Int J Entric Pathog. 2014 February; 2(1): e15195. DOI: 10.17795/ijep15195

Published online 2014 February 08. **Research Article**

Phylogenetic of Shiga Toxin-Producing *Escherichia coli* and a typical Enteropathogenic *Escherichia coli* Strains Isolated From Human and Cattle in Kerman, Iran

Hesam Alizade [1,*]; Reza Ghanbarpour [2]; Mojdeh Nekoubin [2]

[1]Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, I.R. Iran
[2]Department of Molecular Microbiology, Faculty of Veterinary Medicine, Shahid Bahonar University, Kerman, I.R. Iran

*Corresponding author: Hesam Alizade, Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, I.R. Iran, Tel: +98-3412112794, Fax: +98-3412112794, E-mail: Alizade.h2000@yahoo.com

Received: October 1, 2013; **Revised:** November 5, 2013; **Accepted:** November 25, 2013

Background: Shiga toxin-producing *Escherichia coli* (STEC) have emerged as the important zoonotic food-borne pathogens and confirming the risk to public health. Enteropathogenic *Escherichia coli* (EPEC) is a major cause of children diarrhoea in developing countries. *E. coli* strains can be assigned to four main phylogenetic groups, A, B1, B2 and D.
Objectives: The aim of the current study was to analyze the distribution of phylogenetic groups and presence of STEC and atypical EPEC pathotypes in *E. coli* isolated from human diarrhea and fecal samples of healthy cattle in Kerman, Iran by PCR.
Materials and Methods: A total of 188 *E. coli* isolates were isolated from human diarrheic (94 isolates) and fecal healthy cattle (94 isolates) samples. The isolates were identified by standard bacteriological tests. The confirmed isolates were examined to detect the phylogenetic groups and a selection of virulence genes including *stx1, stx2* and *eae* by PCR.
Results: Phylotyping of isolates from diarrheic human showed that 38.29% belonged to A, 20.21% to B1, 14.89% to B2 and 26.59% to D phylo groups. The isolates of healthy cattle distributed in A (34.04%), B1 (47.88%), B2 (7.44 %) and D (10.64%) phylo-groups. Prevalence of *eae* gene in human diarrheic isolates was 5.32% (5 isolates), whereas none of the human diarrheic isolates were positive for *stx1* and *stx2* genes. Among cattle isolates 7.44% (7 isolates) were positive for *stx1* gene and 5.32% (5 isolates) possessed *eae* gene. Of the all isolates examined, none were positive for the *stx2* gene. The *eae* gene were positive for isolates of human diarrhea distributed in A and B2 phylo-groups and isolates possessed *stx1* and *eae* genes from healthy cattle fell into A (4 isolates), B1 (7) and B2 (one isolate).
Conclusions: The isolates of human diarrhea samples and fecal healthy cattle were distributed into different phylogenetic groups, which mostly distributed in A and B1 phylo-groups. In addition, results of this study revealed the lower prevalence of SETC and aEPEC in isolates.

Keywords: Escherichia coli; Diarrhea; Shiga-Toxigenic *Escherichia coli*

1. Background

Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) produce the characteristic attaching and effacing (A/E) lesions in the gut mucosa of humans and animals (1). Based on the molecular studies, currently EPEC is responsible, on average, for 5-10% of pediatric diarrheal episodes in the developing world. Diarrheagenic *E. coli* have been classified into six categories based on epidemiologic, clinical, and molecular criteria: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); Shiga toxin–producing *E. coli* (STEC), also known as enterohemorrhagic *E. coli* (EHEC) or verotoxin-producing *E. coli* (VTEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAEC or EAggEC); and diffusely adherent *E. coli* (DAEC) (2). Domestic ruminants, especially cattle and sheep harboring STEC may constitute an important reservoir for STEC infection of humans (3). STEC can cause severe diseases in humans, such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombocytopenic purpura (TP), which may prove fatal in immunodeficiency patients (4). Typical EPEC strains express *eae* gene, which encodes intimin, and the bundle-forming pili (BFP) responsible for enterocyte attaching and effacing lesions, whereas strains with the A/E genotype which do not possess bfpA2 gene are classified as atypical EPEC (aEPEC) (5). STEC with and without the *eaeA* genotype, may expresses one or two shiga-like toxin encoding genes, *stx1* and *stx2* (6). *E. coli* strains can be classified to one of the main phylogenetic groups: A, B1, B2 or D. The

Implication for health policy/practice/research/medical education:
This study carried out in order to determine the prevalence of shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *Escherichia coli* pathotypes and also determined phylogenetic groups of isolates in cattle and human. Transmission of shiga toxin-producing *Escherichia coli* strains is important from food specially cattle to human.

diarrheagenic *E. coli* strains belong to groups A, B1 and D, the commensal strains to groups A and B1, whilst the extra-intestinal pathogenic strains usually belong to groups B2 and D (7, 8).

2. Objectives

Healthy asymptomatic bovine are the best-recognized animal reservoir for STEC strains. Sources of human infection include primarily foods of cattle origin; mainly undercooked beef products, unpasteurized milk, and direct contact with bovine and person to person transmission. The aim of this study was to analyze the distribution of phylogenetic groups (A, B1, B2 and D) and occurrence of STEC and atypical EPEC pathotypes encoding genes *stx1*, *stx2* and *eaeA* in *E. coli* isolated from patients with diarrhea and fecal samples of healthy cattle in Kerman, Iran by PCR.

2. Materials and Methods

2.1. Source of the E. coli Isolates

A total of 94 *E. coli* isolates were obtained from diarrheic samples of human and 94 isolates were taken from rectal swabs of the healthy cattle. The human samples were related to both male (n=51) and female (n=43). The isolates were collected during 2010 to 2011 in Kerman province, Iran. In the laboratory, samples were cultured on Mac Conkey agar and EMB (Biolife Laboratories, Milano, Italy). *E. coli* isolates were isolated and identified by standard biochemical and bacteriological methods. Isolates were stored in Luria-Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at -20°C.

2.2. Reference Strains

In this study two *E. coli* strains were used as positive controls: *E. coli* Sakai for EHEC and atypical EPEC (*stx1*+, *stx2*+ and *eaeA*+) and *E. coli* ECOR62 for (*chuA*+, *yjaA*+ and TspE4.C2+). *E. coli* strain MG1655 was used as a negative control for virulence genes and as a positive control for phylogenetic ECOR group A (*chuA*, *yjaA*- and TspE4.C2). All the reference strains were from the bacterial culture collection of Microbiology Department of Ecole Nationale Veterinaire Toulouse, France.

2.3. Pathotype and Phylotype Determination by PCR Assay

DNA of *E. coli* isolates and reference strains was extracted by lysis method (9). The phylogenetic analyses of the isolates were carried out by combinations of three genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2 by a triplex PCR method (10). All isolates were tested by multiplex PCR assay for the presence of the genes encoding intimin, *stx1* and *stx2* described by China et al. (11). The primers used for amplification of the virulence genes to determine STEC and atypical EPEC pathotypes and phylogenetic groups are presented in Table 1.

4. Results

PCR assays for phylotyping of isolates indicated that 188 *E. coli* isolates from diarrheic samples of human and fecal samples of healthy cattle fall into four phylogenetic groups, whereas 36.17% (68 isolates) fell into A, 34.04% (64) B1, 11.17% (21) B2 and 18.61% (35) D phylogenetic groups. The combinations of three genetic markers *chuA* (279 bp), *yjaA* (211 bp) and Tspe4.C2 (152 bp) used in determination of phylogenetic groups. Among 94 *E. coli* isolates from diar

Table 1. Oligonucleotide Primers Used in This Study

	Primer Sequence (5′-3′)	Product Size (bp)	Reference
EPEC & STEC [a]			
Gene			
eaeA	AGG CTT CGT CAC AGT TG	570	China et al. (11)
	CCA TCG TCA CCA GAG GA		
stx1	AGA GCG ATG TTA CGG TTT G	388	
	TTG CCC CCA GAG TGG ATG		
stx2	TGG GTT TTT CTT CGG TAT C	807	
	GAC ATT CTG GTT GAC TCT CTT		
Phylo-group			
Gene or probe name			
yjaA	TGA AGT GTC AGG AGA CGC TG	211	Clermont et al. (10)
	ATG GAG AAT GCG TTC CTC AAC		
TspE4.C2	CTG GCG AAA GAC TGT ATC AT	152	
	CGC GCC AAC AAA GTA TTA CG		
chuA	GAC GAA CCA ACG GTC AGG AT	279	
	TGC CGC CAG TAC CAA AGA CA		

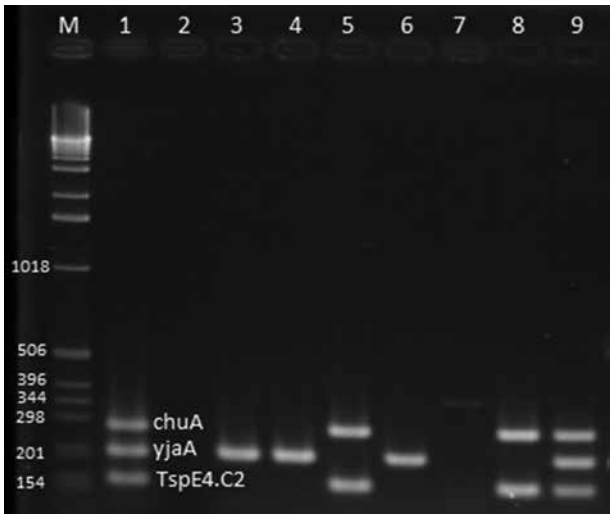
[a] Abbreviations: EPEC, enteropathogenic *E. coli*; STEC, shiga toxin-producing *Escherichia coli*

rheic human 36 isolates (38.29%) belonged to A, 19 (20.21%) to B1, 14 (14.89%) to B2 and 25 (26.59%) to D phylo groups. PCR results showed that 94 isolates from healthy cattle distributed into four phylo groups including 32 isolates (34.04%) in A, 45 (47.88%) in B1, 7 (7.44 %) in B2 and 10 (10.64%) in D group (Figure 1). Overall seventeen (9.04%) of the 188 *E. coli* isolates analyzed carried the STEC and aEPEC encoding genes, while *stx1* gene (388 bp) were detected in 3.72% (7 isolates) and *eae* gene (570 bp) in 5.32% (10 isolates) of isolates. Of the all isolates investigated, none were positive for the *stx2* gene. Prevalence of *eae* gene in human diarrheic isolates was 5.32% (5 isolates), whereas none of the human diarrheic isolates were positive for *stx1* and *stx2* genes. Among 94 *E. coli* isolates of cattle 7.44% (7 isolates) were positive for *stx1* gene and 5.32% (5 isolates) possessed *eae* gene (Figure 2). Among isolates examined 9.04% isolates were positive for STEC and aEPEC pathotypes, which distributed in A (5 isolates), B1 (7 isolates) and B2 (5 isolates) phylogenetic groups. Among *E. coli* isolates from human diarrheic samples 5 isolates were positive for *eae* gene, which distributed in two phylogenetic groups A (one isolate) and B2 (4 isolates) (Table 2). Seven *stx1* positive isolates from healthy cattle fell into A (3 isolates), B1 (3 isolates) and B2 (one isolate) and five isolates were positive for *eae* gene belonged to A (one isolate) and B1 (4 isolates) phylo-groups (Table 2).

Table 2. Distribution of STEC and Atypical EPEC Pathotypes in Phylogenetic Groups

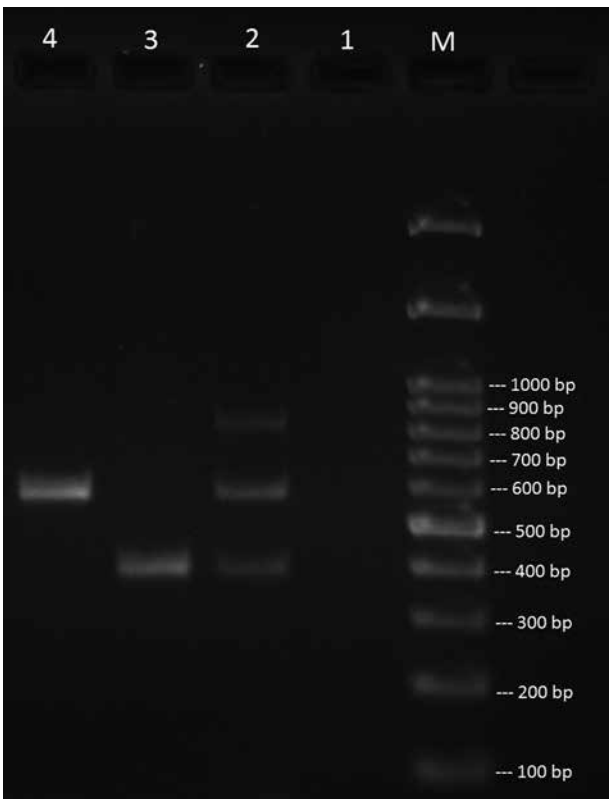
Gene	Human Diarrhea					Fecal Healthy Cattle				
	A	B1	B2	D	Total	A	B1	B2	D	Total
stx1	-	-	-	-	-	3	3	1	-	7
stx2	-	-	-	-	-	-	-	-	-	-
eae	1	-	4	-	5	1	4	-	-	5
Total	1	-	4	-	5	4	7	1	-	12

Figure 1. Positive Multiplex PCR Results for the Detection of *E. coli* Phylogenetic Groups Among the Patients with Diarrhea and Fecal Samples of Healthy Cattle.



M: ladder 1Kb - 1: positive control *E. coli* ECOR62 - 2: negative control *E. coli* MG1655 - 2, 3, 6, 7: A phylo-group – 5, 8: D phylo-group – 9: B2 phylo-group.

Figure 2. The Multiplex PCR Results for *stx1* and *eaeA* Genes.



M: the marker – 1: negative control *E. coli* MG1655 – 2: positive control *E. coli* Sakai - 3: the positive isolate for *stx1* gene – 4: the positive isolate for *eaeA* gene.

5. Discussion

STEC strains, the cause of human infections, occur after consumption of contaminated food or contact with an infected animal. Cattle are thought to be the main reservoir of STEC and often carry this pathotype in their intestinal flora and serve as source of food contamination (12).

Prevalence of EPEC varies in human related to differences in study population, age group, diagnostic criteria and methods used for diagnosis. Recent studies suggest that atypical EPEC are more prevalent than typical EPEC in both developed and developing countries (2). In the present study, STEC and atypical pathotypes occurred at lower frequencies in human with diarrhea and fecal of healthy cattle in the studied region of Kerman (Iran).

In studies on the capital of Iran (Tehran) 808 isolates which obtained from patients with acute diarrhea 7.92% isolates were positive for STEC and 1.48% for aEPEC, which *stx1* and *stx2* genes were detected in 34.37% and 43.75% of SETC strains, respectively (13). In the study of Aktan et al. (14) on faeces of healthy cattle, sheep and pigs entering abattoirs 8.06% isolates were *eae* probe positive that presumptively identified A/E. All isolates were positive for *eae* gene, five of these isolates possessed *stx1* gene and none of them were positive for *stx2* gene. The results from the present study are in accordance with the mentioned study by Aktan et al. (14), which showed none of the faeces of healthy cattle isolates were positive for *stx2* gene. In a study on Iran, 29 STEC strains were isolated in northern (Mazandaran province) and southwest (Ilam province), which 28 of them revealed the presence of *stx1* and one strain possessed *stx2* gene. None of the strains carried the *eae* gene (15). Reports revealed discrepancies in prevalence of STEC in different countries because this pathogen has not been isolated from diarrhea specimen in human (13, 16). Food-borne outbreaks caused by STEC can affect large numbers of people. Since there is currently no specific treatment for infections of this pathotype, an understanding of the epidemiology of STEC infections is urgently needed. In the current study frequency of aEPEC in human was 5.32%, as was found in study conducted in Thailand (5.5%) (17); however, in Brazil (34%) and Korea (56%) EPEC were detected with high frequency (18). Alikhani et al. (6) reported that aEPEC strains possess the *eaeA* gene are a common cause of children with diarrhea in three Iranian provinces, Tehran, Ilam and Mazandaran of Iran. In Spain, distribution of the types of the *eae* gene among a collection of AEEC strains isolated from healthy cattle and healthy sheep was investigated, which healthy sheep isolates were high percentage types of *eae* gene than healthy cattle isolates (18).

In the current study indicated the distribution of the main phylogenetic groups among *E. coli* strains isolated from human diarrhea and healthy cattle. Carlos et al. (19) concluded that geographic variation of the *E. coli* population structure related to different phylogenetic groups. One of the major forces that shape the genetic structure of *E. coli* populations among the hosts is domestication. Escobar-Paramo et al. (20) analyzed fecal strains isolated from humans and animals indicated the prevalence of groups A and B2 in humans and A and B1 in animals. In this study phylogenetic groups of human diarrhea isolates mostly fell into group A, followed by D and B1, and isolates from healthy cattle mostly distributed in phylogroups B1, A and D. Phylogenetic analysis showed that EPEC strains of human are clustered mostly in groups B1 and B2 (21), whereas in the current study mostly aEPEC positive isolates of human diarrheic samples distributed in B2 and A phylo-groups. Phylogenetic analysis of STEC positive isolates of human showed that the isolates belonged to A (3 isolates), B1 (3 isolates) and B2 (one isolate) groups. Phylogenetic study on human diarrheagenic *E. coli* isolates indicated that STEC strains were segregated in A and B1 phylo-groups (22), whereas Phylotyping of STEC strains from animals mostly distributed in B1 (38.7%) and A (35.5%) phylogenetic groups (1).

In conclusion, the *E. coli* isolates of human diarrhea samples and fecal healthy cattle were distributed into different phylogenetic groups, which A and B1 phylogenetic groups were represented the majority of isolates. In addition, results of this study revealed the lower prevalence of SETC and aEPEC in isolates. In order to determine detailed genetic studies of other diarrheagenic *E. coli* pathotypes in human and also typical and atypical EPEC in animal's future research will still be needed.

Acknowledgements

The authors are thankful to Dr. Eric Oswald (Ecole Nationale Veterinaire Toulouse, France) for providing the reference strains. This work was supported by a grant from the Shahid Bahonar University of Kerman, Molecular Microbiology Research Nucleus.

Authors' Contribution

All the authors participated in the manuscript preparation and experiment procedures equally.

Financial Disclosure

There is no conflict of interest.

Funding/Support

This work was supported by a grant from the Shahid Bahonar University of Kerman.

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