Multilocus Variable Number of Tandem Repeat Analysis for molecular typing of uropathogenic *Escherichia coli*

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Abstract

Background: The aim of this study was genotyping of Uropathogenic *Escherichia coli* (UPEC) based on Variable Number of Tandem Repeats (VNTRs) sequences.

Methods: *E. coli* strains isolated from urine samples were included in this study. Seven VNTR loci were subjected to Multilocus variable-number tandem repeat analysis (MLVA) based on PCR amplification. Then data was analyzed via online mlvaplus software and the information was displayed in the form of MST analysis.

Results: A total of 100 *E. coli* strains were isolated and subjected to the study. MLVA was able to differentiate 56 different genotypes. Also, the technique could classify *E. coli* isolates in 5 clonal complexes. Based on UPGMA dendrograms, *E. coli* isolates were classified into 4 clusters (clusters A to D). The strains associated with Complex No. 1 appeared to be dominant pathogens of UPEC in Tehran's patients. The present study provides valuable insights into the genetic relationships of *E. coli* isolates recovered from clinical cases in a major hospital in Iran.

Conclusions: The analysis of MLVA profiles using the MST algorithm showed the usefulness of the MLVA method in the classification of uropathogenic *E. coli* collected in different periods. We evaluated MLVA in a laboratory equipped with simple molecular equipment. Based on these results, it has been assumed that the *E. coli* strains were derived from a limited number of clones that have undergone a small genetic change during this period.

Background

Escherichia coli (*E. coli*), a flagellated member of *Enterobacteriaceae*, is an essential member of the normal flora in the human gut, but many kinds of symptoms are caused by various *E. coli* pathotypes with a wide range of virulence factors. For instance, septicemia causing *E. coli* (SCEC) and neonatal meningitis producing *E. coli* (NMEC) are ExPEC pathotypes as the main cause of septicemia and pediatric meningitis respectively. Another important member of EXPEC pathotypes is uropathogenic *E. coli* (UPEC) which causes urinary tract infections (UTIs) [1–6].

There are several methods for typing of *E. coli* from environmental samples including traditional and modern molecular methods [7–13]. With the advent of the molecular age, scientists changed their approach from phenotyping to genotyping methods [14, 15].

Different molecular techniques such as Repetitive element sequence-based PCR (rep-PCR), Random Amplified Polymorphic DNA (RAPD)-PCR, Ribotyping, Pulsed-field gel electrophoresis (PFGE), and Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) have been used for genotyping of different strains of *E. coli* up to now. Among them, the MLVA method has been proposed as a new molecular method for bacterial genotyping [16, 17]. In MLVA technique, only a PCR-based method is required, while other sophisticated method such as PFGE need specialized and expensive equipment's. In

this way, MLVA is a low-cost alternative to PFGE, especially in developing countries [18]. It can also be used in simple laboratories that are only equipped with a PCR machine [19]. In this study, we tried to design a new, fast, and low-cost method for genotyping of uropathogenic *E. coli* by choosing suitable VNTR repeat sequences.

Methods

Sample collection and *E. coli* isolation

Over a 12-month sampling period (from October 2018 to October 2019), *E. coli* isolates were recovered from patients with UTI in a major hospital in Tehran, Iran and subjected to the current study. Isolation of the *E. coli* was done by conventional standard biochemical and serological methods. Well-isolated colonies of purified *E. coli* were resuspended in trypticase soy broth with 20% glycerol and stored in 70 °C for long-term storage [8].

DNA preparation

A pure culture of *E. coli* was plated on nutrient agar (Merck, Germany) and incubated overnight at 37 °C. A single colony was removed from the plate, suspended in 200 µl of sterile deionized water, and boiled for 15 min. After centrifugation at 6,000 g for 8 min, the supernatant was transferred into a new tube for subsequent PCR analysis [20]. Purity (A260/A280) and concentration of extracted DNA were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA). The truth of the DNA was assessed on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany).

MLVA assay

For genotyping, using the MLVA technique, seven loci VNTRs were used based on the protocol of O Gorgé, S Lopez, V Hilaire, O Lisanti, V Ramisse and G Vergnaud [21]. These seven VNTR loci were: ms06, ms07, ms09, ms11, ms21, ms23 and ms32. The primer sets for PCR amplification of these VNTR loci were previously reported by O Gorgé, S Lopez, V Hilaire, O Lisanti, V Ramisse and G Vergnaud [21] (Table 1). PCR was performed in 25 µl volume including 1X PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH = 9), 2.5 mmol/L MgCl₂, 0.2 mmol/L of each primer with 1 U of TaqDNA polymerase (CinnaGen Co., Iran), and 4 µl of the crude DNA extract.

Table 1
MLVA primers and annealing temperatures for PCR reactions.

MLVA locus	Forward primer 5' to 3'	Reverse primer 5' to 3'	bp	Annealing temperature (°C)
ms06	AAACGGGAGAGCCGGTTATT	TGTTGGTACAACGGCTCCTG	39	55
ms07	GTCAGTTCGCCCAGACACAG	CGGTGTCAGCAAATCCAGAG	39	
ms09	GTGCCATCGGGCAAATTAG	CCGATAAGGGAGCAGGCTAGT	179	
ms11	GAAACAGGCCCGAGGCTACAC	CTGGCGCTGGTTATGGGTAT	96	
ms21	GCTGATGGCGAAGGAGAAGA	GGGAGTATGCGGTCAAAAGC	141	
ms23	GCTCCGCTGATTGACTCCTT	CGGTTGCTCGACCACTAACA	375	
ms32	TGAGATTGCCGAAGTGTTGC	AACTGGCGGCGTTTATCAAG	101	

We introduced the number of replicates for each locus in each isolate, along with other characteristics of the isolates into Microsoft Excel, and then put these data on the WWW.mlvaplus.net site. The frequency of VNTR loci repeats is entirely different from each other by electrophoresis on the agarose gel. MST is a convenient complementary tool to cluster multiple isolates and visualize the relative diversity within different lineages.

A dendrogram of genetic relationships was also generated using the unweighted pair group method with arithmetic averages (UPGMA) method [22]. Furthermore, Simpson's index of diversity (D) and 95% confidence intervals (CI) for each VNTR locus were calculated using version 2.0 phyloviz software (<http://www.phyloviz.net>). For dendrogram analysis, the UPGMA method and Dice similarity coefficient were used to analyze VNTR data [23, 24].

Results

MLVA analysis

One-hundred *E. coli* strains were identified, of these, 70 isolates were belonged to the patients with complete biographical information and subjected to MLVA. Figure 1 describes the polymorphism of 3 VNTR loci in different *E. coli* isolates. As the result of PCR, the DNA band size distributions of the 70 *E. coli* strains for each VNTR were 301–379 bp (ms06), 392–821 bp (ms07), 445–1432 bp (ms09), 718–958 bp (ms11), 233–1492 bp (ms21), 584–959 bp (ms23), and 355–810 bp (ms32). Simpson's index of diversity, confidence Interval 95%, number of different alleles per locus and Tandem repeat (TR) size have been counted (Table 2).

Table 2
VNTR locus, tandem repeat size, Simpson's index of diversity and confidence interval 95%.

VNTR locus	TR size (bp)	Simpson's index of diversity	confidence Interval95%	No. of alleles	Null alleles
ms06	39	0.6301	0.587, 0.6732	2 (3, 4)	yes
ms07	39	0.5921	0.498, 0.6862	3 (7, 8, 9)	No
ms09	179	0.8066	0.7453, 0.868	5 (2, 2.5, 5, 1, 4)	Yes
ms11	96	0.681	0.6234, 0.7387	3 (5, 4, 6)	no
ms 32	101	0.7038	0.6315, 0.7761	7 (1, 1.5, 2, 4, 4.5, 5, 5.5)	No
ms 23	375	0.2532	0.1271, 0.3792	2 (1, 2)	no
ms 21	141	0.8073	0.747, 0.8676	3 (5, 7, 4)	no

VNTR analysis

The analysis of the number of VNTR repetitions using the MST algorithm showed that in 70 isolates of *E. coli*, 56 different genotypes were observed. However, due to the high similarity of the profile of isolated *E. coli* with each other and also for the better separation of complex colonies, the criterion of difference in a locus for grouping isolates (multiple clones) was considered. Accordingly, *E. coli* strains were classified in 5 clonal complexes.

Evolutionary relationship between isolates

Each complex group represents the evolutionary relationship between isolates. In other words, isolates within each group have a genetic correlation that is closer to each other and is likely to have the same origin. In our study, Complex No. 1 was the largest clonal group (Fig. 2). *E. coli* isolates were classified in 4 clusters (A cluster to D) based on the similarity (Fig. 3).

Discussion

In this study, we successfully applied the MLVA method to analyze uropathogenic *E. coli* strains isolated from clinical samples.

MLVA method does not require a high level of expertise and is capable to provide reasonable results about monitoring of outbreaks and clonal spread of bacterial isolates over a short period of time. It has been successfully applied to investigate the clonal relationship and epidemiology of clinical *E. coli* isolates [21, 25, 26]. In this study, the MLVA technique was able to detect 56 different genotypes among 70 *E. coli* strains isolated from patients with UTI in a major hospital in Tehran, Iran, indicating a high diversity of UPEC isolates genotypes.

Also, MLVA technique could classify the stains in 5 clonal complexes. In examining UPGMA dendrograms, uropathogenic *E. coli* isolates were classified according to the similarity in 4 clusters (clusters A to D). Each complex group represents an evolutionary relationship between the isolates. In other words, isolates belonging to each group have a genetic association with each other and are likely to have the same origin. In our study, Complex No. 1 was the largest clonal group. The strains associated with Complex No. 1 appeared to be the dominant pathogens of UPEC in the hospital under study.

In the present study, we used markers that can be easily separated by electrophoresis on the agarose gel. On the other hand, the evolution rate of different VNTRs differentiated the clonal relationships. Typically, loops that have larger replication sizes are slower than circuits that have smaller duplicate sizes. But loci with a slower evolutionary velocity are suitable for the investigation of clonal relationships between isolates that have evolved over a more extended period. Therefore, the type of VNTR locus is also in the analysis [27, 28].

The applied technique could classify *E. coli* isolates in 5 clonal complexes. Each complex group represents the evolutionary relationship between isolates. In other words, isolates belonging to each group have a genetic link with each other and are likely to have the same origin. In our study, Complex No. 1 was the largest clonal group. The strains in Complex No. 1 appeared to be the prevalent strains of UPEC disease, with about two-thirds of the population receiving all strains.

The ms32 has the highest number of alleles. The ms06 and ms23 loci have the smallest amount of alleles, and the ms06, ms09, ms21, and ms23 loci have null alleles. In the dendrogram analysis, The UPGMA of uropathogenic *E. coli* isolates is classified according to the similarity in 4 clusters (clusters A to D). We also found that to better evaluate VNTR loci for the study of epidemics or genotyping, more and more numbers of *E. coli* are needed. Besides, the use of a higher number of VNTR loci will be more beneficial for better differentiation of clonal groups.

Mellor et al. used the MLVA method to determine the genetic diversity of *E. coli* O157 producing Shiga toxin venom. There was some evidence that *E. coli* O157 strains collected from different regions may also show genetically diversity. To study the amount of this variation, Shiga toxin bacteriophage insertion sites (SBI), lineage-specific polymorphisms (LSPA-6), multilocus variable-number tandem repeat analysis (MLVA), and a *tir* 255T > A polymorphism were used to enquire 606 isolates from Australian and U.S. cattle and human. All analyses of collected data showed a significant connection between the source of origin and multilocus genotypes ($P < 0.0001$) and it confirms the evolution of enterohemorrhagic *E. coli* O157 in Australia and the United States [29].

MLVA was also used by Bustamante et al. for subtyping of 202 STEC isolates from different sources to get information about the genetic diversity of native STEC in Argentina. Two different MLVA protocols were used in their investigation, one for O157 and the other for a generic *E. coli* assay. As a result of their studies, MLVA was mentioned as a very sensitive tool to STEC subtyping and it showed the diversity in many serotypes [30].

Naseer et al. used the MLVA and multi-locus sequence typing (MLST) for subtyping of a total of 100 *E. coli* isolates from Spain, UK, Sweden, and Norway (n = 19, 20, 24 and 37 respectively) and the obtained data showed equivalence between the MLST profile and MLVA typing results [31].

In a study in Norway by Sekse et al, on 142 *E. coli* O26 isolated from 491 fecal, PFGE and MLVA was performed for investigation of the genetic relationship between the strains and 63 different PFGE profiles and 22 MLVA profiles were identified. Despite some differences in result between MLVA and PFGE, comparison by partition mapping showed good overall agreement between two methods. A few isolates of EPEC O26: H11 had a close relationship to STEC O26: H11 [32].

VNTRs regions are widely used for bacterial subtyping but it should not be overlooked that the hyper variability in VNTR loci can be problematic in trying to predict the relationships between isolates. It is also worth noting that knowing the rate of VNTR mutation and its effective factors can introduce the MLVA for legal epidemiological or microbiological research [33].

In a study in Japan on a total of 57 isolates from patients, 20 types were identified by using MLVA while they were classified into 23 PFGE types and the results were almost the same. In another study in the same city, a total of 24 isolates from different sources and one outbreak occurred in central parts of the city was investigated by MLVA and PFGE. The results showed a good correlation between these both methods. However, the MLVA typing proved to be a much more comfortable and more rapid method for the analysis of *E. coli* O157: H7 strain relatedness to identify transmission paths. Hence, MLVA method was appeared to be a convenient method for typical typing in many laboratories, including public health agencies and even in hospitals [33–36].

Conclusions

The study population consisted of *E. coli* strains isolated from patients with UTI in a major Hospital in Tehran. The analysis of MLVA profiles using the MST algorithm showed the usefulness of the MLVA method in the classification of uropathogenic *E. coli* collected in different periods. We also found that MLVA technique was a fast, easy and low-cost method for genotyping of uropathogenic *E. coli*. We evaluated this technique in a laboratory equipped with simple molecular equipment. The present study provides valuable insights into the genetic relationships of *E. coli* isolates recovered from clinical cases in a major hospital in Tehran, Iran. Based on these results, it has been assumed that the uropathogenic *E. coli* strains were derived from a limited number of clones that have undergone a small genetic change during this period.

Abbreviations

E. coli: *Escherichia coli*; UPEC: Uropathogenic *Escherichia coli*; VNTRs: Variable Number of Tandem Repeats; MLVA: Multilocus variable-number tandem repeat analysis; UTIs: urinary tract infections; UPGMA: unweighted pair group method with arithmetic averages

Declarations

Ethics approval and consent to participate

The present investigation was confirmed by the ethical supervision of Scientific Research of the Baqiyatallah University of Medical Sciences, Tehran, Iran. Sampling licenses were issued by Prof. Reza Ranjbar (Ref Number 3607614).

Consent for publication

There was no consent for publication.

Availability of data and materials

All data generated or analyzed throughout this research are included in this published article.

Competing interests

The authors declare that they have no competing interests

Funding

Not applicable.

Authors' contributions

RR and FSD designed the study and carried out the PCR genetic alignment and typing. MM and OF supported the study and carried out the samples collection, bacterial isolation and statistical analysis. RR carried out the writing and drafting of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Polymorphism of 3 VNTR loci in different *E. coli* isolates. This image illustrates how the number of repeats can be directly deduced by manual reading.

Figure 2

Data analysis by using the Minimum Spanning Tree (MST) algorithm with a difference of 2 loci. each circle is showing an isolate with a unique profile.

Figure 3

Clustering of the MLVA profiles by UPGMA with the categorical coefficient of similarity.