Multilocus Variable-Number Tandem-Repeat Analysis for Genotyping of *Escherichia coli* Strains Isolated from Hospital Wastewater, Tehran, Iran

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(Received 14 Apr 2019; accepted 17 Jun 2019)

Abstract

Background: *Escherichia coli* is one of the most frequent causes of many common bacterial infections. As a potential reservoir, hospital wastewater is considered for the dissemination of bacterial pathogens such as *E. coli*. Therefore, research on hospital waste's bacteria by low-cost, rapid and easy molecular typing methods such as multilocus variable-number tandem-repeat analysis (MLVA) can be helpful for the study of epidemics.

Methods: *E. coli* strains were isolated from hospital wastewater sources in Tehran, Iran, over a 24-month sampling period (Jun 2014–Jun 2016) and identified by standard bacteriological methods. The diversity of repeated sequences of seven variable-number tandem-repeat (VNTR) loci was studied by MLVA method based on polymerase chain reaction (PCR).

Results: Overall, 80 *E. coli* isolates were discriminated into 51 different genotypes. Analysis of the MLVA profiles using a minimum spanning tree (MST) algorithm showed two clonal complexes with 71 isolates and only nine isolates were stayed out of clonal complexes in the form of a singleton. High genotypic diversity was seen among *E. coli* strains isolated from hospital wastewaters; however, a large number of isolates showed a close genetic relationship.

Conclusion: MLVA showed to be a rapid, inexpensive and useful tool for the analysis of the phylogenetic relationships between *E. coli* strains under the study.

Keywords: *Escherichia coli*; Multilocus variable number tandem repeat analysis; Hospital wastewater; Genotyping; Phylogenetic relationships

Introduction

Hospital wastewater is hazardous for organisms’ life since it contains many pollutants such as chemical products, drugs, and pathogenic microorganisms (1). Contamination of surface waters with hospital wastewater will become a threat to public health (2). If the hospital wastewater is discharged into the environment, it can spread pathogenic microorganisms such as *Escherichia coli* (3, 4). Generally, *E. coli* bacteria are known as a part of the natural gut microflora of humans, but they also have pathogenic strains (5, 6). Some *E. coli* classes
including enteroaggregative E. coli (EAEC), enterohemorrhagic E. coli (EHEC), enteropathogenic E. coli (EPEC), enteroinvasive E. coli (EIIEC) and enterotoxigenic E. coli (ETEC) are a significant cause of diarrhea in the world, especially in developing countries and some others are associated with extraintestinal infections like uropathogenic E. coli (UPEC) and meningitis-associated E. coli (MAEC) that cause urinary tract infection meningitis in neonates, respectively (7-11). E. coli has different strains with genetic and genotype diversity (6).

Therefore, their genotyping is essential. There are some methods for genotyping, such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), repetitive sequence-based PCR (Rep-PCR) and multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) (12-14). Although PFGE is a gold standard method for determining the diversity of bacteria, as an excellent tool for outbreaks analyzing, it is expensive, time-consuming with professional skill requirements. The ability of MLVA in discrimination of bacterial strains is not less than PFGE, and its discriminatory power is much higher than MLST. Moreover, compared to other methods, MLVA is a low-cost and straightforward method (15, 16).

The current study aimed to use the MLVA method for molecular typing of E. coli strains isolated from hospital wastewater sources in Tehran, Iran.

Materials and Methods

Wastewater sample collection and E. coli isolation

The present study included non-duplicate E. coli isolates originated from hospital wastewater of twenty hospitals in Tehran, Iran over a 24-month sampling period (Jun 2014- Jun 2016).

After sampling, bottles were rapidly placed in a lightproof insulated box including icepacks to ensure rapid cooling. The samples were shipped to the laboratory for further analysis. To isolate the E. coli, conventional biochemical methods were used according to Standard Methods for the Examination of Water and Wastewater 22nd edition (17) and then samples were cultured in different environments. E. coli colonies from agar plates were picked and streaked for purity on EMB agar. Identification of E. coli strains was made by standard bacteriological procedures. Well-isolated colonies of purified E. coli were resuspended in trypticase soy broth with 20% (v/v) glycerol and stored in -70 °C for long-term storage, as described previously (18).

DNA preparation

A pure culture of E. coli was plated on LB agar 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 minutes. After centrifugation at 8,000 gr for 6 min, the supernatant was transferred into a new tube for subsequent PCR analysis.

MLVA assay

These seven VNTR loci were selected: ms06, ms07, ms09, ms11, ms21, ms23 and ms32. The primer sets for PCR amplification of these VNTR loci were previously reported (Table 1) (19).

Table 1: MLVA primers and annealing temperatures for PCR reactions

| MLVA locus | Forward primer 5’ to 3’ | Reverse primer 5’ to 3’ | Ta°C |
|-----------|-------------------------|-------------------------|------|
| ms06      | AAACGGGAGAGCGGTTATT     | TGGTGCTACAAGGCTCTCT    | 55°C |
| ms07      | GTCAGTTGCCCCAGACACAG   | CGGTGTCAGCAAATCCAGAG   | 55°C |
| ms09      | GTGGCACGCGGCAAAATTAG   | CCCGATAAGGAGCAGCTAGT   | 55°C |
| ms11      | GAAAACGGCCAGGCTACAC    | CTGGGCGTGTTATGCTATG    | 55°C |
| ms21      | GCTGATGGCGAAGGAGAAGAGA| GGAGATGTCGAGAGTCAAAAGC| 55°C |
| ms23      | GCCTCCGCTAGTGTAGCTCCT  | CGGTTGCTGAGCCACTAAAGC  | 55°C |
| ms32      | TGAGATTGCGAGTGGTTGCG   | AACTGGCCGCGCTTTATCAAG  | 55°C |
PCR was performed in 25 μl volume containing 1X PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH9), 2.5 mmol/L MgCl₂, 0.2 mmol/L of each primer with 0.5 U TaqDNA polymerase (CinnaGen Co., Iran), and 3 μl of DNA extract. Cycling conditions for PCR reactions were 93 °C for 5 min, followed by 34 cycles of 93 °C for 30 sec, 55 °C for 1 min, and 72 °C for 1 minute. The PCR products were run on agarose gels, stained with ethidium bromide, and visualized under UV transillumination by gel doc.

**Data analysis**

The number of repetitions was calculated according to the size of the amplicon by manual reading (Fig.1).

**Results**

Overall, 91 isolates suspected to *E. coli* were recovered based on culture characteristics. Eighty isolates were definitely identified as *E. coli* by standard biochemical and serological procedures and subjected to the study. As the result of PCR, the size distributions of the 80 *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 were counted (Table 2). Analysis of VNTR showed different genotypes in isolates. 38 *E. coli* isolates were divided into 2 clonal complexes, and nine isolates were out of the clonal complexes in the form of Singleton (Fig. 2).
According to the UPGMA method and Dice similarity coefficient 80, *E. coli* isolates were classified in three clusters, namely cluster number 1(C1) contained 7 isolates which none of them were from a same hospital, cluster number 2(C2) included the maximum numbers of isolates (70 out of 80) and all hospitals had isolated in this cluster, finally cluster number 3(C3) that consisted of just 3 isolates from two different hospitals. Most of the isolates were in cluster 2(C2), for this reason, this cluster was divided into two sub-clusters C1a with 25 isolates and C1b with 45 isolates (Fig. 3).
Fig. 3: Clustering of the MLVA profiles by UPGMA with the categorical coefficient of similarity.
Discussion

Now, it is the first study of using MLVA method among *E. coli* strains isolated from hospital wastewaters. Accordingly, seven VNTR loci were analyzed by MLVA method for genotyping of the strains.

One of the achievements of this research refers to describing the ability of MLVA to differentiate the tested isolates. It can offer valuable information about the relationship between hospital wastewater *E. coli* strains.

In recent years, various studies based on VNTR have been performed on several bacteria, such as *Shigella* (13, 14, 21), *Brucella* (22, 23), *Salmonella* (24, 25), *Coxiella* (26) and *E. coli*, especially O157:H7 serotype (27-30). However, according to the source of isolation, no VNTR research has been done on *E. coli* bacteria isolated from hospital wastewater.

One most important step in subtyping protocols is the TR loci choosing for analysis. In France, study on 19 *Shigella flexneri*, 26 *S. boydii*, 9 *S. sonnei*, 23 *S. dysenteriae*, 1 *Salmonella enterica* and 11 *E. coli* isolates, 15 VNTR loci such as ms06, ms07, ms09, ms11, ms21, ms23 and ms32 were chosen and evaluated by designed primers. The selected loci VNTR in their study had suitable repeat sizes, so it was easy to estimate repeat sizes by electrophoresis on agarose gel (19). The mentioned genes and primers also were used by others (13, 18, 21, 31) and they reached to their goals. We targeted these genes by using mentioned primers in MLVA. The results of this study showed that these primers are precise and appropriately selected.

Typing of *E. coli* strains contains older and newer objectives. More former purposes are related to epidemiological studies, local and regional strains identification and the possible changes in the native strains. In new targets, the study of evolution and phylogenetic tree were added to the previous aims. In older studies, selected loci had small repeat size. For this reason, accurate calculation of PCR amplicon sizes is needed to capillary electrophoresis or DNA sequencing that they are costly. In this study, the selected sizes are perfectly adequate, and results are accurate.

Simplicity, low cost, rapid and the ability to store information and comparison with results from other researchers are the advantages of the MLVA technique (32). The results of this study confirmed the above-mentioned reports. Based on the obtained data of the study, the diversity of seven TR loci was calculated with values ranging from 0.253 to 0.807 (Table 2). In addition, 51 different alleles were observed in this study. The highest level of diversity was related to ms21 and this was the most polymorphic locus in the current study with 14 different alleles. The lowest level of diversity was related to ms23. Similar observations were reported (19).

| 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          | 3 (2, 3, 4)    | No          |
| ms07       | 39          | 0.5921                     | 0.498, 0.6862          | 7 (1, 3, 6, 7, 8, 10,12) | No          |
| ms09       | 179         | 0.8066                     | 0.7453, 0.868          | 9 (0, 1, 5, 2.5, 3, 4, 4.5, 5, 6, 7) | Yes         |
| ms11       | 96          | 0.681                      | 0.6234, 0.7387         | 6 (0, 4.5, 5, 5.5, 6, 7) | Yes         |
| ms21       | 141         | 0.8073                     | 0.747, 0.8676          | 14(0, 1, 2, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10) | Yes         |
| ms23       | 375         | 0.2532                     | 0.1271, 0.3792         | 5 (0, 1, 2, 3, 4) | Yes         |
| ms32       | 101         | 0.7038                     | 0.6315, 0.7761         | 7 (1, 2, 3, 4, 4.5, 5, 5.5) | No          |

In a study on EPEC and STEC in Norway 2011, seven VNTR loci were investigated (33). Although it’s obtained data were not similar to the data of the current study but both studies were common in null
allele observation so that null alleles were seen in both studies. This can occur because of a mutation in the primer-binding site on the DNA.

One of the essential aims of this study was pointed to investigate genetic relationship between E. coli strains isolated from hospitals' wastewater in Tehran, Iran. As the result of this study, 86% of E. coli isolates had a high molecular affinity and gathered in a common clonal complex (CC1), and only 11% of isolates did not collect in a group. The remaining isolates owned another clonal complex (CC2) (Fig. 2) and were closely related isolates.

Since similar studies did not perform on E. coli strains recovered from hospital wastewaters, this study can be considered as a primary base for other studies focusing on the genetic relationship between E. coli and other non-E. coli isolates recovered from hospital wastewater sources in the future.

Conclusion

This study showed low genotypic diversity among E. coli strains isolated from hospital wastewater in Tehran, Iran. MLVA showed to be comfortable, high-operational, rapid and dependable method for genotyping of our strains. Future studies should carefully compare MLVA with older genotyping techniques such as PFGE that is the gold standard genotyping method.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

We would like to thank from the “Clinical Research Development Center of Baqiyatallah hospital” for their kindly cooperation. This study was financially supported in part by “Clinical Research Development Center of Baqiyatallah hospital”.

Conflict of interest

The authors declare that there is no conflict of interests.

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