Serogroups, subtypes and virulence factors of shiga toxin-producing Escherichia coli isolated from human, calves and goats in Kerman, Iran

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ABSTRACT

Aim: The present study was conducted to detect the occurrence, serogroups, virulence genes and phylogenetic relationship of shiga toxin-producing Escherichia coli (STEC) in human, clave and goat in Kerman (southeast of Iran).

Background: STEC have emerged as the important foodborne zoonotic pathogens causing human gastrointestinal disease and confirming the risk to public health.

Methods: A total of 671 fecal samples were collected from diarrheic patients (n=395) and healthy calves (n=156) and goats (n=120) and screened for the presence of stx gene. Furthermore, the prevalence of stx1 and stx2 variants, serotypes (O157, O145, O103, O26, O111, O91, O128, and O45), phylogenetic groups and the presence of ehxA, eae, hylA, iha and saa virulence genes were studied.

Results: Prevalence of STEC in human diarrheic isolates was 1.3% (5 isolates), in calves was 26.3% (41 isolates) and in goats was 27.5% (33 isolates). stx1 gene was the most prevalent variant and detected in 75 isolates. Furthermore, stx1c was the most predominant stx subtype, found in 56 isolates. The ehxA identified in 36 (45.6%) isolates, followed by iha 5 (6.3%), eaeA 4 (5.1%), hylA 2 (2.5%) and saa 2 (2.5%). Most of the isolates belonged to phylogroup B1. Only two O26 and one O91 isolates were detected in our study.

Conclusion: Our results show that STEC strains were widespread among healthy domestic animals in the southeast of Iran

Keywords: Shiga toxin-producing E. coli, serogroup, virulence factors.

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Introduction

Shiga toxin-producing by Escherichia coli (STEC) is an important enteric pathogen, has been reported in several outbreaks with clinical manifestations including mild diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (1, 2). The disease in human is primarily a food-borne infection. Although STEC strains have been isolated from other animals such as goats, sheep, swine, wild animals and humans, cattle are the major source of food contamination (3). The ability of STEC strains to cause human disease is mainly due to the production of shiga-like toxins (stx) which are classified into two closely related subgroups, stx1 and stx2 (encoded by the stx1 and stx2 genes). Stx1 is a homologous group with only three variants (stx1a, stx1c, and stx1d), while stx2 is a more heterogeneous group and is comprised of several subtypes (stx2a, stx2b, stx2c, stx2d, stx2e, stx2f and stx2g) (4, 5). STEC strains producing stx2a, stx2c, or stx2d subtypes are more associated with HC and HUS in humans. In contrast, stx2b, stx2e, stx2f and stx2g are related to animal infections (6). Additional factors that contribute to virulence have also been described, including intimin (encoded by the eae gene), involved
in the attachment of E. coli to the enterocyte, plasmid-encoded enterohemolysin (encoded by ehxA gene) which acts as a pore-forming cytolysin, alpha-hemolysin (encoded by the hlyA gene), IrgA homologue adhesin (ihA) which is a STEC adherence-conferring molecule and Saa which is an autoagglutinating adhesin produced by LEE-negative STEC (3, 7-9). Epidemiologic investigations demonstrated that O157 is the main cause of HC and HUS in human; however, additional serogroups that have been reported in human clinical cases are O26, O45, O91, O103, O111, O128 and O145, and others in recent years (10, 11).

E. coli can also be assigned to one of the four major phylogenetic groups (A, B1, B2 and D) based on the presence or absence of chuA, yjaA and TspE4.C2 (12). Bearing in mind the importance of E. coli as foodborne pathogens, as vehicle of human disease, the objectives of this study were to investigate the distribution of subtypes, serotypes, virulence factors and phylogenetic groups among STEC strains from healthy domestic animals (calves and goats) and patients with diarrhea in Kerman, southeast of Iran.

Methods

Specimen collection and microbiological processing

In a prospective study, from October 2014 to November 2015, a total of 671 fecal samples were collected from diarrheic patients (n=395) and fecal healthy calves (n=156) and goats (n=120). The human samples were related to both male (n=215) and female (n=180). Their age ranged from <5 years old (n=107), 5 to 15 years old (n=146), 15 to 40 years old (n=75) and 40 to 90 years old (n=67). The human isolates obtained from the rectal swab of the patient with diarrhea referred to Afzalipour and Payambar-Azam hospitals. All animal samples were collected by veterinarians from School of Veterinary Medicine, Shahid Bahonar University, Kerman, Iran. All samples were placed into Amies medium (Becton Dickinson, BBL, and USA) and were sent out to the laboratory in ice-cooled containers. The samples were taken to the microbiology laboratory, Kerman University of Medical Sciences and identified as E. coli by biochemical characteristics and conventional diagnostic tests (13). All strains were stored at −70°C in Trypticase Soy broth (Difco Laboratories, Detroit, Mich.) containing 30% glycerol for further study.

Detection of STEC strains

For the detection of stx gene, DNA template was obtained by boiling method (14). Presence of stx gene in the selected E. coli colonies was verified by PCR method (15). In addition, stx-positive isolates were examined for the presence of stx1 and stx2 genes by using duplex-PCR (16). A positive control for PCR was E. coli strain MG1655 was used as a negative control for virulence genes. Details of the primers and the length of the expected amplification product are listed in Table 1.

Identification of subtype genes

We used PCR method for determination of stx1 and stx2 subtypes. PCR for detection of stx1a, stx1c, stx1d, stx2a, stx2b, stx2c, stx2d and stx2e and stx2f subtypes was carried out by methods described previously (17-19) (Table 1).

Identification of serogroup genes

Furthermore, PCR assay was used for the identification of O157, O145, O103, O26, O111, O91, O128 and O45 as described by Hemmatinezhad et al. (20) (Table 1).

Identification of virulence genes

The presence of following virulence genes ehxA, eae, hlyA, iha and saa were detected by PCR assay (21-24) (Table 1).

Determination of STEC strains phylogenetic groups

Strains assigned to one of the four main phylogenetic group of E. coli (A, B1, B2 and D) by using a PCR-based method as described previously (12). The genomic DNA of bacterial strains amplified by triplex-PCR using primers targeted at three markers, chuA, yjaA and TspE4.C2.

Statistical analysis

SPSS version 15.0 software for Windows (SPSS Inc., Chicago) was used for statistical analysis. P values of less than 0.05 were considered to be significant.

Results

Among 671 E. coli isolates isolated from healthy farm calves, goats and patients with sign of diarrhea, 79 strains were positive for the presence of stx gene and identified as STEC. Among STEC strains 41 strains were positive in calves, 33 strains in goats and 5 strains
**Table 1.** Oligonucleotide Primers Used in this Study.

| Target gene | Primer sequence (5'-3') | Size (bp) | Annealing temp (°C) | References |
|-------------|--------------------------|-----------|---------------------|------------|
| stx         | GAGCGAAATAATTTATATGGTG   | 518       | 55                  | 15         |
|             | TGGATGATGGCAATTTCAATGAT  |           |                     |            |
| stx1        | TAAAACGCACTCGTGTACAC     | 180       | 58                  | 16         |
|             | AGAAGCCTCCTGAGATCATC     |           |                     |            |
| stx2        | GGCACCTGTCGGAACTGTCGCC   | 255       | 60                  | 16         |
|             | TGGCCAGTTATTCTGACATTCTG  |           |                     |            |
| stx1a       | CAGGTTACACGCGTGTGGCA     | 219       | 57                  | 18         |
|             | CCGCCGACTGAATCATCC       |           |                     |            |
| stx1c       | TTTCACATGTACCTCTCTCTCT   | 498       | 54                  | 17         |
|             | CATAGAAAGGAAACTCTATTAGG  |           |                     |            |
| stx1d       | CTTTTTCAGTTAACTGGATGCT   | 192       | 57                  | 18         |
|             | AACCCTATATGACGTACTG      |           |                     |            |
| stx2a       | AGATATCGACCCCTCTTGGAA    | 969       | 55                  | 18         |
|             | GTGCAACCTCAGTAAATG       |           |                     |            |
| stx2b       | AAATATGAGAAAGATTTTGGAGGC| 251       | 60                  | 19         |
|             | CAGCAGAAACTCTGAACTGAG    |           |                     |            |
| stx2c       | GCCGTITTTATTTGCATTGT     | 124       | 55                  | 17         |
|             | AGTACTCTTCTTCCGACCT      |           |                     |            |
| stx2d       | GGTAAAATGTTTCTCTAAATGAT  | 175       | 58                  | 17         |
|             | CAGCAGAAACTCTGAACTGAG    |           |                     |            |
| stx2e       | CAGAAGGAAGTGTATTTAAGCA   | 267       | 56                  | 17         |
|             | TCGAGAAGCTTCACCTGAGGC    |           |                     |            |
| stx2f       | CTTTTCAGTTAATGGATGCT     | 192       | 57                  | 18         |
|             | AACCCTATATGACGTACTG      |           |                     |            |
| stx2g       | CACCGGGTATTTATATTT CCTCTGATATC | 573 | 62              | 19         |
|             | GATGCCAATTTCAAGATAACCCGT |           |                     |            |
| chuA        | GAGCGAAACTCGGACAGCT     | 279       | 59                  | 12         |
|             | TGCCGCAAGTAACCCGAGCA     |           |                     |            |
| YjaA        | TGGGCGTCAGGAGAGCTG       | 211       | 59                  | 12         |
|             | TGGGCGTCAGGAGAGCTG       |           |                     |            |
|             | TGGGCGTCAGGAGAGCTG       |           |                     |            |
|             | CTCCCTAAGCTCCCGCCGCTGA  | 1087      | 65                  | 22         |
|             | TCAGCGTGGGTTGGATCAACCT   |           |                     |            |
| TspE4.C2    | CTGGCG CAAAGACTGATCATCT | 152       | 59                  | 12         |
|             | CCGCGCAAGAAGATATTA CG    |           |                     |            |
| ehxA        | GGTGCGAGCAAAAAAAGTGTGA  | 1551      | 61                  | 24         |
|             | TCTCGCTGTAAGTGGTGTTGTA   |           |                     |            |
| hylA        | AACAAAGGATAGGCACCTGTTCGCT| 1177     | 61                  | 21         |
|             | ACCATATAAGCGTGCTATCCGCTA |           |                     |            |
| saa         | CGTGTAGAAGAAGCTGTAATGC  | 119       | 59                  | 23         |
|             | ATGGGACCTGGTGCGCACAC   |           |                     |            |
| iha         | CTGGCGGAGGCTCGAGTACGA   | 827       | 59                  | 23         |
|             | TCTTAAAGCTCCCGCCGCTGA   |           |                     |            |
| eaeA        | CAGGTCGCTGATGCTGCTTAAA  | 1087      | 65                  | 22         |
|             | TCACGCGTGTTGGATCAACCT   |           |                     |            |
| O157        | CGGACATCCATGTTAGATGG    | 259       | 58                  | 20         |
|             | TTGCTATGTACAGCTAATTCC   |           |                     |            |
| O145        | CCATACCAAGATTAGGAGTGA   | 609       | 58                  | 20         |
|             | TTTCCTCCGCCAAATCTAC    |           |                     |            |
| O111        | TAGAAAGGAATATCAAGTTAGTCC| 406       | 58                  | 20         |
|             | ATAGTTATGAACATTTGTTGAGC |           |                     |            |
| O91         | GCTGAACCTTATGATGTTGTA   | 291       | 58                  | 20         |
|             | TAATTTAACCCTGTAGAATCTGCT |           |                     |            |
| O128        | GCTTTTGCGTATATTGCGC     | 289       | 58                  | 20         |
|             | CCGACAGATGATGCCGAGTAGT  |           |                     |            |
| O45         | CCGGGTTGCTGATGTTGGAAGTGG | 527    | 58                  | 20         |
|             | CACAAGCACTACTAAGCCAGAAA |           |                     |            |
| O103        | TGCCGCGTAATCGGACCT     | 321       | 58                  | 20         |
|             | CTCGCCGAGACGCTATAAG     |           |                     |            |
| O26         | CAGAATTGTATGCTAATGT     | 423       | 58                  | 20         |
|             | CTTACATTGTTCCTGCAGCATC  |           |                     |            |
in human samples. Our results showed that 54 (68.4%) of the strains carried \( stx_1 \) only, 4 (5.1%) contained \( stx_2 \) only, and 21 (26.6%) possessed both \( stx_1 \) and \( stx_2 \).

Two \( stx_1 \) subtypes (\( stx_{1a} \) and \( stx_{1c} \)) and four \( stx_2 \) subtypes (\( stx_{2a} \), \( stx_{2b} \), \( stx_{2c} \) and \( stx_{2d} \)) were detected with a total of 13 different \( stx_1 \) and \( stx_2 \) subtypes combinations as shown in Table 2. Among the subtypes, \( stx_{1c} \) was detected in 56 strains, followed by \( stx_{1a} \) (25 strains), \( stx_{2b} \) (20 strains), \( stx_{2c} \) (19 strains) and \( stx_{2a} \) (1 strain). In addition, \( stx_{2d} \) (11 strains) was detected only in combination with other \( stx \) genes (Table 2). There was no correlation between \( stx \) subtypes and animal sources \( (P \leq 0.05) \).

The \( STEC \) strains were further tested for five putative virulence factors, including \( eaeA \), \( hlyA \), \( iha \), \( ehxA \) and \( saa \). Out of 79 strains, 49 (62%) carried at least one virulence gene tested. The \( ehxA \) was detected in 36 (45.6%), \( eaeA \) in 4 (5.1%), \( iha \) in 5 (6.3%), \( hlyA \) in 2 (2.5%) and \( saa \) in 2 (2.5%) of the isolates.

Phylogroup B1 was the most prevalent (62/79; 78.5%) among the \( STEC \) strains, followed by phylogenotypes A (12/79; 15.2%) and D (5/79; 6.3%). As shown in Table 3, all isolates of human origin belonged to the D Phylogroup. In this study, phylogenetic group B2 was not detected in \( STEC \) strains.

Serogroup analysis showed that none of the isolates belonged to O45, O103, O111, O128, O145 and O157 serogroups, while O26 and O91 were detected in two (clave and goat) and one (clave) isolate, respectively.

**Discussion**

\( STEC \) can be found in various food sources, transmission of this pathotype from undercooked or unpasteurized animal products to human is problematic \((1, 10)\). It is estimated that \( STEC \) to cause more than 265,000 illnesses each year in the USA, with more than 3,600 hospitalizations and 30 deaths \((25)\). In the present study, \( STEC \) strains were isolated just from 1.2% of patients with diarrhea which was consistent with previous studies \((26-28)\). According to a survey, high variability of genes-encoding \( stx \) was detected in the \( E. coli \) isolates in HIV and thalassemia patients in Kerman, south-east of Iran. Among \( E. coli \) isolates from faecal samples, 30.8% isolates were positive for \( stx \) genes \((34)\). However, 26.8% of \( E. coli \) isolated from goats and calves carried at least one of the \( stx \) genes.
This frequency was, lower compared to reports from Spain and Brazil (37% and 44%) (29, 30), but higher from results reported in Iran (8.5%) (31). Another study from West Azerbaijan province in Iran revealed that 21.92% of the E. coli isolates recovered from fecal healthy calves harbored stx genes (32). These variations may likely be due to geographical and climatic conditions and differences in the natural intestinal flora present in animal’s gastrointestinal tract (33).

In STEC strains characterized in this study, stx1 was the most common stx gene identified, a result which is similar to previous reports (31, 34). In contrast, some studies have detected stx2 as a dominant stx gene in fecal samples of animals (35, 36). Although, this variant mainly found in strains isolated from healthy human carriers and most likely does not cause severe diseases in human (36).

In the present study, stx1c was the predominant variant among the STEC strains isolated. Stx1c Subtype has also frequently been reported in previous studies (5, 37). However, stx1c-encoding strains are associated with asymptomatic human carriage or mild illness (38). Stx2c and stx2d are associated with HUS. However, they are less toxic on Vero cells compared to stx2a. STEC strains with stx2a are associated with several clinical symptoms, such as HUS and HC (39). Stephan and Hoelzl suggested that stx2a was not associated with severe human diseases, because most strains carrying stx2a were isolated from healthy human carriers (40). In the present study, two strains carrying only stx2a were isolated from human and it was possible that these two STEC strains were not the main causative agent of diarrhea. In this study, two strains isolated from calves carried 5 subtypes of stx1a, stx1c, stx2b, stx2c, stx2d simultaneously. The combination of five stx genes in one isolate had not been previously reported. In the study of Bertin et al. strains with a combination of stx1 and/or stx2 subtypes were found to be more toxic toward Vero cells than other strains (41). In our study, other stx2 subtypes such as stx2a, stx2b and stx2d were not found. These subtypes are related to animal infections (42).

In addition, we studied the distribution of eight important serotypes in the above isolates which associated more frequently with HUS and HC. None of the isolates belonged to O45, O103, O111, O128, O145 and O157 serogroups, while O26 and O91 were detected in two and one isolates respectively. This finding is in agreement with the failure to find these serotypes in yaks and cattle (8, 43). It seems that in some regions, ruminants are not important reservoirs for the outbreak isolates. Although, human infections with stx-producing E. coli O26 is uncommon and has resulted in less severe illness, but is a major cause of HUS in Europe continent (44).

In this study, only four strains contained eaeA gene; however, none of the isolates carried the stx2 subtypes. The low frequency of the eaeA gene found in the present study may be related to the low frequency of certain serogroups, as it has been reported that the presence of the eaeA gene is associated with specific O serogroups of STEC, such as the O157, O145, O103, O26, and O111 (33). Since the majority of the STEC strains lacked eaeA gene, we investigated other factors associated with adherence including iha and saa. These two virulence factors have been reported to be highly important for pathogenicity of eae-negative STEC strains (8). Only 2.5% and 6.35% of strains were positive for saa and iha respectively. It is possible that other virulence factors, that were not investigated in the present study like lpfa and paa play important role in the adherence of STEC strains. Also, we detected ehxA and hlyA genes in 45.6% and 2.5% of strains respectively. Overall, the frequency of virulence factors in STEC isolates was lower than that observed in other studies (8, 45). Carriage of stx gene positive E. coli isolates in the gastrointestinal tract of healthy ruminants proposes that these are transient commensal bacteria in these animals and the virulence genes of these isolates were either not or very poorly expressed (32).

Investigation on STEC phylogroups indicated that majority of commensal and diarrhetic strains are belonged to group B1 and A, while extra intestinal E. coli strains belong mainly to group B2 and D (46). In this study, phylogenetic group B2 were not detected in STEC isolates, which was consistent to previous study (46). However, like in many studies, phylogenetic group B1 was predominant among isolates from animals (47, 48). All of the human strains belonged to phylogenetic group D2, while it was not found in strains isolated from animals.

In conclusion, although STEC strains were widespread among healthy domestic animals in the southeast of Iran, prevalence of STEC in patient with diarrhea was
low and most of the STEC strains did not belong to O
serogroups that are commonly associated with severe
disease in humans. Furthermore, these strains were
mainly belonged to phylogenetic group B1. These facts
together with the high prevalence of stx1c, stx2b, stx2c
subtypes and low prevalence of stx2a, suggest that most
of STEC in Iranian calves and goats may not pose a
serious public health concern.

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Conflict of interests

The authors declare that they have no conflict of
interest.

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