample size (20, 28). An issue that arises a great concern about ESBL-producing bacteria is the high rate of cross-resistance to non-ß-lactam antibiotics such as trimethoprim-sulfamethoxazole, aminoglycoside, and ciprofloxacin (30).

It is noteworthy that in this study, the bla_{CTXM} and the bla_{TEM} were the most common ESBL enzymes detected in the majority of ESBL producing isolates. Our data are consistent with several other studies indicating that the bla_{CTXM} and the bla_{TEM} are more prevalent than the bla_{SHV} among *E. coli* isolates (9, 31-36). In the current study, while 40.2% of the isolates showed ESBL activity, no cassette encoding ESBLs was found, indicating that ESBL genes were rarely carried by integrons (36). However, we found a correlation between ESBL production and the presence of class I integron. This finding could be due to the co-carriage of ESBL genes and integrons on the same plasmids (37). The results also showed that three isolates that carried bla_{TEM} were not phenotypically ESBL producers. These three bla_{TEM} types were assumed to be $bla_{\text{TEM-1}}$, although sequence analysis would be needed to confirm this possibility.

Integrons play an important role in the dissemination of antimicrobial resistance and the formation of MDR bacteria. Therefore, we evaluated the prevalence of class I and II integrons among the 102 UPEC isolates. In this study, the frequency of integrons of class I and II was 63.7% and 9.8%, respectively. Of the 65 class I integron–positive isolates, 47 had gene cassettes, and six different gene cassette arrays were detected. Sequence analysis revealed the different variants of *aadA* (*aadA1, aadA2*) and *dfrA* (*dfrA5, dfrA7, dfrA17, dfrA32*) gene cassettes. *aadA* and *dfrA* gene cassettes are the most commonly identified class I integrons in Gram-negative bacteria, accounting for up to 63% of all *E. coli* class I integrons (38). These two gene cassettes are extremely stable, even in the absence of selective pressure, because of their negligible fitness costs (38). All isolates that were positive for class I integrons harbored cassettes with *dfrA* gene. The *dfrA* gene encodes a dihydrofolate reductase that confers resistance to trimethoprim, an antibiotic largely used for the treatment of UTIs (19).

We observed *dfrA17-aadA5* as the predominant class I integron gene cassette array in *E. coli* isolates, which is similar to some previous studies (17, 39, 40). Sunde et al. found *dfrA17-aadA5* as the most prevalent gene cassette array among *E. coli* isolates of human origin but not isolates from meat. They concluded that this class of integron array may be linked

to *E. coli* subpopulations, which can cause severe infection, or highly adapted to the colonization of the human host (39).

In contrast to class I integrons, class II integrons are less common in enteric bacteria and are usually associated with genes encoding resistance to trimethoprim (*dfrA1*), streptothricin (*sat2*), and streptomycin (*aadA1*) (41). Only two isolates containing the most prevalent class II integrons gene cassette array (*dfrA1-sat2- aadA1*) were identified. The low diversity of class II integrons could be due to a non-functional integrase gene with a stop codon at position 179, which produces an inactive 178-amino-acid polypeptide (38, 42). Of the all integron-positive isolates, 27.7% of class I positives and 80% of class II positives harbored empty integrons and did not carry any gene cassettes. The presence of empty integrons indicates the potential of these strains to capture resistance gene cassettes and increase the ability of bacteria to adapt to different ecological niches, especially in hospital settings due to antibiotic selective pressures (17, 43).

MLVA is an inexpensive and easy-to-perform genotyping method that can be used for the rapid and efficient genotyping of *E. coli* strains (15). In this study, MLVA based on a set of seven VNTR loci, was successfully applied to type 102 *E. coli* isolates. Sixteen MLVA clusters (clusters A to P), each containing two or more than two isolates, and six singletons were identified. MLVA patterns represented high heterogeneity and genetic diversity among *E. coli* isolates. Previous studies have also revealed that UPEC strains are genetically heterogeneous (36, 44). High levels of genetic diversity among these strains showed that multiple clones within the community have acquired the resistance genes. No obvious correlation was found between ESBLs or integrons carriage with MLVA clusters. This may be due to the fact that many of the resistance genes are found on plasmids, transposons, or integrons, which can be disseminated by horizontal gene transfer (45).

CONCLUSION

In conclusion, the current study demonstrated the simultaneous presence of class I integrons and ESBLs involved in the resistance of UPEC isolates to several antibacterial agents. Our finding also revealed that the *E. coli* isolates belonged to diverse clones.

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