Shiga Toxigenic *Escherichia coli* in Iranian Pediatric Patients With and Without Diarrhea: O-Serogroups, Virulence Factors and Antimicrobial Resistance Properties

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Abstract

**Background:** Shiga-toxigenic *Escherichia coli* is an important human pathogen cause of diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura in humans. 

**Objectives:** The aim of this study was to determine the molecular characteristics and antimicrobial resistance properties of Shiga toxigenic *Escherichia coli* (STEC) strains with respect to their seasonal, age and geographical distributions in Iranian pediatric patients with and without diarrhea.

**Patients and Methods:** Four hundred and eighty swab samples were taken from patients with and without diarrhea of four major provinces of Iran. Swab samples were immediately cultured and the positive culture samples were analyzed by the polymerase chain reaction (PCR) method. Finally, antimicrobial susceptibility testing was performed using the disk diffusion method in Mueller-Hinton agar.

**Results:** In total, 118 out of 200 diarrheic stool samples (59%) and 77 out of 280 non-diarrheic stool samples (27.5%) were positive for *E. coli*. Samples taken from one to ten months old cases (73.33%) and from Shiraz province (81.13%) were the most commonly infected. Samples taken in the summer season (91.66%) were the most commonly infected. A significant difference was shown between AEEC and EHEC strains of *E. coli*. The genes encoding Shiga toxins and intimin protein were the most commonly detected in all strains. O26 (33.33%), O111 (38.18%) and O91 (12.12%) serogroups had the highest frequency in patients with and without diarrhea. Prevalence of the genes that encode resistance against ampicillin (*aac(3)-IV*), gentamicin (*tet(A)*), and tetracycline (*tet(A)*) were 80.30%, 75.75% and 65.15%, respectively. The STEC strains harbored the highest levels of resistance against ampicillin (84.84%), gentamicin (78.78%), tetracycline (50%) and sulfamethoxazole (40.90%) antibiotics. We found that 55.0% of diarrheic and 1.29% of non-diarrheic *E. coli* isolates were resistant to more than six antibiotics.

**Conclusions:** Accurate control programs should be organized for antibiotic prescription especially during warmer seasons in Iran. Primary treatment of diarrheic patients with co-trimoxazole, cefotaxime and ceftriaxone is effective.

Keywords: Microbial Sensitivity Test, Virulence Factors, Iran, Shiga Toxigenic *Escherichia coli*.

1. Background

Shiga toxigenic *Escherichia coli* (STEC) is a significant cause of gastrointestinal disease, diarrhea, bloody diarrhea, hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura, hemolytic anemia, hemorrhagic colitis (HC) and acute renal failure in humans (1-3). It has been estimated that the STEC strains are one of the most prevalent causes of gastrointestinal disorders in children (3-6). To appraise the pathogenicity of STEC strains, assessment of latent virulence factors is a prerequisite. The factors that are most frequently associated with STEC infections and diarrhea are Shiga toxins (*stx1* and *stx2*), intimin (*eaeA*) and hemolysin (*hlyA*) (1-3, 7). Most outbreaks and sporadic cases of bloody and non-bloody diarrhea, HUS and even HC have been attributed to the O157 serogroup of the STEC strains. In line with this, the roles of non-O157 serogroups like O111, O103, O26, O145, O113, O121, O91 and O128 have been recognized as causes of HUS, HC, bloody and non-bloody diarrhea and other gastrointestinal disorders (1, 2, 8, 9). In addition to O-serogroups and virulence factors, treatment is a critical point to assess the epidemiological contents of STEC strains in the cases of diarrhea, while therapeutic options have become somewhat limited because of the presence of multi drug resistant strains of these bacteria (1-3, 10). Antibiotic resistant strains of STEC can cause more severe diseases in humans and animals. Antibiotic resistance in STEC strains is associated with the presence of some antibiotic resistance genes (1-3, 10). The genes that...
encode resistance against tetracycline (tetA and tetB), trimethoprim (dfrA1), aminoglycosides (aadA1), fluoroquinolone (qnr), gentamicin (aac(3)-IV), sulfonamide (sulI), cephalexin (blaSHV), ampicillin (CITM), erythromycin (ermA) and chloramphenicol (cmlA and cmlB) are the most commonly detected antibiotic resistance genes in the resistant isolates of STEC strains (1,3,10). Based on Iranian epidemiological researches, STEC strains have been known as the most commonly detected pathogens in pediatric patients with diarrhea and show a high incidence of resistance (85-100%) against commonly used antimicrobial agents (3,11,12).

Imperious data about the prevalence of O-serogroups, virulence factors and antibiotic resistance properties in STEC strains isolated from pediatric patients are rare in Iran.

2. Objectives

The present research was carried out in order to study the distribution of virulence factors, O-serogroups, antibiotic resistance genes and pattern of antibiotic resistance of STEC strains isolated from diarrheic and non-diarrheic pediatric patients with respect to the role of season, age and geographical area of sample collection.

3. Patients and Methods

3.1. Samples and Escherichia coli Identification

From January 2014 to January 2015 during various seasons of the year, a total of 480 stool samples from diarrheic (n = 280) and non-diarrheic (n = 280) pediatric patients were collected from educational hospitals of various provinces of Iran. Individuals of the diarrheic group were classified into six groups based on their age (less than a month old, 1-10 months, 11-21 months, 22-33 months, 34-45 months, 46-57 months and 58-69 months old). Clinical histories of pediatric patients were obtained through questionnaires. Stool specimens were collected using sterile rectal swabs. Samples were placed in tubes containing Stuart medium. Samples were transferred to the laboratory at 4°C in cooler with iced-packs.

All samples were diluted using phosphate buffer saline (PBS, Merck, Germany). Samples were then plated onto MacConkey agar (Merck, Germany) and incubated overnight at 37°C. From each sample, a typical lactose positive isolate was selected and placed onto eosin methylene blue (EMB; Merck, Germany) and incubated over night at 37°C. Next, 1.5 mL of a saturated NaCl solution was added and the culture was centrifuged for five minutes, at 10000 rpm. The cell pellet was resuspended and lysed using 1 mL of lysis buffer (40 mM tris-acetate, pH 7.8, 20 mM sodium chloride, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µL of 5 M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12000 rpm for 10 minutes at 4°C. After transferring the clear supernatant to a new eppendorf tube, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely formed. Following centrifugation at 14000 rpm for five minutes, the supernatant was transferred to another eppendorf tube and double volume of 100% ethanol was added. The tubes were gently inverted five to six times, then centrifuged at 10000 rpm for five minutes. The supernatant was discarded and 1 ml of ethanol (70%) was added to the pellet, and tubes were centrifuged at 10000 rpm for five minutes. Finally, the supernatant was discarded and the pellet was dried for 10 minutes at room temperature, and the pellet was resuspended by 100 µl of H2O. The stock was kept at -20°C until use. The DNA concentration was determined by measuring absorbance of the sample at 260 nm using a spectrophotometer (14).

3.3. DNA Extraction

Total genomic DNA was extracted from the bacterial colonies. A single colony was suspended in sterile Tryptic soy agar (TSA, Merck, Germany) and incubated over night at 37°C. Next, 1.5 mL of a saturated NaCl solution was added and the culture was centrifuged for five minutes, at 10000 rpm. The cell pellet was resuspended and lysed using 1 mL of lysis buffer (40 mM tris-acetate, pH 7.8, 20 mM sodium chloride, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µL of 5 M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12000 rpm for 10 minutes at 4°C. After transferring the clear supernatant to a new eppendorf tube, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely formed. Following centrifugation at 14000 rpm for five minutes, the supernatant was transferred to another eppendorf tube and double volume of 100% ethanol was added. The tubes were gently inverted five to six times, then centrifuged at 10000 rpm for five minutes. The supernatant was discarded and 1 ml of ethanol (70%) was added to the pellet, and tubes were centrifuged at 10000 rpm for five minutes. Finally, the supernatant was discarded and the pellet was dried for 10 minutes at room temperature, and the pellet was resuspended by 100 µl of H2O. The stock was kept at -20°C until use. The DNA concentration was determined by measuring absorbance of the sample at 260 nm using a spectrophotometer (14).

3.4. Detection of Serogroups, Virulence Factors and Antibiotic Resistance Genes

The oligonucleotide primers and PCR program used for detection of O-serogroups, virulence factors and antibiotic resistance genes of E. coli isolates are shown in Table 1. All PCR reactions were amplified in a programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device. Finally, 15 µl of PCR products were resolved on a 1.5% agarose gel containing 0.5 mg/mL of ethidium bromide in tris-borate- Ethylenediaminetetraacetic acid (EDTA) buffer at 90 V for one hour, using suitable molecular weight markers. The products were examined under ultraviolet illumination. Strains of E. coli O157: 8884ac: H9, CAPM 5933 and E. coli O159: H20, CAPM 6006 were used as positive controls and distilled water was used as the negative control.
Table 1. The Oligonucleotide Primers and the Polymerase Chain Reaction Programs Used for Amplification of O-Serogroups, Virulence Factors and Antibiotic Resistance Genes of *Escherichia coli* Isolates From Pediatric Patients With and Without Diarrhea

| Target Gene | Primer Sequence (5’ - 3’)
|-------------|---------------------------------|
| **O157** b  | F: CGGACATCCATGTTGATATGG R: TTTCTACCGCGAATCTATC |
| **O145** b  | F: CCATCAACAGATTTAGGAGTG R: CACACACACACACTAAGGC |
| **O103** b  | F: TTGGAGCGTTAACTGGACCT R: GCTCCCGAGCACGTATAAG |
| **O26** b  | F: CAGAATGTTATGCTACGTG R: CTACATTGTGGTCGGCAT |
| **O111** b  | F: TAGAGAAATATCAACGTATTC R: ATATATGAGGAGACCTT |
| **O91** c  | F: GCTGACCTTCATGATCTGTTGA R: TAATTTAACCCGTAGAATGG |
| **O128** c  | F: GTGATTCTGCGGATATGGGC R: CCGACGGACTGATGCCGGTGAT |
| **O113** c  | F: GGGTATGGGAGCAGCTATGGGA R: AGGTCAACCITCTGAATATTGGGA |
| **O45** c  | F: CCGGTTTCGATTTGTGAAGGTTG R: CACAACACACTAAGCCAGC |
| **stx1** d  | F: AAATCGCCATTCGTTGACTACTTCT R: TGCCATTCTGCAACTCAGGAGA |
| **stx2** d  | F: CGTGTCACTCAGCCTTCATC R: GGAATTTTCCCCCCGAG |
| **eaeA** d  | F: TGGGACACAGGCGGTCAGA R: CGGACTCTAGGTGGAA |
| **ehly** d  | F: CAGATCTGTGGCTTGTGGGG R: GGAGTTTTGAGGCTGGG |
| **aadA1** e  | F: GCTGACCCAGTCAAATGTTCA R: AGTATCACCCCCATGGTT |
| **tetA** e  | F: GTTATCCTCAACCGGCTGT R: GTGCTACCCGTAGCAGAT |
| **tetB** e  | F: CTGTCCGACAAGCTCGGTAC R: GTGCTATACCCGAGCTAG |
| **dfraT** e  | F: GGATGTCGCAAAAGTGAAACAGC R: GAGGCGAAGTCTTGGGTAAAAC |
| **qnr** e  | F: GGTTATGGGAGCAGCTATGGGA R: ATATATGAGGAGACCTT |
| **aac(3)-IV sul2** e  | F: CTTGAACAGATGTTGACCGGT R: TTTCCTCGCTCCGCTTCC |
| **blaSHV** e  | F: TGCCGGATCAGCCATGACCT R: ATATATGAGGAGACCTT |
| **CITM** f  | F: CGGACATCCATGTTGATATGG R: TTTCTACCGCGAATCTATC |
| **cat1** f  | F: CGGACATCCATGTTGATATGG R: TTTCTACCGCGAATCTATC |
| **aac(3)-IV sul2** f  | F: CGGACATCCATGTTGATATGG R: TTTCTACCGCGAATCTATC |

**Note:** Oligonucleotide primers and PCR programs were obtained from previous studies.

- **PCR Program:** 1 cycle: 95°C, 3 minutes. 30 cycles: 95°C, 20 seconds; 58°C, 40 seconds; 72°C, 30 seconds. 1 cycle: 72°C, 8 minutes. PCR Volume (50 µL): 5 µL PCR buffer 10×, 1.5 mM MgCl²
- **PCR Program:** 1 cycle: 94°C, 6 minutes. 34 cycle: 95°C, 30 seconds; 58°C, 30 seconds; 72°C, 10 minutes. PCR Volume (50 µL): 5 µL PCR buffer 10×, 2 mM MgCl
- **PCR Program:** 1 cycle: 95°C, 3 minutes. 34 cycle: 94°C, 60 seconds; 56°C, 45 seconds; 72°C, 60 seconds. 1 cycle: 72°C, 10 minutes. PCR Volume (50 µL): 5 µL PCR buffer 10×, 2 mM MgCl
- **PCR Program:** 1 cycle: 94°C, 8 minutes. 32 cycle: 95°C, 60 seconds; 55°C, 70 seconds; 72°C, 2 minutes. 1 cycle: 72°C, 8 minutes. PCR Volume (50 µL): 5 µL PCR buffer 10×, 2.5 mM MgCl

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Table 2. Age, Seasonal and Geographical Distribution of *Escherichia coli* in Pediatric Patients With and Without Diarrhea

| Different Criteria              | No. Samples Collected | Positive Samples |
|---------------------------------|-----------------------|-----------------|
| **Age distribution, mon**       |                       |                 |
| <1                              | 32                    | 19 (59.37)      |
| 1 - 10                          | 30                    | 22 (73.33)      |
| 11 - 21                         | 30                    | 21 (70)         |
| 22 - 33                         | 27                    | 16 (59.25)      |
| 34 - 45                         | 29                    | 16 (55.17)      |
| 46 - 57                         | 26                    | 14 (53.84)      |
| 58 - 69                         | 26                    | 10 (38.46)      |
| **Geographical distribution**   |                       |                 |
| Tehran                          | 59                    | 30 (50.84)      |
| Isfahan                         | 46                    | 32 (69.56)      |
| Shiraz                          | 53                    | 43 (81.13)      |
| Mashhad                         | 42                    | 13 (30.95)      |
| **Seasonal distribution**       |                       |                 |
| Spring                          | 50                    | 31 (62)         |
| Summer                          | 60                    | 55 (91.66)      |
| Autumn                          | 45                    | 48 (88)         |
| Winter                          | 45                    | 10 (22.22)      |
| **Total diarrheic samples**     | 200                   | 118 (59)        |
| **Total non-diarrheic samples** | 280                   | 77 (27.5)       |
| **Total**                       | 480                   | 195 (40.62)     |

aData are presented as No. (%).

3.5. Statistical Analysis

The data were analyzed using the SPSS (statistical package for the social sciences) software and P Value was calculated using the chi-square test to find any significant relationship between various seasons, ages, clinical symptoms and distribution of O-serogroups, virulence genes and antibiotic resistance properties of STEC strains isolated from pediatric patients with and without diarrhea. A P Value of less than 0.05 was considered statistically significant.

3.6. Ethical Issues

The present study was approved by the ethical committee of educational hospitals of Tehran, Isfahan, Shiraz and Mashhad. The life, health, dignity, integrity, rights to self-determination, privacy and confidentiality of personal information of research subjects were all protected. All patients or their parents signed the written informed consent.

4. Results

All of the swab samples of pediatric patients with and without diarrhea were studied using the culture method and positive results were confirmed to be *E. coli* by the PCR technique. Age, seasonal and geographical distribution of *E. coli* in the pediatric patients with and without diarrhea are shown in Table 2. Of the 480 studied samples, 195 (40.62) samples were positive for *E. coli*. On the other hand, 118 out of 200 diarrheic stool samples (59%) and 77 out of 280 non-diarrheic stool samples (27.5%) were positive. A significant difference was observed between the prevalence of *E. coli* from diarrheic and non-diarrheic patients (*P* < 0.027). Overall, 1 - 10 month old patients had the highest incidence of *E. coli* (73.33%) yet 58 - 69 month old patients had the lowest incidence of *E. coli* (38.46%). *Escherichia coli* strains had the highest prevalence in the Shiraz (81.13%), followed by Isfahan (69.56%) and Tehran (50.84%). The swab samples, which were taken in the summer season had the highest prevalence of *E. coli* (91.66%), while those that were taken in the winter season had the lowest prevalence (22.22%). There were significant differences in the incidence of *E. coli* between hot and cold seasons (*P* = 0.038).

Total distribution of virulence genes in the *E. coli* subtypes of pediatric patients with and without diarrhea is shown in Table 3. *Stx1* and *eaeA* were the most commonly detected virulence factors in the diarrheic and non-diarrheic pediatric patients. The majority of *E. coli* strains harbored *stx1* and *eaeA* genes together, while the prevalence of *E. coli* isolates harboring the *stx2* and *eaeA* factors together were low. The AEEC were the most commonly detected subtype yet EHEC was the least commonly detected. The EHEC subtype was only detected in less than a month (7.69%), 1 - 10 (12.5%) and 34 - 45 (10%) month old pediatric patients. Significant statistical differences were observed between the incidence of *stx1* and *stx2* genes (*P* = 0.022) and also between the incidence of AEEC and EHEC subtypes (*P* = 0.031). Of the 195 *E. coli* strains, 66 isolates (33.84%) were confirmed to be STEC. Total distribution of O-serogroups in the STEC strains of pediatric patients with and without diarrhea is shown in Table 4. We found that the most commonly detected O-serogroups in the diarrheic and non-diarrheic pediatric patients were O26 (33.33%), O111 (18.18%) and O91 (12.12%). There were no positive strains for O157 serogroup in the non-diarrheic pediatric patients. The STEC O-serogroups had the highest incidence in 1 - 10 month old pediatric patients. Significant difference was seen between the age of...
Pediatrics and incidence of STEC O-serogroups (P = 0.043). Total distribution of antibiotic resistance genes in the STEC strains from pediatric patients with and without diarrhea is shown in Table 5. Regarding resistance, CITM (80.30%), aac(3)-Iv (75.75%) and tetA (65.15%) were the most commonly detected antibiotic resistance genes in pediatric patients with and without diarrhea. Non-diarrheic pediatric patients had the lowest prevalence of antibiotic resistance genes when compared to diarrheic pediatric. There were no positive results for the cmlA gene. A significant difference was seen between the age of pediatric patients and incidence of antibiotic resistance genes (P = 0.035).

Susceptibility of STEC strains against 14 commonly used antimicrobial agents is shown in Table 6. The STEC strains of our study harbored the highest levels of resistance against ampicillin (84.84%), gentamycin (78.78%), tetracycline (50%) and sulfamethoxazole (40-90%) antibiotics. Levels of antibiotic resistance in the STEC strains of pediatric patients without diarrhea were lower than those with diarrhea. Figure 1 shows the total distribution of multi-drug resistance in the STEC strains of pediatric patients with and without diarrhea. All of the E. coli strains of pediatric patients with and without diarrhea harbored resistance against one antibiotic. We found that 65 out of 118 (55.08%) diarrheic and one out of 77 (1.29%) non-diarrheic E. coli isolates were resistant to more than six antibiotics.

**Table 3.** Total Distribution of Virulence Factors in *Escherichia coli* Subtypes Isolated From Diarrheic and Non-Diarrheic Pediatric Patients.a,b

| Diarrhea Status and Age, mon | No. Positive | Positive Virulence Factors |
|-----------------------------|-------------|---------------------------|
| **Positive**                |             |                           |
| <1                          | 19          | stx1: 7 (87.5), stx2: 3 (37.5), eaeA: 5 (62.5), stx1; stx2: 2 (25), stx1; stx2; eaeA: 2 (25) |
| 1 - 10                      | 22          |                           |
| Non detected                | 5 (31.25)   | stx1: 8 (88.88), stx2: 4 (44), eaeA: 6 (66.66), stx1; eaeA: 5 (55.55), stx2; eaeA: 3 (33.33), stx1; stx2; eaeA: 1 (11.11) |
| EHEC                        | 2 (12.5)    | stx1; eae; ehly: 2 (100) |
| AEEC                        | 9 (56.25)   | stx1; eae: 7 (70), stx1; eaeA: 6 (60), stx2; eaeA: 2 (20), stx1; stx2; eaeA: 2 (20) |
| Total                       | 16 (71.43)  |                           |
| II - 21                     | 21          |                           |
| Non detected                | 5 (33.33)   |                           |
| EHEC                        | -           | stx1; eae; ehly: -       |
| AEEC                        | 10 (66.66)  | stx1; eae: 7 (70), stx1; eaeA: 6 (60), stx2; eaeA: 2 (20), stx1; stx2; eaeA: 2 (20) |
| Total                       | 15 (71.42)  |                           |
| 22 - 33                     | 16          |                           |
| Non detected                | 4 (36.36)   |                           |
| EHEC                        | -           | stx1; eae; ehly: -       |
| AEEC                        | 7 (63.64)   | stx1: 5 (83.33), stx2: 3 (42.85), eaeA: 4 (57.14), stx1; eaeA: 4 (57.14), stx2; eaeA: 2 (28.57), stx1; stx2; eaeA: 1 (14.28) |
| Total                       | 11 (68.75)  |                           |
| 34 - 45                     | 16          |                           |
| Non detected                | 3 (18.75)   |                           |
| EHEC                        | -           | stx1; eae; ehly: 1 (100) |
| AEEC                        | 10 (62.5)   | stx1; eae: 5 (83.33), stx2: 2 (33.33), eaeA: 4 (66.66), stx1; eaeA: 3 (50), stx2; eaeA: 2 (33.33), stx1; stx2; eaeA: 1 (16.66) |
| Total                       | 13 (81.25)  |                           |
| 46 - 57                     | 10          |                           |
| Non detected                | 2 (20)      |                           |
| EHEC                        | -           | stx1; eae; ehly: -       |
| AEEC                        | 6 (66.66)   | stx1: 6 (100), stx2: 2 (33.33), eaeA: 4 (66.66), stx1; eaeA: 3 (50), stx2; eaeA: 2 (33.33), stx1; stx2; eaeA: 1 (16.66) |
| Total                       | 9 (90.91)   |                           |
| 58 - 69                     | 10          |                           |
| Non detected                | 2 (20)      |                           |
| EHEC                        | -           | stx1; eae; ehly: -       |
| AEEC                        | 4 (66.66)   | stx1: 3 (75.00), stx2: 1 (25), eaeA: 2 (50), stx1; eaeA: 1 (25), stx2; eaeA: 1 (25) |
| Total                       | 6 (60)      |                           |
| **Negative**                | 77          |                           |
| Non detected                | 13          |                           |
| EHEC                        | -           | stx1; eae; ehly: -       |
| AEEC                        | 12          | stx1; stx10 (83.33), stx2: 5 (41.66), eaeA: 8 (66.66), stx1; eaeA: 7 (58.33), stx2; eaeA: 3 (25), stx1; stx2; eaeA: 2 (16.66) |
| Total                       | 25 (32.46)  |                           |

*aAbbreviations: AEEC: attaching and effacing. bValues are presented as No. (%).*
Table 4. Total Distribution of O-Serogroups in the Shiga Toxigenic Escherichia coli Strains From Pediatric Patients With and Without Diarrhea

| Diarrhea Status and Age, mon | No. STEC Strains | Distribution of O-Serogroups a |
|-----------------------------|------------------|--------------------------------|
|                             | O157 | O26 | O103 | O111 | O145 | O45 | O91 | O113 | O121 | O128 |
| Positive                    |      |     |      |      |      |     |     |      |      |      |      |
| < 1                         | 9    | 1   | 3    | 1    | 2    | -   | -   | 1    | -    | 1    | -    |
| 1-10                        | 11   | 2   | 4    | 1    | 3    | -   | -   | 1    | -    | -    | -    |
| 11-21                       | 10   | -   | 3    | 1    | -    | 2   | 1   | -    | 2    | -    | 1    |
| 22-33                       | 7    | -   | 2    | 1    | 1    | -   | -   | 1    | -    | -    | -    |
| 34-45                       | 7    | 1   | 2    | 1    | 1    | -   | -   | 1    | -    | -    | -    |
| 46-57                       | 6    | -   | 2    | 1    | 1    | -   | -   | 1    | -    | -    | -    |
| 58-69                       | 4    | -   | 1    | -    | 1    | -   | 1   | 1    | -    | -    | -    |
| Negative                    | 12   | -   | 5    | 1    | 1    | 1   | 1   | 1    | 1    | 1    |
| Total                       | 66   | 4 (6.06) | 22 (33.33) | 6 (9.09) | 12 (18.18) | 2 (3.03) | 2 (3.03) | 8 (12.12) | 1 (1.51) | 19 (28.88) | 3 (4.54) |

aValue’s unit is %.

Table 5. Total Distribution of Antibiotic Resistance Genes in the Shiga Toxigenic Escherichia coli Strains From Pediatric Patients With And Without Diarrhea

| Diarrhea Status and Age, mon | No. STEC Strains | Distribution of Antibiotic Resistance Genes a |
|-----------------------------|------------------|-----------------------------------------------|
|                             | aadA1 | tetA | tetB | dfrA1 | qnr | aac(3)-IV | sul1 | blaSHV | CITM | cat1 | cmlA |
| Positive                    |       |      |      |       |     |           |      |        |     |      |      |
| < 1                         | 9     | 3    | 7    | 2     | 6   | 8         | 6    | 4      | 8   | -    | -    |
| 1-10                        | 11    | 3    | 8    | 3     | 6   | 8         | 7    | 6      | 9   | -    | -    |
| 11-21                       | 10    | 2    | 6    | 1     | 6   | 8         | 8    | 5      | 9   | 1    | -    |
| 22-33                       | 7     | 1    | 5    | 1     | 6   | 4         | 7    | 5      | 3   | 7    | -    |
| 34-45                       | 7     | 1    | 5    | 2     | 6   | 4         | 2    | 6      | -   | -    | -    |
| 46-57                       | 6     | 1    | 4    | 4     | 2   | 4         | 3    | 2      | 5   | -    | -    |
| 58-69                       | 4     | -    | 3    | 1     | 2   | 4         | 3    | 1      | 4   | 1    | -    |
| Negative                    | 12    | 1    | 5    | 1     | 2   | 1         | 5    | 2      | 3   | 5    | -    |
| Total                       | 66    | 12 (18.18) | 43 (65.15) | 11 (16.66) | 33 (50) | 29 (43.93) | 50 (75.75) | 38 (57.87) | 25 (37.87) | 53 (80.30) | 2 (3/03) |

aValues unit is %.

Table 6. Antibiotic Resistance Pattern of Shiga Toxigenic Escherichia coli Strains Isolated From Pediatric Patients With and Without Diarrheaa

| Diarrhea Status and Age, mon | No. STEC Strains | Pattern of Antibiotic Resistance b |
|-----------------------------|------------------|-----------------------------------|
|                             | SXT | GMt0 | CIP5 | TMP5 | AMt0 | COT25 | Cef10 | Cfr30 | Cfx5 | F/M300 | Nor10 |
| Positive                    |     |      |      |      |      |      |      |      |      |      |        |       |
| < 1                         | 9    | 6    | -    | 5    | 8    | 4    | 5    | 4    | 8    | 2    | 3      | 1      |
| 1-10                        | 10   | 1    | 5    | 3    | 4    | 9    | 2    | 3    | 3    | 3      | 3      | 1      |
| 11-21                       | 4    | -    | 6    | 2    | 2    | 3    | 7    | 1    | 2    | 2      | 1      | 2      |
| 22-33                       | 10   | 6    | 1    | 5    | 9    | 3    | 4    | 5    | 9    | 2      | 3      | 3      |
| 34-45                       | 7    | 3    | -    | 3    | 6    | 2    | 2    | 3    | 6    | 1      | 1      | 3      |
| 46-57                       | 6    | 2    | -    | 2    | 5    | 1    | 2    | 2    | 6    | 1      | 2      | 1      |
| 58-69                       | 4    | 2    | -    | 1    | 3    | 1    | 1    | 1    | 4    | -      | 1      | 1      |
| Negative                    | 12   | 3    | -    | 3    | 6    | 3    | 3    | 2    | 6    | 1      | 2      | 3      |
| Total                       | 66   | 33   | 1    | 27   | 52   | 18   | 23   | 25   | 56   | 11    | 17     | 20     |

aIn this table: TE30: tetracycline (30 µg/disk); C30: chloramphenicol (30 µg/disk); SXT: sulfamethoxazole (25 µg/disk); GMt0: gentamycin (10 µg/disk); CF30: cephalothin (30 µg/disk); CIP5: ciprofloxacin (5 µg/disk); TMP5: trimethoprim (5 µg/disk); AMt0: ampicillin (10 µg/disk); COT25: co-trimoxazole (25 µg/disk); Cef5: cefotaxime (30 µg/disk); Cfr: ceftriaxone (30 µg/disk); Cfx5: cefixime (5 µg/disk); F/M300: nitrofurantoin (300 µg/disk); Nor10: norfloxacin (10 µg/disk) antimicrobial agents.

bValue’s unit is %.

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5. Discussion

The present investigation focused on the study of the prevalence of O-serogroups, virulence factors and antimicrobial resistance properties of STEC strains isolated from diarrheic and non-diarrheic pediatric patients with respect to age, seasonal and geographical distribution. We found that the 1-10 month old patients from the Shiraz during the summer season were the group at highest risk of infection with STEC strains. The main reason for the higher prevalence of E. coli in the summer season in Iran is that during this time climatic events, heat, rain and thunderstorms, as well as variation of barometric pressure may influence the autonomic nervous system. These events cause a reduction in the levels of human immunity; therefore, several infections may occur. Furthermore, E. coli has better growth and surveillance in warm conditions. The levels of public and individual health are decreased in warm climates, such as during summer. After analyzing the average temperatures of the infections in the study area (19°C for spring, 23°C for summer, 14°C for autumn and 5°C for winter), it was recognized that the prevalence of E. coli in each season is related to the average temperatures. A significant difference (P = 0.037) was seen between the average temperature of hot and cold seasons. Similar researches have also reported on the seasonal distribution of E. coli infections (3, 15-18). Available data between years 2005 and 2011, revealed the higher prevalence of E. coli in males during the hot months (18). Higher prevalence of both gastrointestinal infections in the warm season of the year has also been reported previously (3, 15-18). We found that 1-10 month old pediatric patients were the most commonly infected group, which was similar to the results of Momtaz et al. (3). They showed that 13-24 month old patients were the most infected age group (77.63%).

Our results revealed that the E. coli isolates of patients with diarrhea had a high prevalence of virulence factors. We found that the majority of E. coli strains harbored all stx1, stx2 and eaeA genes together, indicating higher levels of pathogenicity and infection. The relatively high occurrence of the stx1 gene compared to stx2 in the diarrheic E. coli isolates suggests that E. coli carrying a combination of the eaeA and stx1 genes is more common than the combination of eaeA and stx2 genes. Some of the E. coli strains of our study harbored the stxt, stx2 and eaeA genes together.

The importance of these data lies in the fact that eae-positive strains are considered more virulent for humans than eae-negative strains, as well as strains carrying only the stx2 genes (19). This observation is of concern, as the former combination of genes is known to cause more severe diarrhea in humans (19-21). Momtaz et al. reported on the multiple presences of stx1, eaeA and other genes in STEC strains of one to 60 month old pediatric patients (3). High presence of stx1, stx2, eaeA and other genes, in the E. coli isolates of diarrheic patients from Korea, Brazil, Australia and Kenya, has also been reported previously (21-24). Sang et al. showed that 37.1% of diarrheic patients were positive for E. coli, of which 24.1% had stx1 (3). Sang et al. reported that 52.9% of STEC isolates carried stx1, 29.4% possessed stx2, 14.7% carried both stx1 and stx2, and 2.9% had stx2, and 23.5% carried stx1. All 3.8% of the isolates possessed the eaeA gene, which was similar to our results (23).

Both O157 and non-O157 strains had a high prevalence in diarrheic pediatric patients of our investigation. On the other hand, O26 (33.33%), O111 (18.18%) and O91 (12.12%) were the most commonly detected O-serogroups in pediatric patients with and without diarrhea. In a study conducted in Iran, O26 (27.04%) had the highest incidence amongst STEC serogroups of diarrheic pediatrics, followed by O111 (18.85%) (3). High prevalence of non-O157 serogroups of STEC strains in the cases of diarrhea has been reported by various studies (15-17, 25). High prevalence of non-O157 strains and especially the O26 strain of STEC in the cases of diarrhea was reported from Germany (26), Japan (27) and South Africa (28).

Extreme and highly irregular prescriptions of antimicrobial agents in our study area caused the high levels of resistance of STEC strains against ampicillin (84.84%), gentamycin (78.78%), tetracycline (50%) and sulfamethoxazole (40.90%) antibiotics. These high levels of resistance have been derived from the high presence of certain antibiotic resistance genes including CITM (80.30%), aac (3)-IV (75.75%) and tetA (65.15%). We also found that 55.08% of diarrheic and 12.9% of non-diarrheic E. coli isolates were resistant to more than six antibiotics, which was considerably high. We also recognized that O26 serogroup had the highest levels of antibiotic resistance and antibiotic resistance genes, which was similar to the results of Kijima-Tanaka et al. (27) and Momtaz et al. (3). Our results showed that the STEC strains of our study were also resistant to chloramphenicol (1.51%) and nitrofurantoin (12.12%) antibiotics. Chloramphenicol and nitrofurantoin are forbidden antibiotics and the slight antibiotic resistance to these antimicrobial agents indicated their irregular and unauthorized use in medical treatment in Iran. Similarly, chloramphenicol and nitrofurantoin resistance have also been
reported previously (3, 29, 30). Another Iranian study (31) showed that the STEC strains of diarrheic patients were resistant to amoxicillin (72.4%), trimethoprim-sulfamethoxazole (65.5%) and tetracycline (58.6%) antibiotics. Motmaiz et al. (2) reported that the STEC strains had the highest levels of resistance against sulfisoxazole (36%), tetracycline (32%), streptomycin (29%), ampicillin (10%), trimethoprim (8%), cotrimoxazole (8%), chloramphenicol (7%), kanamycin (7%), pipercillin (6%), and neomycin (5%) antibiotics, which was similar to our results.

In conclusion, we identified E. coli with defined pathotypes, which originated mainly from diarrheic and non-diarrheic pediatric patients of four major provinces of Iran. We also found a large number of virulent and resistant strains of E. coli with higher prevalence of stx1 and eaeA genes, O26, O111 and O91 serogroups, CTnT, aaeC(3)-IV and tetA antibiotic resistance genes and resistance to ampicillin, gentamycin, tetracycline and antibiotics. Shiraz due to its high temperature and moisture had the highest prevalence of E. coli. Simultaneous presence of stx1 and eaeA, and stx2 and eaeA virulence factors in some strains of E. coli in diarrheic children warned about an important public health problem. Prescription of co-trimoxazole, cotefoxime, cephalothin and ceftriaxone can be effective for treatment of the cases of infection due to STEC strains.

Footnote
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