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ORIGINAL ARTICLE

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 claves 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%), *hlyA* 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, cattles 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

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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 food-borne 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 stx_1 and stx_2 genes by using duplex-PCR (16). A positive control for PCR was *E. coli* Sakai (stx_1+/stx_2+/*eaeA*+). The *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 stx_1 and stx_2 subtypes. PCR for detection of stx_{1a}, stx_{1c}, stx_{1d}, stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f} and stx_{2g} 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*, *hylA*, *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 claves, 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	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	518	55	15
stx$_1$	TAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	58	16
stx$_2$	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	60	16
stx$_{1a}$	CACGTTACAGCGTGTTGCA CGCCCACTGAGATCATCC	219	57	18
stx$_{1c}$	TTTTCACATGTTACCTTTCCT CATAGAAGGAAACTCATTAGG	498	54	17
stx$_{1d}$	CTTTTCAGTTAATGCGATTGCT AACCCCATGATATCGACTGC	192	57	18
stx$_{2a}$	AGATATCGACCCCTCTTGAA GTCAACCTTCACTGTAAATG	969	55	18
stx$_{2b}$	AAATATGAAGAAGATATTTGTAGCGGC CAGCAAATCCTGAACCTGACG	251	60	19
stx$_{2c}$	GCGGTTTTATTTGCATTAGT AGTACTCTTTTCCGGCCACT	124	55	17
stx$_{2d}$	GGTAAAATTGAGTTCTCTAAGTAT CAGCAAATCCTGAACCTGACG	175	58	17
stx$_{2e}$	ATGAAGAAGATGTTTATAGCG TCAGTTAAACTTCACCTGGGC	267	56	17
stx$_{2f}$	TGGGCGTCATTCACTGGTTG TAATGGCCGCCCTGTCTCC	424	60	19
stx$_{2g}$	CACCGGGTAGTTATATTTCTGTGGATATC GATGGCAATTCAGAATAACCGCT	573	62	19
*chu*A	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	59	12
*Yja*A	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	59	12
TspE4.C2	CTGGCG AAAGACTGTATCAT CGCGCCAACAAAGTATTA CG	152	59	12
*ehx*A	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	1551	61	24
hylA	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177	61	21
saa	CGTGATGAACAGGCTATTGC ATGGACATGCCTGTGGCAAC	119	57	23
iha	CTGGCGGAGGCTCTGAGATCA TCCTTAAGCTCCCGCGGCTGA	827	57	23
*eae*A	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	1087	65	22
O157	CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC	259	58	20
O145	CCATCAACAGATTTAGGAGTG TTTCTACCGCGAATCTATC	609	58	20
O111	TAGAGAAATTATCAAGTTAGTTCC ATAGTTATGAACATCTTGTTTAGC	406	58	20
O91	GCTGACCTTCATGATCTGTTGA TAATTTAACCCGTAGAATCGCTGC	291	58	20
O128	GCTTTCTGCCGATATTTGGC CCGACGGACTGATGCCGGTGATT	289	58	20
O45	CCGGGTTTCGATTTGTGAAGGTTG CACAACAGCCACTACTAGGCAGAA	527	58	20
O103	TTGGAGCGTTAACTGGACCT GCTCCCGAGCACGTATAAG	321	58	20
O26	CAGAATGGTTATGCTACTGT CTTACATTTGTTTTCGGCATC	423	58	20

Table 2. Summary of *stx* subtyping in 79 non O157-STEC strains isolated from calves, goats and human fecal samples.

No (%) of strains	source			*stx* subtype					
	humans	calves	goats	stx_{1a}	stx_{1c}	stx_{2a}	stx_{2b}	stx_{2c}	stx_{2d}
35 (44.3)	2	20	13	-	+	-	-	-	-
16 (20.3)	1	6	9	+	-	-	-	-	-
8 (10.1)	0	4	4	-	+	-	+	+	+
5 (6.3)	0	5	0	-	+	-	+	+	-
5 (6.3)	0	2	3	+	+	-	-	-	-
2 (2.5)	2	0	0	-	-	-	+	-	-
2 (2.5)	0	2	0	+	+	-	+	+	+
1 (1.3)	0	0	1	+	-	-	-	+	-
1 (1.3)	0	1	0	-	+	-	+	-	-
1 (1.3)	0	0	1	+	-	+	-	-	-
1 (1.3)	0	0	1	-	-	-	+	+	-
1 (1.3)	0	1	0	-	-	-	+	+	+
1 (1.3)	0	0	1	-	-	-	-	+	-
Total= 79	5	41	33	25	56	1	20	19	11

No = Number of non O157-STEC strains, % = percent of non O157-STEC strains; – = negative, + = positive.

Table 3. Distribution of STEC in phylogenetic groups from calves, goats and human fecal samples

Phylogenetic group	No. (%) of STEC strains isolated from			Total
	humans	calves	goats	
A	0 (0)	6 (14.6)	6 (18.2)	12 (15.2)
B1	0 (0)	35 (85.4)	27 (81.8)	62 (78.5)
B2	0 (0)	0 (0)	0 (0)	0 (0)
D	5 (100)	0 (0)	0 (0)	5 (6.3)

NO = Number of STEC strains isolated from calves, goats and human fecal samples, % = percent of STEC strains isolated from calves, goats and human fecal samples

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 *eae*A, *hly*A, *iha*, *ehx*A and *saa*. Out of 79 strains, 49 (62%) carried at least one virulence gene tested. The *ehx*A was detected in 36 (45.6%), *eae*A in 4 (5.1%), *iha* in 5 (6.3%), *hly*A 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 phylogroups 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, stx_1 was the most common *stx* gene identified, a result which is similar to previous reports (31, 34). In contrast, some studies have detected stx_2 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, stx_{1c} was the predominant variant among the STEC strains isolated. Stx_{1c} 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). Stx_{2c} and stx_{2d} are associated with HUS. However, they are less toxic on Vero cells compared to stx_{2a}. STEC strains with stx_{2a} are associated with several clinical symptoms, such as HUS and HC (39). Stephan and Hoelzle suggested that stx_{2b} was not associated with severe human diseases, because most strains carrying stx_{2b} were isolated from healthy human carriers (40). In the present study, two strains carrying only stx_{2b} 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 stx_{1a}, stx_{1c}, stx_{2b}, stx_{2c}, stx_{2d} 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 stx_1 and/or stx_2 subtypes were found to be more toxic toward Vero cells than other strains (41). In our study, other stx_2 subtypes such as stx_{2e}, stx_{2f} and stx_{2g} 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 *eae*A gene; however, none of the isolates carried the stx_2 subtypes. The low frequency of the *eae*A 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 *eae*A 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 *eae*A 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 *ehx*A and *hly*A 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 diarrhogenic 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 stx_{1c}, stx_{2b}, stx_{2c} subtypes and low prevalence of stx_{2a}, 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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