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ORIGINAL RESEARCH

Distribution of Genes Encoding Virulence Factors and the Genetic Diversity of Enteroinvasive *Escherichia coli* (EIEC) Isolates from Patients with Diarrhea in Ahvaz, Iran

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Background: Entero-invasive *E. coli* (EIEC) is one of the causes of bacillary dysentery in adults and children. The ability of EIEC to invade and colonize the surface of epithelial cells is influenced by many virulence factors. This study aimed to investigate the distribution of virulence factor genes in EIEC strains isolated from patients with diarrhea in Ahvaz, Iran, as well as the genetic diversity between these isolates by Multilocus variable-number tandem repeat analysis (MLVA).

Materials and Methods: A total of 581 diarrheic stool samples were collected from patients with diarrhea attending two hospitals, in Ahvaz, Iran. The *E. coli* strains were identified by biochemical methods. Subsequently, all *E. coli* isolates were identified as EIEC by polymerase chain reaction (PCR) for the *ipaH* gene. The EIEC isolates evaluated by PCR for the presence of 8 virulence genes (*ial, sen, virF, invE, sat, sigA, pic*, and *sepA*). All EIEC strains were genotyped by the MLVA typing method.

Results: A total of 13 EIEC isolates were identified. The presence of *ial, virF, invE, sen, sigA, pic*, and *sat* genes was confirmed among 92.3%, 84.6%, 84.6%, 76.9%, 69.2%, and 15.3% of EIEC isolates, respectively. On the other hand, none of the isolates were positive for the *sepA* gene. The EIEC isolates were divided into 11 MLVA types.

Conclusion: Our results showed a high distribution of virulence genes among EIEC isolates in our region. This study showed that MLVA is a promising typing technique for epidemiological studies. MLVA can supply data in the form of codes that can be saved in the database and easily shared among laboratories, research institutes, and even hospitals.

Keywords: entero-invasive *Escherichia coli*, diarrhea, virulence factor, MLVA

Introduction

Entero-invasive *Escherichia coli* (EIEC) is one of the pathotypes of diarrheagenic *Escherichia coli* (DEC) that cause shigellosis-like symptoms in both children and adults.[1] Although EIEC infections occur worldwide, these are particularly common in low-income countries with poor hygiene.[2] EIEC, like *Shigella*, is responsible for bacillary dysentery. Bacillary dysentery is characterized by fever, abdominal cramps, diarrhea, sometimes vomiting, and the stool contains blood and pus.[3] EIEC can cause invasion and penetration of the epithelial cells of the colon. After the penetration into the colonic mucosa, EIEC proliferates intracellularly and spread to adjacent cells and destroying the intestinal epithelial barrier.[2] The genes related to the invasion of EIEC

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are located on a large virulence *inv* plasmid and chromosome. The *inv* plasmid is an essential virulence determinant of EIEC which encodes the type III secretion system necessary for attachment, invasion of the host cell and intercellular spread.[4] Clinical phenotypes are determined by different virulence genes and host immune system activity. They are often multifactorial and coordinately regulated, and the genes tend to be clustered in the genome.[5] The epithelial cell penetration and modification of the host response for dissemination from cell to cell are mediated by an invasion-associated locus (*ial*), which is located on a plasmid and the invasion plasmid antigen H (*ipaH*) genes present in both chromosome and plasmid. Because the *ipaH* gene is present as multiple copies on *the inv* plasmid and the chromosome, it is used as a diagnostic marker for EIEC detection.[6,7] *virF* and *invE* are two regulatory proteins that control the transcriptional of invasion genes.[7] The serin autotransporters of proteins Enterobacteriaceae (SPATEs) are present in EIEC strains. The SPATEs family has been classified into 2 classes. Class 1 SPATEs are toxic for epithelial cells, include the *Shigella* IgA-like protease homolog gene (*sigA*) and secreted autotransporter toxin gene (*sat*) which are directly cytotoxic for epithelial cells. The *sat* gene is located in the chromosome and promotes serine protease activity and displays cytopathic activity in several intestinal cell lines. Class 2 SPATEs members, including the protease involved in colonization of the intestine *(pic*) and the extracellular protein *Shigella* A (*sepA*), contribute to intestinal inflammation and colonization.[8] Two enterotoxins, *Shigella* enterotoxin 1 (ShET1) and *Shigella* enterotoxin 2 (ShET2), transport water and electrolyte in the small intestine and cause diarrhea. ShET-2 which encoded by the *sen* gene is found commonly among EIEC strains and believed to be involved in the invasion process.[9] EIEC isolates harboring these virulence genes that can induce the inflammation and extensive mucosal damages in intestinal infections, especially when these strains encode more than one of the virulence factors. Therefore, understanding the distribution of virulence genes in EIEC isolates could be useful for researchers designing new antibiotics against them. Virulence therapies do not damage the normal flora of the host, because this therapy targets virulence pathways that do not exist in the nonpathogenic bacteria. For example, because of the importance of *virF* in regulating viral genes, new antibiotics targeting the *virF* gene have received much attention.[10] Furthermore, the molecular epidemiology of such isolates is very helpful. Multilocus variable-number tandem repeat analysis (MLVA) is an effective molecular typing method based on counting the number of Variable Number Tandem Repeat (VNTR) loci. In recent years, MLVA has been applied to investigate the clonal relationships among isolates of *E. coli* strains.[11] Despite many reports about the prevalence and antimicrobial resistance of EIEC isolates from different parts of the world including Iran, investigations about the occurrence of virulence factors are still rare worldwide. Previous studies showed that EIEC is not endemic in Iran and our region.[7,12,13] There is no information about the genetic diversity of EIEC strains in the southwest of Iran. Therefore, this study aimed to investigate the distribution of genes encoding virulence factors and the genetic diversity of EIEC strains isolated from patients with diarrhea by MLVA.

Materials and Methods

Ethics Statement

The study design was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Iran (IR.AJUMS.REC.1396.434). As a part of the Ahvaz Jundishapur University of Medical Sciences policy, written informed consent was obtained from all of the adult patients and children's parents or legal guardians of any patient under the age of 18 years. The study was conducted in accordance with the Declaration of Helsinki.

The Collection of Samples

In this cross-sectional study, 581 stool specimens were collected from patients with diarrhea referring to the teaching hospitals (Golestan and Abouzar hospitals), in Ahvaz, southwest of Iran, from September 2016 to August 2017. Patients with a history of fever, nausea, vomiting, abdominal cramps, watery, mucoidal and bloody diarrhea were included in our study. Also, they had not used the antibiotic nearly 2 weeks before. If patients had taken antibiotics in the last two weeks, they would be excluded from our study. Stool samples initially were cultured on MacConkey agar (Merck, Germany), and incubated for 24 hrs at 37°C. The lactose positive colonies were tested by standard biochemical and bacteriological tests such as Triple Sugar Iron Agar, Indole test, Methyl red and Voges-Proskauer tests, and Simmons citrate agar (Merck, Germany) for detection of *E. coli* isolates.[13] All isolates that confirmed as *E. coli*, were preserved in Tryptic Soy Broth (TSB) (Merck, Germany), containing glycerol (30%) at −70°C.

Molecular Confirmation of EIEC Strains

All *E. coli* isolates were evaluated for the presence of the *ipaH* gene by PCR. DNA extraction of *E. coli* isolates was performed by the boiling method previously described.[14] The sequences of primers used for the detection of the *ipaH* gene are shown in Table 1. The reaction volume was performed in a final volume of 25 µL containing 1X PCR buffer, 1U Taq polymerase, 1 µM $MgCl_2$, 200 µM of dNTPs (CinnaGen, Iran), 0.2 µL of each primer, and 3 µL of DNA template. The amplification reaction was programmed by a thermal cycler (Eppendorf, Germany) as follows: Initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, annealing 58°C for 45 s, extension 72°C for 1min and final extension 72°C for 5 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and finally visualized in the gel documentation system (Protein simple, USA). *Shigella flexnery* ATCC 12122 was used as a positive PCR control for the *ipaH* gene.

Amplification of Virulence Factor Genes

PCR was carried out on all EIEC strains to evaluate the prevalence of the *ial, virF, invE, sen, sat, sigA, pic*, and *sepA* genes.[8,9] The sequences of primers and annealing temperatures of virulence factor genes are shown in Table 1. The total volume of the PCR mixture was 25 µL, containing 0.5 µL of DNA template, 1X PCR buffer, 2.5 Mm of $MgCl_2$, 0.5 µL each virulence gene primer, 0.5 µL Taq DNA polymerase. The PCR conditions for the amplification of virulence genes included an initial denaturation at 94°C for 60 seconds, 35 cycles of denaturation at 94°C for 60 seconds, annealing (variable) for 60 seconds, and extension at 72°C for 60 seconds, as well as a final extension at 72°C for 7 mins. After performing PCR, the size of each locus was easily determined on 1.5% gel agarose. Positive controls for each gene were as follows: *S. flexnery* ATCC 12122 for *virF*, EIEC strain 44825 for *invE*, EIEC strain 43893 for *ial, S. flexneri* 2a strain 2457T is for *sat, sepA, pic* and *sigA, S. flexnery* 4a strain 12023 for *sen*.

Genotyping of EIEC Isolates by MLVA Analysis

MLVA was performed for all EIEC isolates. Seven VNTR loci were selected for the genetic typing of the EIEC isolates. PCR was carried out as described by a previous study.[15] The primers and repeat sizes for each locus are shown in Table 2. The PCR products were electrophoresed

Table 1 Primers Used to Identify Virulence-Associated Genes of EIEC

Gene	Primer Sequence (5´-3´)	Amplicon Size (bp)	Annealing Temperature (°C)	Ref.
ipaH	F-GAAAACCCTCCTGGTCCATCAGG R-GCCGGTCAGCCACCCTCTGAGAGTAC	437	61	[7]
Sen	F- ATGTGCCTGCTATTATTTAT R- CATAATAATAAGCGGTCAGC	799	53	[9]
virF	F- TCAGGCAATGAAACTTTGAC R- GGGCTTGATATTCCGATAAGTC	618	58	[9]
invE	F-CGATAGATGGCGAGAAATTATATCCCG R-CGATCAAGAATCCCTAACAGAAGAATCA	766	59	[9]
Sat	F-TCAGAAGCTCAGCGAATCATTG R-CCATTATCACCAGTAAAACGCACC	930	58	[8]
sigA	F- CCGACTTCTCACTTTCTCCCG R- CCATCCAGCTGCATAGTGTTTG	430	58	[8]
sepA	F- GCAGTGGAAATATGATGCGGC R- TTGTTCAGATCGGAGAAGAACG	794	58	[8]
Pic	F- ACTGGATCTTAAGGCTCAGGAT R- GACTTAATGTCACTGTTCAGCG	572	58	[8]
ial	F- CTGGATGGTATGGTGAGG R- GGAGGCCAACAATTATTTCC	320	59	[9]

Table 2 Locus-Specific PCR Primers Selected for MLVA

Locus	Primer Sequence	Repeat Size (bp)	Annealing Temperature (°C)	Ref.
ms06	F-AAACGGGAGAGCCGGTTATT R-TGTTGGTACAACGGCTCCTG	39	57	[15]
ms07	F-GTCAGTTCGCCCAGACACAG R-CGGTGTCAGCAAATCCAGAG	39	57	[15]
ms09	F-GTGCCATCGGGCAAAATTAG R-CCGATAAGGGAGCAGGCTAGT	179	57	[15]
ms11	F-GAAACAGGCCCAGGCTACAC R-CTGGCGCTGGTTATGGGTAT	96	57	[15]
ms21	F-GCTGATGGCGAAGGAGAAGA R-GGGAGTATGCGGTCAAAAGC	141	57	[15]
ms23	F-GCTCCGCTGATTGACTCCTT R-CGGTTGCTCGACCACTAACA	375	57	[15]
ms32	F-TGAGATTGCCGAAGTGTTGC R- AACTGGCGGCGTTTATCAAG	101	57	[15]

on a 1.5% agarose gel containing ethidium bromide and visualized in a gel documentation system (Protein simple, USA). The copy numbers of the repeat for each isolate was calculated by the following formula:

$$\text{Number of repeats} = \text{size of each locus (bp)} - \text{flanking regions (bp)} / \text{repeat size (bp)}.$$

The results were imported into Microsoft Excel 2010 software and analyzed with the Bionumerics Software v.6.6 (Applied maths, Sint-Martens-Latem, Belgium). For clustering, a cut-off value of 90% similarity was used. The dendrogram of genetic relationships was generated using the Unweighted Pair Group Method with Average linkages (UPGMA).[16]

Statistical Analysis

The results were analyzed, using SPSS software version 22 to obtain frequencies and comparisons among clones. A nonparametric Chi-square test was used and a P-value of < 0.05 was considered statistically significant.

Results

Study Population

Of 581 fecal specimens 43.2% (n=251), were DEC strains confirmed by standard biochemical and microbiological tests. The patient's age range was between 0 to 81 years.

Distribution of EIEC

Out of 251 *E. coli* isolates, 5.1% (n=13) were positive for *ipaH*. All the EIEC strains have been isolated from children. Of 13 strains of EIEC, 84.6% (n=11) isolated from children under the age of 2 years (P<0.05). Children were categorized into five different groups according to their age: (0–12), (13–24), (25–36), (35–48), and (49–60) months. Distribution of EIEC strains according to age, gender, and clinical symptoms are shown in Table 3.

Prevalence of Virulence Factors Genes Among EIEC Strains

The detection of the virulence genes from 13 EIEC isolates showed that 92.3% (n=12) of isolates were positive for *ial*, whereas 84.6% (n=11) were positive for the *invE* and *virF* genes. The data revealed that 76.9% (n==10), 69.2% (n=9), 30.7% (n=4), and 15.3% (n=2) of strains were positive for the *sen, sigA, pic*, and *sat* genes, respectively. None of the isolates were positive for the *sepA* gene. There were six distinct virulence patterns in our isolates. The most prevalent Virulence patterns were, $ipaH^+$, ial^+, $virF^+$, $invE^+$, sen^+, $sigA^+$ found in 30.7% (n=4) strains, followed by $ipaH^+$, ial^+, $virF^+$, $invE^+$, sen^+ 23.1% (n=3). The virulence patterns of all EIEC isolates are shown in Figure 1.

MLVA Assay

MLVA performed for all EIEC isolates. Analysis of the MLVA profiles using UPGMA showed that all EIEC isolates were

Table 3 Distribution EIEC Strains According to Seasons of Sampling, Age, Gender and Clinical Symptoms

Strain ID	Sex	Age (Month)	Seasonality	Clinical Symptoms
EIEC 1	Female	0–12	Spring	Watery stool, abdominal pain, vomiting
EIEC 2	Female	0–12	Spring	Watery stool, abdominal pain, fever
EIEC 3	Male	0–12	Summer	Watery stool, vomiting, fever
EIEC 4	Male	13–24	Summer	Watery stool, abdominal pain,
EIEC 5	Male	0–12	Summer	Watery stool, abdominal pain, fever
EIEC 6	Female	49–60	Summer	Watery stool, abdominal pain
EIEC 7	Male	0–12	Summer	Abdominal pain, vomiting
EIEC 8	Female	0–12	Spring	Watery stool
EIEC 9	Male	13–24	Winter	Watery stool, abdominal pain
EIEC 10	Male	0–12	Summer	Watery stool, abdominal pain, vomiting
EIEC 11	Female	49–60	Summer	Watery stool
EIEC 12	Male	13–24	Winter	Abdominal pain, vomiting
EIEC 13	Male	0–12	Spring	Watery stool, fever

grouped into 11 distinct MLVA types with 2 clusters and 9 singletons, and 2 multitone genotypes. The six virulence patterns were showed with their MLVA pattern in Figure 1. Some EIEC isolates with the same MLVA type (M8 and M10) had different virulence patterns. Minimal spanning tree (MST) of virulence genes distribution among different MLVA patterns is shown in Figure 2. Each circle denotes an MLVA type, with the number of isolates in each type, as indicated within the circle.

Discussion

EIEC is one of the most important *E. coli* pathotypes that cause watery diarrhea and dysentery. Unfortunately, there are limited reports on the prevalence of EIEC in Iran. Due to the inability of conventional culture methods to detect pathogenic *E. coli* from non-pathogenic isolates, EIEC is usually ignored.[13] In the current study, the *ipaH* gene was used for detecting of EIEC isolates. The invasive genes *ipaH* was positive for all (100%) the isolates which agree with various other studies done so far.[7,13] The *ipaH* gene is more stable because it presents multiple copies on both chromosomes and the *inv* plasmid and seemed to be less compromised by plasmid loss and/or deletions. In our study, the prevalence of EIEC among DEC isolates was (5.1%) by PCR. This was in agreement with the findings in the previous study from Shiraz (5.5%).[17] Compared with our findings, lower prevalence rates of EIEC have been reported from Northern Iran (0.5%),[18] Nigeria and Vietnam (0.8%).[19,20] The high frequencies of *the ipaH* gene could explain frequencies of fever, vomiting, and dehydration in infected children. The EIEC isolates are similar to *Shigella* in the pathogenesis. Several virulence factors associated with EIEC pathogenesis have been identified. However, very little research has been done to identify their virulence factors profiles. This study, for the first time in Ahvaz, investigated the prevalence and distribution of virulence genes in EIEC isolates. The severity of the disease depends on the virulence factors of the bacterial strains. Among virulence factors in EIEC isolates, the *ial* gene which is located on a large plasmid facilitates the penetration of this bacterium into epithelial cell.[21] In the current study, (92.3%) of isolates were positive for the *ial* gene. In research conducted by Hosseini Nave et al all isolates were positive for the *ial* gene.[7] The *ial* gene cluster is located near a region of the plasmid, which is a hot spot for spontaneous deletions. This probably explains the lower prevalence of *ial* than *ipaH* in the EIEC strains. The expression of virulence genes is regulated by the heat-stable nucleotide structural protein (H-NS), which regulates their transcription for invasion. Transcription starts from the *virF* gene, which then turns on the transcription of the *invE* regulatory genes. Subsequently, the *invE* protein reverses the H-NS induced inhibition of transcription, which eventually elicits virulence genes on the plasmid.[3,22] In our study, both *virF* and *invE* were found in (84.6%) of EIEC isolates. These results are approximately consistent with the previous study.[7] Since *virF* and *invE* genes are located on the virulence plasmid, they are susceptible to deletion. This may be the reason for the reduced frequency of *virF* and *invE* among the isolates. A new study has shown the use of regulatory genes like *virF* as a target for novel antibiotics.[10] ShET-1 and ShET-2 play an important role in stimulating fluid secretion into the small intestine, thus, contributing to the watery phase of diarrhea. ShET-2 is encoded by the *sen* gene. This gene is located on a plasmid and originally discovered in EIEC isolates. Studies have shown that ShET-2 contributes to

% Similarity	MLVA allelic profile									virulence genes pattern								
80 85 90 95 100	ms 06	ms 07	ms 09	ms 11	ms21	ms23	ms32	Strain ID	M type	*ipaH*	*ial*	*invE*	*virF*	*sen*	*sigA*	*pic*	*sat*	*SepA*
	3	7	1	5	5	2	5	1	M1	+	+	+	+	+	+	-	-	-
	3	8	2	5	5	2	5	4	M2	+	-	+	-	-	+	-	-	-
	2	7	2	6	6	2	5	6	M3	+	+	+	+	+	-	-	-	-
	3	7	1	5	4	1	1	11	M4	+	+	+	+	+	+	-	-	-
	3	8	1	5	4	1	1	2	M5	+	+	+	+	+	+	-	-	-
	3	7	1	5	4	2	1	8	M6	+	+	+	+	+	+	+	-	-
	3	8	1	5	4	1	2	13	M7	+	+	+	+	+	-	-	-	-
	3	7	1	6	5	1	2	3	M8	+	+	+	-	+	-	-	-	-
	3	7	1	6	5	1	2	5	M8	+	+	+	+	+	+	+	-	-
	2	7	1	6	5	1	2	12	M9	+	+	-	+	-	+	+	+	-
	2	7	2	5	5	2	1	10	M10	+	+	+	+	+	+	-	-	-
	2	7	2	5	5	2	1	7	M10	+	+	-	+	-	+	+	+	-
	2	7	2	6	5	2	1	9	M11	+	+	+	+	+	-	-	-	-

Figure 1 UPGMA dendrogram based on MLVA type of isolates in regard to their virulence gene. +: Present, -: Absent.

epithelial inflammation and diarrhea.[9,23] In our study, the *sen* gene was found in (76.9%) of EIEC strains. Therefore, it can be predicted that *sen* is the major player to cause the symptoms of electrolyte imbalance and water loss causing dehydration among EIEC strains in our region. The SPATE genes are virulence factors that secreted autotransporters in gram-negative bacteria. There is very little information about the distribution of SPATE genes in EIEC isolates. In the present study, the *sigA* gene was recognized as the most common SPATE gene among EIEC isolates. In agreement with our study, Hosseini Nave et al and Boisen et al found the high rates of this gene among EIEC isolates.[7,8] These results implied that *sigA* may play an important role in the pathogenesis of EIEC. The *pic* gene was detected in 30.7% of the EIEC isolates. The frequency of this gene was relatively similar to the previous study from Kerman, Iran.[7] In this study, all isolates were negative for the *SepA*. Our results matched with the findings of studies conducted by Hosseini Nave et al and Boisen et al[8] The proteases are encoded by the *sepA* gene located in the virulence plasmid, and by the *sigA* and *pic* genes located in the chromosome. Due to storage/subculturing, the plasmid might have been lost together with the *sepA* gene. The results of the distribution of the *sat* gene in the present study showed that (15.3%) of EIEC isolates were positive for this gene which is different from prior.[7,8]

Molecular typing methods are used to determine genetic relationships between pathogenic strains for epidemiological surveillance. MLVA can provide data in the form of codes that can be saved in the database and transferable between different laboratories. This method, compared with other typing

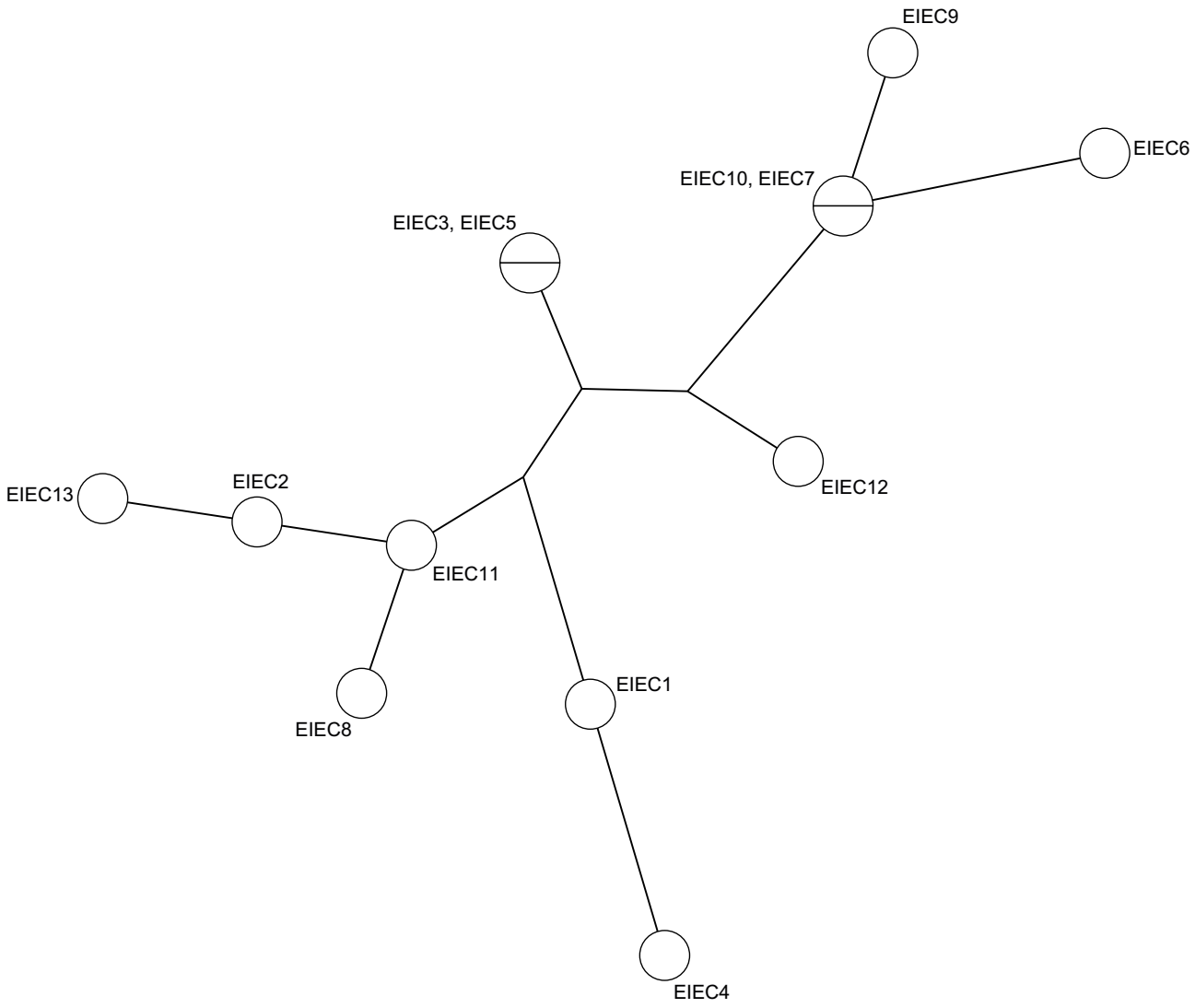

Figure 2 Spanning tree of virulence genes distribution among different MLVA patterns.

methods, such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) is a rapid and low-cost genotyping method that has differentiated closely related strains of bacteria from each other.[11,24] The MLVA also allowed us to establish associations between genotypes and parameters such as virulence characteristics.[25] In this study, we used the MLVA method for the typing of EIEC isolates. We selected VNTR loci which can produce the most discriminatory power for *E. coli* and easily analyzed on the agarose gel. Therefore, this method can be performed for epidemiological purposes in a laboratory with simple molecular tools. The UPGMA clustering of the MLVA data showed that there were 2 major clusters with different 11 MLVA types. In agreement with our study, Hosseini Nave et al showed that EIEC isolates belonged to two clonal complexes.[7] The current study findings demonstrated heterogeneity among EIEC isolates and it seemed to be due to the horizontal spread of mobile genetic elements. In the current investigation, some EIEC isolates with the same MLVA type had different virulence gene patterns. This might be because many virulence genes only located on the virulent plasmid that is susceptible to deletion and loss during storage and subculturing. Understanding these patterns of EIEC infection and transmission would provide important information on how best to design intervention and control strategies targeted at EIEC.

Conclusion

The present study provided insights into some baseline information about the distribution of some virulence genes of EIEC isolates in Ahvaz Province in Iran. This study indicated that epidemiological programs are necessary to monitor the distribution of virulence genes locally

for the prevention of the spread of the EIEC isolates harboring them. As mentioned above, MLVA typing is a much easier and more rapid technique for the analysis of *E. coli* strains relatedness. This study showed that MLVA is a promising typing technique for epidemiological studies. MLVA can supply data in the form of codes that can be saved in the database and easily shared among laboratories, research institutes, and even hospitals.

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Disclosure

The authors report no conflicts of interest in this work.

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