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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