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Virulence profiling and genetic relatedness of Shiga toxin-producing *Escherichia coli* isolated from humans and ruminants

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ABSTRACT

In the present study the occurrence, genotypic characteristics and relatedness of Shiga toxin-producing *Escherichia coli* (STEC) isolated from 235 fecal samples of diarrheic children ($n=75$), sheep ($n=80$), and cattle ($n=80$) were investigated. Overall, STEC was found in 4%, 61.2%, and 18.7% of diarrheic children, sheep and cattle, respectively. Three of the four STEC isolates from diarrheic children yielded the *stx1/ehly* profile. The predominant virulence profile of sheep isolates was *stx1/ehly* (85.2%), but cattle isolates were heterogeneous. Genetic relatedness and diversity of 36 selected isolates were analyzed by enterobacterial repetitive consensus sequences fingerprinting (ERIC) and phylogrouping. In total, 19 ERIC-types were observed in humans ($n=2$), sheep ($n=5$), and cattle ($n=12$) isolates. The majority of the sheep STEC were assigned into B1 phylogroup (83.3%), but cattle isolates belonged to different phylogroups with B1 predominance. Three human STEC isolates had the major characteristics of sheep isolates but revealed distinct fingerprint. These findings indicate that cattle can potentially carry a diverse group of STEC strains.

1. Introduction

Escherichia coli strains comprise a broad group of bacteria that generally inhabit as commensal in gastrointestinal tract of human and warm-blooded animals. However, some *E. coli* strains cause significant diseases in humans and different animal species [1]. Pathogenic strains that potentially target the gastrointestinal tract, are called diarrheagenic *E. coli* (DEC). These intestinal pathogens have been classified based on virulence factors, and pathogenic characteristics into at least six pathotypes including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC) [2,3]. Recently, after the emergence of highly pathogenic O104:H4 serotype in Europe, a new pathotype has been suggested: EAHEC (Entero-aggregative-hemorrhagic *E. coli*) [4].

Shiga toxin-producing strains can cause a broad spectrum of human diseases including hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans [1]. Generally, STEC strains can be identified by the presence of one or more genes encoding Shiga toxins. However, some STEC strains, often termed "enterohemorrhagic *E. coli* (EHEC)" have the potential to also harbor a locus of enterocyte effacement (LEE). The genes responsible for induction of attaching and effacing lesions are located within the LEE.

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The prominent gene in LEE is *eae*, which encodes intimin [5]. A number of other virulence genes have been described in STEC, of which *saa* encodes an important adhesion in *eae*-negative STEC strains (STEC-autoagglutinating adhesin), and *astA* mediates the production of enteroaggregative heat-stable enterotoxin [6,7]. Cattle are considered as the main reservoir for STEC strains [8], but isolation has not been limited to cattle, and other ruminants like sheep and goats can also act as reservoirs of STEC [9,10].

The strains that only harbor LEE, but not Shiga toxin genes are called EPEC [11]. EPEC strains, which possess the *E. coli* adherence factor (EAF) plasmid, are known as typical EPEC (tEPEC) and the strains lacking this plasmid, called atypical EPEC (aEPEC). The tEPEC are identified by the presence of bundle forming pilus (*bfp*) gene, which is carried within the EAF plasmid. Although typical strains have been isolated only from humans, atypical strains have been found in both humans and different animal species [12,13]. Intimin gene (*eae*) of STEC and EPEC strains can be divided into more than 17 subtypes by different methods based on variations at 3′ end of the *eae* gene [14]. Determination of intimin subtypes has great value for clonal comparison LEE-positive strains in epidemiological studies [14].

As an important food-borne pathogen, STEC strains are of major public health concern worldwide [10]. The major sources of food-borne infections can be different in diverse geographical areas of the world due to various dietary habits and culinary procedures. Garmsar region in the east of Tehran is one of the most important suppliers of meat and animal products for its population and other cities. Meat and other foodstuffs of animal origin are produced and consumed locally in Garmsar; therefore, this city with more than 40,000 populations has been chosen to compare the epidemiology of STEC in humans and ruminants in Iran. The aim of the present study was to investigate the occurence and virulence gene profiles of STEC among healthy ruminants and children with diarrhea in Garmsar region. Thirty-six strains were additionally compared by different genotypic criteria including, subtyping of the initimin gene, ERIC-types and phylogenetic groups.

2. Materials and methods

2.1. Sampling and E. coli strains

A total number of 235 fecal samples were collected using sterile swabs from 80 cattle, 80 sheep and 75 diarrheic children in Garmsar district in Semnan province of Iran. The animal samples were obtained from ruminants in the only local slaughterhouse in Garmsar. Human fecal samples were obtained from children, 2–10 years old, referred to Imam Khomeini hospital of Garmsar due to diarrhea. Both human and animal samples were taken at the same period of time (February–April). Samples from animals were collected in five times slaughterhouse visit across this time period and care was taken to ensure that animals of the same flock/herd were not sampled twice. The samples were transported to the laboratory in Amies medium (Becton Dickinson, BBL, USA) within 4 h. Then, they were inoculated into 3 ml buffered peptone water (Merck, Germany) and incubated at 37 °C for 5–6 h for primary enrichment. Next, the enriched samples were streaked on MacConkey agar (Merck, Germany) and sorbitol MacConkey agar (SMAC) (Merck, Germany) and incubated at 37 °C for 18–20 h. Up to two suspect lactose-positive and/or sorbitol-negative colonies were sub-cultured to obtain pure cultures. Confirmation of *E. coli* strains was done by conventional biochemical tests [15].

2.2. PCR for virulence genes (stx1, stx2, eae, ehly, bfp, saa, astA)

Total genomic DNA of the confirmed *E. coli* strains was extracted by boiling method. In brief, a loopful from confluent growth area in LB agar culture was suspended in 350 μl molecular grade water and boiled for 10 min. Then, samples centrifuged at 10,000 × *g* for 5 min, and the supernatants were used as templates. All strains were subjected to multiplex-PCR detecting *stx1*, *stx2*, *eae* and *ehly* virulence genes according to Paton and Paton [6]. Amplification was carried out in a total volume of 25 μl containing: 3 μl prepared DNA, 0.3 μM of each oligonucleotide primer, 0.2 mM dNTP mix, 2 mM $MgCl_2$, 2.5 μl of 10× PCR buffer, 1 unit *Taq* DNA polymerase (Cinnagen, Iran) and PCR grade water. Samples were subjected to 35 cycles of touchdown PCR, each consisting of 1 min denaturation at 95 °C, 2 min annealing at 65 °C for first 10 cycles, decreasing to 60 °C by cycle 15 and 1.5 min elongation at 72 °C, incrementing to 2.5 min from cycles 25–35. The PCR products were electrophoresed on 1.5% agarose gel for 90 min at 85 v and visualized by staining with ethidium bromide. Strains were additionally tested for the presence of *saa* (STEC autoagglutinating adhesion) and *astA* (enteroaggregative heat stable enterotoxin) virulence genes by PCR as previously described [6,7]. Positive PCR reactions were recorded by comparing the specific bands with positive-controls and 100 bp-plus molecular size marker (Vivantis, Malaysia). Positive control (O157:H7 strain, University of Tehran, collection strain No. 297) and negative control (sterile water) were included in all PCR reactions. Strains harboring the *eae* gene were also investigated for the presence of *bfp* gene as described previously [16]. For comparison of the occurrence of STEC in humans and ruminants, the frequencies were calculated with 95% confidence interval according to the normal distribution formula. A list of all primer sets used in this study is provided in Table 1.

2.3. Subtyping of the eae gene

Intimin subtypes were determined in all *eae*-positive strains by PCR–RFLP (polymerase chain reaction-restriction fragment length polymorphism). For typing of intimin the 3′-polymorphic end of *eae* gene was amplified using one forward and three reverse primers (Eae VF, Eae VR, Eae Zeta VR and Eae Iota) detecting most of the known intimin subtypes according to Ramachandran et al. [17]. After PCR optimization, the 840–880 bp specific products digested by *Cfo*I, *Rsa*I and *Alu*I restriction enzymes separately according to the manufacturer's instruction (Vivantis, Malaysia). After electrophoresis at 75 V for 100 min in 2% agarose gel, the *eae* subtypes were

Table 1
Primers used in this study.

Gene/method	Primer sequence (5′–3′)	Product size (bp)	References
stx1	ATA AAT CGC CAT TCG TTG ACT AC AGA ACG CCC ACT GAG ATC ATC	180	Paton and Paton [6]
stx2	GGC ACT GTC TCT CTG AAA CTG CTC C TCG CCA GTT ATC TGA CAT TCT G	255	
eae	GAC CCG GCA CAA GCA TAA GC CCA CCT GCA GCA ACA AGA GG	384	
ehly	GCA TCA TCA AGC GTA CGT TCC AAT GAG CCA AGC TGG TTA AGC T	534	
saa	CGT GAT GAA CAG GCT ATT GC ATG GAC ATG CCT GTG GCA AC	119	
astA	CCA TCA ACA CAG TAT ATC CGA GGT CGC GAG TGA CGG CTT TGT	111	Yamamoto and Nakazawa [7]
bfp	AAT GGT GCT TGC GCT TGC TGC GCC GCT TTA TCC AAC CTG GTA	324	Gunzburg et al. [16]
eae/RFLP	AGY[a] ATT ACT GAG ATT AAG AAA TTA TTY[a] TAC ACA RAY[a] AGT TTA TTT TAC GCA AGT TTA AAT TAT TTT ATG CAA AC	840-876[b]	Ramachandran et al. [17]
ERIC	ATG TAA GCT CCT GGG GAT TCA C AAG TAA GTG ACT GGG GTG AGC G	Random[c]	Versalovic et al. [18]
chuA	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279	Clermont et al. [19]
yjaA	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	211	
tspE4.C2	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	152	

[a] Guanine or cytosine.
[b] Variable based on intimin type.
[c] Variable based on the primer bindings to enterobacterial repetitive sequences.

determined by comparison with the described patterns [17].

2.4. Fingerprinting by ERIC-PCR

Based on different criteria including virulence gene profile, time of sampling, and animal source, 36 virulence-positive isolates were selected and subjected to fingerprinting by enterobacterial repetitive intergenic consensus sequences polymerase chain reaction (ERIC-PCR). The strains consisted of 5 human isolates, 13 isolates from cattle, and 18 from sheep. For ERIC-typing, DNA was extracted from 18 h LB broth culture using a commercial DNA extraction kit (Cinnagen, Iran), and concentration of DNA was adjusted to 50 ng/μl using a spectrophotometer. ERIC-PCR was performed with two primer sequences of ERIC1 and ERIC2 as described by Versalovic et al. [18]. PCR was conducted in a 25 μl volume containing 50 ng template DNA, 2 mM $MgCl_2$, 0.4 μM primers, 1.25 unit *Taq* DNA polymerase (Cinnagen, Iran) and 200 μM dNTP mix in 1× PCR buffer. The applied thermal cycles were as follow: initial denaturation at 94 °C for 7 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 3 min followed by a final extension at 72 °C for 15 min. Electrophoresis was performed using 2% agarose gel at 75 v for 3 h.

2.5. Phylogenetic group analysis

Thirthy-six *E. coli* isolates (the same strains as described above) were subjected to phylogenetic group analysis according to Clermont et al. [19]. Strains were assigned into one of the four phylogenetic groups (A, B1, B2, D) based on the possession or absence of three genes (*chuA*, *tspE4.C2*, *yjaA*). Multiplex-PCR conducted in 25 μl using 50 ng template DNA, 1.5 mM $MgCl_2$, 0.4 μM of each primers, 1 unit *Taq* DNA polymerase (Cinnagen, Iran) and 200 μM dNTP mix in 1× PCR buffer. The applied thermal cycles were consisted of 94 °C for 35 s, 59 °C for 15 s, and 72 °C for 20 s. The annealing temperature in the initial 5 cycles was 61 °C for 30 s, decreasing to 60 in the next 5 cycles, and 59 °C in the remaining 23 cycles.

3. Results

3.1. Virulence genes and eae-subtypes

A total number of 312 *E. coli* isolates were obtained from 235 fecal samples of cattle, sheep and diarrheic children and were subjected to PCR assays for detection of seven virulence genes. In total, 82 isolates harbored at least one on the tested virulence genes. Table 2 shows the distribution and frequency of different virulence gene profiles in 82 PCR-positive isolates in this study. Four samples (5.3%; 95% CI: 1.5–13.1%) out of 75 from diarrheic children contained five PCR-positive isolates, of which one isolate was EPEC (harbored only the *eae* gene) and the remaining four isolates from three samples were STEC (4%; 95% CI: 0.8–11.2) that three of them harbored the *stx1*/*ehly* (Table 2). Intimin subtyping revealed that the only human EPEC isolate possessed the Int-*θ*. This *eae*-positive isolate was negative for *bfp* gene and was considered aEPEC.

In 80 samples from cattle, 15 animals (18.7%; 95% CI: 10.9–29) carried STEC strains. One cattle isolate only

Table 2
Distribution of virulence gene profiles among *E. coli* isolates from different sources.

Gene profile	No. of isolates			Total (%)
	Cattle	Sheep	Human	
stx1	1	1		2(2.4)
stx2	2			2(2.4)
eae			1	1(1.2)
ehly		2		2(2.4)
ehly/astA	1			1(1.2)
stx1/stx2			1	1(1.2)
stx1/stx2/ehly	2	6		8(9.7)
stx1/stx2/ehly/astA	2			2(2.4)
stx1/stx2/ehly/saa	2			2(2.4)
stx1/eae/ehly	1			1(1.2)
stx1/ehly		52	3	55(67)
stx1/astA	1			1(1.2)
stx2/eae/ehly/astA	1			1(1.2)
stx2/ehly/astA	2			2(2.4)
stx2/ehly/saa	1			1(1.2)
Total[a]	16	61	5	82

[a] Total number of PCR-positive isolates.

possessed the *ehly*/*astA* genes. Two STEC isolates from cattle were *eae*-positive with one harboring Int-θ, and the other one Int-ι (Table 3). The *stx2* and *ehly* genes were the most frequent virulence genes among PCR-positive bovine *E. coli* isolates with the frequency of 75% (12/16) each. With regard to possession of the virulence genes, cattle isolates showed noticeable diversity and 11 virulence gene profiles were observed in 16 isolates (Table 2).

Sixty-one strains possessing at least one of the tested virulence genes were isolated from 49 out of 80 samples from sheep (61.2%; 95% CI: 49.7–71.9). All of these 49 samples from sheep carried STEC. In total, 59 of the PCR-positive isolates (96.7%; 59/61) were STEC and 2 isolates harbored only the *ehly* gene. The most prevalent virulence genes were *ehly* (98.4%; 60/61) and *stx1* (96.7%; 59/61) in sheep virulence-positive isolates. None of the isolates harbored the *eae*, *saa* and *astA* genes. The *stx1*/*ehly* (85.2%; 52/61) was the most frequent gene profile in sheep isolates (Table 2).

3.2. ERIC-PCR

Molecular fingerprinting of 36 selected isolates from different sources resulted in 19 ERIC-types that were designated as A–S (Table 3). Five isolates from diarrheic children yielded three profiles, of which three STEC isolates from two children yielded the similar fingerprinting pattern (designated as S). The greatest degree of diversity was observed in cattle isolates that yielded 12 profiles in total of 13 tested isolates. In contrast, ERIC-typing of 18 sheep isolates revealed only 5 profiles. One profile (designated as N) was predominant in isolates from sheep and 10 isolates out of 18 revealed this profile. The genotypic characteristics of 36 isolates from different sources are presented in Table 3.

3.3. Phylogenetic groups

Thirty-six isolates from different sources were assigned into three phylogroups including A, B1, and D (Table 3). Stains that not produced any amplicon in phylotyping-PCR were designated as A0. The predominant phylogroup was B1 (63.9%; 23/36) in isolates from three studied sources including cattle (5/13), sheep (15/18) and human (3/5). Three STEC isolates from two children had the similar phylogroup (B1) and harbored *stx1*/*ehly* genes. Half of the isolates from sheep (9/18) yielded the same virulence profile (*stx1*/*ehly*) and phylogroup (B1).

Table 3
Comparison of ERIC-types, phylogroups, intimin types and virulence genes of 36 selected isolates from different sources.

Phylogeny		Virulence genes						Hosts		
ERIC	Phylotype	*stx1*	*stx2*	*eae*	*ehly*	*astA*	*saa*	Cattle	Sheep	Human
A	B1	+	+		+	+		1		
B	B1	+	+		+			1		
C	B1		+					1		
D	B1	+	+		+		+	1		
E	A	+				+		1		
F	B1	+		+ (θ)[a]	+			1		
G	A0	+	+		+		+	1		
G	B1				+				1	
H	A		+	+ (ι)[a]	+	+		1		
I	A0				+	+		1		
J	D		+		+		+	1		
K	A		+		+	+		2		
L	A0	+						1		
M	B1	+	+		+				2	
M	B1	+			+				2	
M	B1				+				1	
N	B1	+	+		+				3	
N	B1	+			+				7	
O	A	+			+				1	
P	A	+							1	
Q	A	+	+							1
R	A			+ (θ)[a]						1
S	B1	+			+					3

[a] Intimin subtype according to the PCR-RFLP results.

4. Discussion

Different animal species could carry the STEC strains, but ruminants have been considered as the main reservoir for infection in humans worldwide [1]. Monitoring the virulence and genotypic characteristics of STEC from different sources provides useful information on epidemiology of disease in different geographical settings. In the present study, STEC was the found in 4% of diarrheic children, but only 1.3% children carried EPEC. Similarly, some previous studies showed the higher prevalence of STEC in Iran [20,21]. In contrast, Alikhani et al. [22] reported the high prevalence of EPEC compared with other pathogenic *E. coli* strains in children with diarrhea in Iran. In other recent study, none of the isolates were positive for *stx* gene, while 5.3% was positive for *eae*, which is in contrast to our observation [23]. Different results regarding the prevalence of STEC and EPEC have been reported in other countries. In Brazil, EPEC was more frequent than STEC [24], and the frequency of EPEC was 8.4% in Kuwait and 15.5% in Bangladesh in two other studies [25,26]. The results of the present study and most of the previous research suggest that geographical area and time of sampling are the most important criteria in the epidemiology of diarrhea in children in developing countries.

In the present study, the frequency of STEC carriage in cattle and sheep was 18.7% and 61.2% respectively. Previously, STEC strains were reported in 12.1% of *E. coli* isolates from cattle in Fars province in Iran [27] that is almost similar to the findings of this study. As other studies showed, STECs are generally more frequent than EPEC in cattle [28,29]. Regarding sheep isolates, the results of the current study were in agreement with Sanchez et al. [30] observation who reported the STEC prevalence in 80.8% of sheep isolates in Spain. Overall, based on the applied screening method in the present research, EPEC strains were not isolated from ruminants. In contrast, STEC carriage was dramatically high in sheep and noticeable in cattle.

In the current research three gene profiles were identified in *E. coli* isolates from diarrheic children: *stx1*/*ehly* ($n=3$), *stx1*/*stx2* ($n=1$), and *eae* ($n=1$). In animal *E. coli* isolates, *stx1*/*ehly* (85.2%; 52/61) was the most common combination of genes in sheep, but isolates from cattle showed a great degree of diversity and yielded 11 virulence profiles. Cattle strains were also the most diverse group showing different phylogroups and ERIC-types (Table 3). It should be noted that the combination of the most important virulence genes (especially *stx2*/*eae*/*ehly* harboring EHEC) were only observed in *E. coli* isolates from cattle (Table 2). Available literature indicated that sheep can be the natural reservoir for particular STEC strains that mainly harbor *stx1* and *ehly* genes. Novotna et al. [31] reported the *stx1*/*ehly* as the predominant gene profile with the frequency of 57.1% in a sheep farm in Czech that is similar to the findings of the present study. High prevalence of *stx1* (52.8%) and *ehly* (70.6%) genes was similarly observed in sheep isolates from 504 fecal samples in Spain [30]. The predominance of *stx1* in isolates from sheep has been also reported in southeast of Iran [32]. Interestingly, the maintenance of specific non-O157 STEC clones has been observed previously in four independent sheep flocks for a period of time as long as 11 months [30]. The finding of similar gene profiles, phylogroups and ERIC-types in sheep isolates in the present study also indicates the predominant colonization of a particular strain in sheep of different flocks within the studied area. Three STEC isolates from two diarrheic children in this study harbored *stx1*/*ehly* genes, and revealed the B1 phylogroup similar to the predominant sheep isolates. The similar virulence gene combination and phylogroup suggest the probable transmission of STEC of sheep source to humans.

To the best of our knowledge, there is not any data available on intimin subtypes of *E. coli* from humans in Iran. In this study, the only aEPEC strain of human origin harbored the Int-θ. In comparison, one of the cattle isolates harbored Int-θ, and the other one Int-ι (Table 3). Although the intimin subtype of a cattle isolate was similar to humans, these could not be proved to be of the same origin, since the cattle isolate was STEC, and the humans were aEPEC. Beta intimin has been considered as the most frequent subtype in isolates from humans and animals in several studies [17,26,33,34]; however, Int-θ has also been reported as a prevalent subtype [10,17,33]. Recently, the particular significance of Int-β1 has been shown in aEPEC isolates from diarrheic patients in Japan, but the association between other subtypes and diarrhea was not significant [34]. Askari Badouei et al. [33] have shown the particular importance of Int-θ in association with calf diarrhea. The association of intimin subtype and diarrhea are of particular interest and needs further research using a large number of *eae*-positive isolates.

In conclusion, findings of the present research show that ruminants can be the potential reservoirs of STEC in the studied area. Comparison of the genotypic characteristics of the isolates from different sources showed a high degree of genetic diversity in cattle isolates. Sheep in the studied area frequently carried the highly similar STEC strains harboring the minimal virulence genes. Most of the STEC isolates from diarrheic children showed the similar virulence profile and phylogroup to the sheep isolates that suggest the possible transmission from sheep source, but since the ERIC-type of human isolates were not similar to sheep, this cannot be proved by the applied methods. Although the present study highlighted some genotypic differences of STEC strains in the studied source species, for better understanding the epidemiology of STEC, future comparative studies needs to be conducted on a larger sample size from various geographical areas.

Conflict of interest

None to declare.

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