Research Article

Prevalence and Resistance Profiles of Enteropathogenic and Shiga Toxin-Producing *Escherichia coli* in Diarrheic Calves in Mashhad and Garmsar Districts, Iran

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Background: Shiga toxin-producing *Escherichia coli* (STEC) strains are considered as one of the most important widespread food-borne pathogens, which cause diarrhea and life threatening diseases, such as hemolytic uremic syndrome, in humans. More recently, the STEC strains have also been incriminated to cause diarrhea and hemorrhagic colitis in calves; enteropathogenic *E. coli* (EPEC) also causes diarrhea in neonate animals.

Objectives: This study aimed to study the prevalence and antibacterial resistance patterns of STEC and EPEC in fecal samples from diarrheic calves in Mashhad and Garmsar districts, Iran.

Materials and Methods: A total of 115 fecal samples were collected from diarrheic animals, 75 from Mashhad and 40 from Garmsar districts. A total of 146 *E. coli* isolates were obtained from culture and subjected to multiplex-PCR assay targeting *stx1*, *stx2*, *eaeA* and *ehly* virulence genes. The antibacterial resistance patterns of the virulence-positive isolates were determined using disc diffusion method.

Results: Eight samples (6.9%) carried the strains with positive results for at least one of the tested virulence genes. Five samples (4.3%) contained the *stx*-positive strains (STEC) and three (2.6%) carried the *eaeA*-positive and *stx*-negative strains, which were categorized as EPEC. In nine virulence-positive *E. coli* isolates, *stx1* (n = 6) was the predominant virulence gene, followed by *ehly* (n = 5), *eae* (n = 4), and *stx2* (n = 2). Antibacterial resistance patterns of virulence-positive isolates were also determined and nine resistance profiles were discriminated; higher rates of resistance were observed in isolates from Mashhad.

Conclusions: This study indicated that other pathologic factors might play a more important role in calf diarrhea in the studied areas, but public health significance of these strains should not be overlooked.

Keywords: STEC; EPEC; Calf Diarrhea; Antibacterial Resistance

1. Background

Escherichia coli strains that produce Shiga toxins are called Shiga toxin-producing *E. coli* (STEC). Shiga toxins are classified into two types that are encoded by *stx1* and *stx2* genes. Intimin, another important virulence factor encoded by the *eaeA* gene, mediates the intimate attachment of bacteria to the intestinal villi and induces attaching and effacing lesions (A/E) (1-3). The *ehly* gene is located on a 60-MDa virulence plasmid, and encodes for enterohemolysin, a distinct product from alpha hemolysin of *E. coli* (4). Shiga toxin-producing strains, which also possess *eaeA*, and *ehly* genes, are preferably termed enterohemorrhagic *E. coli* (EHEC). In humans, EHEC strains are the potential sources for outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) worldwide (5, 6). STEC not only cause life threatening diseases in humans, but also is incriminated to cause diarrhea and hemorrhagic enterocolitis (HC) in calves (7, 8). Different serotypes of STEC that might be associated with diarrhea in calves (mainly O5, O26, and O118) have been recognized (1). Surveying the prevalence of STEC and EPEC in animals in different geographical areas is important to monitor the epidemiology of infection with pathogenic *E. coli* strains.

2. Objectives

The aim of the present study was to investigate the prevalence of STEC and EPEC strains in diarrheic calves, which were younger than five weeks old, in Garmsar and Mashhad districts, Iran. The major virulence factors of STEC and EPEC and antibacterial resistance profiles were also determined.

3. Materials and Methods

3.1. Specimen Collection and Escherichia coli Strains

Fecal samples were obtained from 115 diarrheic calves in geographically separate farms, located around Mashhad and Garmsar districts. Farms in Garmsar were traditional ones with generally less than 30 animals per farm. All farms in Mashhad were industrial dairy farms. Animals that had recent history of antimicrobial therapy were excluded from sampling. A total of 75 samples were obtained from Mashhad and 40 samples from Garmsar. Specimens were collected using sterile swabs from younger than 30-day-old calves with symptoms of diarrhea or dysentery at the time of sampling. Specimens were sent to the laboratory in Amies transport medium (Difco, USA) and were transferred on MacConkey agar (Merck, Germany) and Sorbitol MacConkey agar (SMAC) (Quelab, Canada). One suspected colony was randomly selected from each culture including lactose fermenting colonies on MacConkey agar and sorbitol-negative colonies on SMAC. All isolates were confirmed by biochemical tests including conventional lactose and glucose fermentation (using TSI medium), urease, indole, methyl red, voges proskauer, citrate, and lysine decarboxylase (9)

3.2. Detection of Virulence Genes by Multiplex-PCR

Confirmed *E. coli* strains were subjected to multiplex-PCR assay, specific for four major virulence genes of STEC and EPEC. Total genomic DNA was extracted from overnight LB agar culture (Merck, Germany) by the boiling method, as was described previously (10). The supernatant was used as template in the PCR mixture. In multiplex-PCR, four pairs of specific primers were used for *stx1, stx2, eae,* and *ehly* genes as described by Paton and Paton (1998) (11). Amplification was performed in a total volume of 25 μL containing: prepared DNA, 3 μL; 0.3 μM of each oligonucleotide primer; dNTP mix, 0.2 mM; $MgCl_2$, 2 mM; 10× PCR buffer, 2.5 μL; Taq DNA polymerase (Cinnagen, Iran), one unit; and PCR grade water, up to 25 μL. Samples were subjected to 35 cycles of touchdown PCR, each consisting of one-minute

denaturation at 95℃, two-minute annealing at 65℃ for first ten cycles, which was decreased to 60℃ by cycle 15, and 1.5-minute elongation at 72℃, which was increased to 2.5 minutes from cycles 25 to 35. The PCR products were electrophoresed on 2% agarose gel for 90 minutes at 85v and were visualized by staining with ethidium bromide. Positive results in PCR reactions were recorded by comparing the specific bands with 100bp-plus molecular size marker (Fermentas, Lithuania). Positive controls (O157:H7, Tehran University, collection strain) and negative control (sterile water) were included in all PCR reactions.

3.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities of the strains that yielded positive results in the PCR assay were determined on Mueller-Hinton agar (Merck, Germany) by Kirby- Bauer method, according to Clinical and Laboratory Standard Institute (CLSI) protocol (12). Fourteen commercial antibacterial discs (Padtan Teb, Iran) from different classes, which were generally used in veterinary and human medicine in Iran, were employed. The discs included amoxicillin-clavulanate (AMC, 30 μg), gentamicin (G, 10 μg), neomycin (N, 30 μg), doxycycline (D, 30 μg), florfenicol (FF, 30 μg), trimethoprim-sulfamethoxazole (STX, 25 μg), trimethoprim (TMP, 5 μg), ceftriaxone (CRO, 30 μg), cefixime (CM, 5 μg), enrofloxacin (NFX, 5 μg), furazolidone (FR, 100 μg), flumequine (FM, 30 μg), lincospectin (LS, 150 μg), and Fosbac (200 μg).

4. Results

A total of 146 isolates were confirmed as *E. coli* through conventional biochemical tests with 96 from 75 samples from Mashhad and 50 from 40 samples in Garmsar. In multiplex-PCR assay, nine isolates from eight calves had at least one of the tested virulence genes. Two isolates from a diarrheic calf in Garmsar had produced positive results in the PCR assay; one isolate harbored only the *stx1* (isolate No. 7) and the other one had *stx1, stx2,* and *ehly* genes (isolate No. 8) (Table 1). Overall, eight calves (6.9%) carried the strains that were positive for at least one of the tested virulence genes (EPEC or STEC). Among nine virulence-

Table 1. Enteropathogenic and Shiga Toxin-Producing *Escherichia coli* Isolated From Diarrheic Calves [a]

Strain No.	Virulence Genes				Pathogenic Type	Age, d	Isolate's Origin
	stx1	*stx2*	*eaeA*	*ehly*			
1	-	-	+	+	EPEC	7	Mashhad
2	-	-	+	-	EPEC	8	Mashhad
3	-	-	+	+	EPEC	8	Mashhad
4	+	-	-	-	STEC	7	Mashhad
5	+	-	-	-	STEC	5	Mashhad
6	+	-	+	+	STEC	10	Garmsar
7	+	-	-	-	STEC	10	Garmsar
8	+	+	-	+	STEC	10	Garmsar
9	+	+	-	+	STEC	17	Garmsar

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

Table 2. Antibacterial Resistance Profiles of Nine *Escherichia coli* Isolated From Diarrheic Calves [a,b]

Strain No.	Antibiotic								
	GM	**CFM**	**FF**	**D**	**STX**	**LS**	**N**	**NFX**	**TMP**
1	S	S	I	R	S	R	S	S	S
2	S	I	R	R	R	R	I	S	R
3	I	S	I	I	R	R	R	I	R
4	S	S	S	R	R	R	I	S	R
5	I	S	I	I	S	R	I	S	S
6	S	S	S	R	S	I	I	S	S
7	S	S	S	I	S	R	I	S	S
8	I	S	S	R	S	S	I	S	S
9	S	S	S	S	S	S	I	S	S

[a] All strains were sensitive to Fosbac, flumequine, furazolidone, and ceftriaxone and resistant to amoxicillin-clavulanate. Therefore, they were excluded from resistance profiles.
[b] Abbreviation: GM, gentamicin; CFM, cefixime; FF, florfenicol; DC, doxycycline; NFX, enrofloxacin; STX, trimethoprim-sulfamethoxazole; LS, lincospectin; N, neomycin; TMP, trimethoprim; S, sensitive; I, intermediate; and R, resistant.

positive *E. coli* isolates, *stx1* (n = 6) was the predominant virulence gene, followed by *ehly* (n = 5), *eae* (n = 4), and *stx2* (n = 2). Five calves (4.3%) carried the *E. coli* strains with a variant of *stx* genes (STEC), and three calves (2.6%) carried the eaeA-positive and stx-negative strains, which were categorized as EPEC. In Mashhad, two calves (2.6%) carried the STEC, and three calves (4%) carried EPEC strains. None of the isolates from Mashhad was positive for stx2. In Garmsar, 10% of cultured fecal samples had positive results for STEC, but EPEC was not detected (Table 1). Antibacterial susceptibility testing of nine isolates revealed nine distinct resistance patterns (Table 2). All strains were sensitive to Fosbac, flumequine, furazolidone, and ceftriaxone, but resistant to amoxicillin-clavulanate.

5. Discussion

In humans, STEC strains are considered as major cause of HC and HUS worldwide. The disease in human is primarily a food-borne infection, but contact with carrier animals might be a secondary route of infection (13). In addition, infections with STEC have been described in a wide range of domestic and wild animal species, but the natural pathogenic role has been demonstrated only in weaning pigs, young calves, and dogs. Typically, the diarrheagenic STEC strains in calves harbor *stx1* and *eaeA* genes (6, 7). This study investigated the presence of major virulence factors of EPEC and STEC among 146 isolates from 115 diarrheic calves in Mashhad and Garmsar districts using an efficient multiplex-PCR assay. The results showed the higher importance of EPEC in Mashhad district while no EPEC was detected in diarrheic calves in Garmsar. Interestingly, two STEC strains from Mashhad had negative results for *eaeA* gene and only harbored the *stx1* gene. One strain from Garmsar harbored *stx1, eaeA,* and *ehly* genes simultaneously, which was categorized as EHEC. It should be noted that EHEC strains have higher pathogenic capacity and are of particular concern in hu-

man diseases and outbreaks (6). Although EPEC are considered to induce diarrhea in different animal species and calves (9), very little information on the importance of EPEC in neonate ruminants is available. In the present study primers for *eaeA* gene were able to target a conserved region of the intimin gene (*eae*) between EHEC and EPEC; therefore, strains with positive results for *eae* (not harboring *stx*) are considered as EPEC (11). The results showed that three calves (2.6%) carried the *eaeA*-positive and *stx*-negative EPEC strains; two of these isolates also carried the *ehly* gene. Presence of *ehly* gene in these *eae*-positive strains suggests that these might be the former EHEC, which lost the Shiga toxin genes during infection or subculture (14, 15). The *stx1* was the predominant virulence gene in the present study and all of the six STEC isolates carried this virulence factor, of which two also carried the *stx2* gene. Interestingly, most of the strains in the current study were isolated from seven to ten-day-old diarrheic calves (Table 1). Our findings support other studies, which reported higher frequency of *stx1* in calves. Leomil et al. (2003) documented higher frequency of carriage of *stx1* in diarrheic calves in Brazil (16). Orden et al. studied isolates from 221 diarrheic calves and found that 69.8% of STEC strains harbor *stx1*, 20.9% *stx2*, and 9.3% *stx1/stx2* genes (17). Wieler et al. evaluated 176 diarrheic calves and found that 61%, 7%, and 1% of STEC strains harbored *stx1, stx2,* and *stx1/stx2*, respectively (18). In our previous study, the combination of *stx1*, *eaeA,* and *ehly* genes was the predominant virulence profile among 200 diarrheic calves in Iran (19). In contrast, some studies have detected *stx2* as a dominant Shiga toxin type among STEC from calves (20-22). It should be noted that geographical area and time of sampling are important factors in epidemiology of STEC in animals.

Antibacterial susceptibility testing of nine isolates revealed nine distinct resistance patterns, which indicated the heterogeneity of isolated strains from calves.

All strains were shown to be sensitive to Fosbac, flumequine, furazolidone, and ceftriaxone, but resistant to amoxicillin-clavulanate. Resistance to doxycycline and lincospectin was also substantial in this study. We recently reported the considerable resistance to amoxicillin and tetracyclines in STEC isolates from pigeons in Iran, which was similar to the results of the present study (23). Comparison of antibacterial resistance between isolates from Mashhad and Garmsar showed that the strains from Mashhad were multiple-resistant to three or more antibacterial agents (except one isolate); this might be the result of limited use of antibiotics in traditional farming system, which is common in Garmsar. Similar to infantile diarrhea in humans, calf diarrhea has also several pathoetiologies such as infection with viral, parasitic, or bacterial agents (8, 24). This study indicated that other causative agents might play a more important role in calf diarrhea in the studied areas, but because of the public health significance of STEC, the characteristics of these isolates from calves should not be overlooked.

Authors' Contributions

The study was designed, drafted, analyzed, and supervised by Mahdi Askari Badouei. The results were analyzed and the draft was reviewed by Samad Lotfollahzadeh. Moein Arman and Masoud Haddadi performed the laboratory procedures.

Funding/Support

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