



Isolation and Molecular Characterization of Enterotoxigenic *Escherichia coli* (ETEC) Strains From Industrial Dairy Farms of Hamedan, Iran

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Abstract

Background: Enterotoxigenic *Escherichia coli* (ETEC) is considered as one of the most common causes of infectious diarrhea in calves, infecting animals during the first week of age. The secretory diarrhea is attributed to the virulence factors of ETEC strains mainly including heat stable toxin (STa), as well as F5 (K99) and F41 fimbriae.

Objectives: The present study was undertaken to investigate ETEC infection in neonatal calves of industrial dairy farms of Hamedan, Iran. Additionally, it was undertaken to investigate the genotypic screening of virulence genes in enterotoxigenic *E. coli* isolated from dairy farms calves of Hamedan county.

Materials and Methods: A total of 120 rectal swab samples were collected from healthy and diarrheic calves at one week of age belonging to eight farms. Conventional bacteriological methods, multiplex PCR, and antibiotic susceptibility test of the ETEC isolates were performed.

Results: Nine *E. coli* isolates were found to be ETEC strains, carrying STa enterotoxin along with F5 and/or F41 fimbriae as the indicators of ETEC cells. Additionally, antibiotic susceptibility test of the ETEC isolates revealed that all of them were sensitive to trimethoprim-sulfamethoxazole, ciprofloxacin, and enrofloxacin, whereas complete resistance was observed against amoxicillin-clavulanic acid (100%) and polymyxin B (100%). The present study, conducted for the first time in Hamedan, indicated a prevalence of 7.5% for Enterotoxigenic *Escherichia coli* in the examined animals.

Conclusion: Regarding economic losses of the infection in calves as well as the zoonotic nature of ETEC cells, it is recommended that measures should be taken, such as immunization of pregnant cows prior to the delivery, feeding of adequate colostrum to newborn calves at the right time, and adherence to hygiene practices on the farms to prevent and/or reduce the incidence of diarrhea cases caused by infection with these bacteria.

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Background

Neonatal calf diarrhea is a worldwide infectious disease with high morbidity and mortality rates, which is basically caused by four major pathogens including Enterotoxigenic *Escherichia coli* (ETEC), *Rotavirus*, *Coronavirus*, and *Cryptosporidium*.¹ ETEC strains are host specific and usually cause watery diarrhea in one-week-old calves, which is also known as colibacillosis. Their pathogenicity is correlated with the presence of adhesins and the production of enterotoxins.² ETEC strains can adhere to the receptors on the small intestinal epithelium of the host by their fimbriae without inducing significant morphological changes. Various fimbriae have been described in pigs and calves including F4 (K88), F5 (K99), F6 (987P), F17, F18, and F41.³ These fimbriae are characterized by their properties such as amino acid composition and ability to agglutinate RBCs.² Hence,

fimbriae are considered as the virulence factors of these bacteria. F5, also known as K99, and F41 are the two major pili which are found on most of the ETEC strains obtained from calves.^{4,5} Consequently, host specificity may be related to the presence or absence of the fimbrial receptors on the intestinal cell surfaces. Besides, it is supposed that by increasing the host age, resistance may be observed because of release of over-expressed free receptors which in turn can attach and neutralize ETEC adhesins and prevent their adherence to the intestinal epithelium.⁶ On the other hand, ETEC cells produce two classes of enterotoxins designated as heat-labile (LT) and heat-stable (STa and STb) enterotoxins, among which STa is produced by ETEC strains obtained from calves.⁷ Although the mechanism of action of these enterotoxins are somewhat different from each other, they totally exert their effect by reducing absorption and increasing

the secretion of water and electrolytes at the villous tips without damaging the intestinal epithelial cells. However, it has been noted that interference with the enteric nervous system may also play role in diarrhea caused by these *E. coli* enterotoxins.⁷

ETEC strains are known as causative agents of traveler's diarrhea in humans and these bacteria may be transmitted to humans via contaminated food and water. Although the pathogenicity of human ETEC strains is almost similar to that of animal ETEC strains, i.e., the production of LT and ST toxins, there are some important differences in adhesion factors. While bovine ETEC strains use the aforementioned specific fimbriae (F5 and/or F41) to attach to the intestinal epithelial cells, human ETEC strains apply their own colonization factors (CFs) (e.g., CFA/I to CFA/IV) for this purpose which in turn result in host-specific infections.^{8,9} However, the ability to bind to human ileal cells has been demonstrated for K88 positive ETEC strains (porcine ETEC strains).¹⁰

Since colibacillosis in neonatal calf may result in huge economic loss in dairy farms, its identification can prevent this and help to control the infection. Therefore, several methods have been introduced for identification of ETEC strains, including serological assays such as enzyme-linked immunosorbent assay, specific DNA probes for detection of enterotoxins and fimbrial genes, and multiplex polymerase chain reaction (mPCR) for the rapid screening of ETEC virulence factors.¹¹ However, some of these experiments have disadvantages, including being time-consuming and costly, as well as showing false-negative results in the detection of F5 and F41 fimbriae and STa enterotoxin.¹² Given the importance of ETEC detection, the present study was conducted to investigate ETEC strains using a multiplex PCR assay which targeted F5, F41, and STa followed by determination of antibiotic resistance profiles of the isolates.

Materials and Methods

Specimen Collection

A total of 120 rectal swab samples (60 from clinically healthy and 60 from diarrheic calves) were collected from 1-7-day old calves which belonged to 8 dairy farms with scours in Hamedan. However, no antibiotic or vaccine had been used for the control of ETEC. The samples were immediately transferred on ice to the microbiology lab for further examinations.

Bacterial Isolation and Identification

The collected swabs were cultured on MacConkey agar. Thereafter, the identity of grown colonies which showed characteristic morphology of *Escherichia coli* (bright-pink colonies) was further confirmed using various biochemical examinations including gram staining, oxidase and catalase tests as well as metabolic characteristics in/on MRVP broth (Methyl Red and Vogues-Proskauer),

SIM (Sulfide indole motility), TSI (Triple Sugar Iron), Simmons' Citrate, and EMB (Eosin Methylene Blue) agar. All of the media were purchased from Merck (Germany).

DNA Extraction and PCR

DNA was extracted from each of the biochemically identified *E. coli* isolates using boiling method. Briefly, about 3 mL of an overnight broth culture of each isolate was transferred into a microtube and the bacterial cells were precipitated at 10000 rpm for 3 minutes. After removing the supernatant, 200 µL of sterile distilled water was added to the pellets and the microtubes were incubated in a boiling water bath (100°C) for 10 minutes, followed by centrifugation at 12000 rpm for 2 minutes.¹³ The supernatants were transferred into sterile microtubes and used as template DNA samples in PCR assay.

A multiplex PCR assay was performed to investigate virulence factors (F5, F41 and STa) in order to identify ETEC strains using previously described protocol as follows:

The multiplex PCR reaction (20 µL) contained 10 µL of a commercial PCR Master Mix (BioScience, Germany), 0.5 µL of each of 6 primers (0.5 µM) (Table 1), and 7 µL of the extracted DNA samples to identify the encoding genes.¹³ A sample that contained no template DNA and *E. coli* ATCC 35218 were used as negative and positive controls, respectively, in each run of PCR. The following thermal cycling program for PCR amplification consisted of pre-denaturing at 94°C for 5 minutes, followed by 32 cycles of denaturing at 94°C for 30 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 90 seconds and a final extension at 72°C for 10 minutes. The amplified PCR products were visualized by electrophoresis on 1.5% agarose gel containing 0.5 µg/mL of ethidium bromide.¹³

Antibiotic Susceptibility Test

Antibiotic susceptibility of the isolates was evaluated using disk diffusion method based on the guidelines of Clinical and Laboratory Standards Institute.¹⁴ Resistance patterns of the isolates were recorded against a panel of antibiotics including co-trimoxazole (trimethoprim-sulfamethoxazole: 1.25/23.75 µg), cefazolin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), amoxicillin-clavulanic acid (20/10 µg), gentamycin (10 µg),

Table 1. The Sequences of the Primers Used to Perform Multiplex PCR Assay

Primer	Sequence 5'-3'	Product Size (bp)
STa-forward	GCTAATGTTGGCAATTTTATTCTGTA	190
STa-reverse	AGGATTACAACAAAGTTCACAGCAGTAA	
F5-forward	TATTATCTTAGGTGGTATGG	314
F5-reverse	GGTATCCTTTAGCAGCAGTATTTTC	
F41-forward	GCATCAGCGGCAGTATCT	380
F41-reverse	GTCCCTAGCTGAGTATTATCACCT	

enrofloxacin (5 µg), streptomycin (10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), and polymyxin B (300 U). All Antibiotic disks were purchased from Padtan Teb (Iran). *E. coli* ATCC 35218 was used as a control in the experiment.

Results

Isolation of *Escherichia coli*

The bacteriological and biochemical examinations led to the identification of 120 *E. coli* isolates. In fact, *E. coli* was isolated from all rectal samples collected from apparently healthy and diarrheic calves.

Molecular Identification of ETEC Strains Using Multiplex PCR

Multiplex PCR was used to investigate the presence of the two fimbrial (F5 and F41) and one enterotoxin (STa) encoding genes. As shown in Figure 1, positive reactions were accompanied by the amplification of DNA fragments of 314, 380, and 190 bp, respectively. The isolates which were positive for STa enterotoxin encoding gene and at least one of the F5 and/or F41 fimbriae encoding genes were considered as ETEC strains. Although 23 (19.17%) out of 120 *E. coli* isolates were positive for at least one of the studied virulence factor genes, only 9 isolates (7.5%) were recognized to be ETEC strains, from which 7 isolates (5.83%) possessed all 3 studied virulence factor genes (Figure 1). All of the ETEC strains belonged to the

samples collected from diarrheic calves, while none of the examined specimens from apparently healthy calves showed infection with ETEC strains. Detailed statistics obtained from the multiplex PCR are presented in Table 2.

Antibiotic Susceptibility Test

Antibiotic susceptibility test was carried out for the 9 ETEC isolates, identified by multiplex PCR assay, using Kirby-Bauer disc diffusion method. The results revealed that all of the ETEC isolates were resistant to amoxicillin-clavulanic acid and polymyxin B; however, the highest susceptibility was observed against ciprofloxacin, trimethoprim-sulfamethoxazole, and enrofloxacin since all of the ETEC isolates were sensitive to these antibiotics (Table 3).

Discussion

ETEC is a known intestinal pathogen in calves,^{15,16} which is widespread around the world.¹⁷ Diarrhea syndrome caused by this bacterium is important in neonatal calves, especially at age of one week.¹⁸ Due to death rates, treatment costs, and adverse effects on the growth of calves, enterotoxigenic *E. coli* is considered to be one of the main causes of economic losses in cattle farming.¹⁹ A prevalence of about 1% to 50% has been reported in several studies carried out to investigate ETEC infection in different countries.²⁰ In a study conducted by Lotfollahzadeh et al in Ghaem-shahr and Babol, Iran, in 2001, 93 stool samples of calves with diarrhea were tested. Their results showed that out of 38 *E. coli* isolates, only one (1.07%) was identified as ETEC.²¹ Shams et al examined 312 rectal swab specimens of diarrheic calves by multiplex PCR in Fars province and found that 5.3% of the isolates were ETEC strains.²² In another study performed by Pourtaghi et al in Alborz province, the researchers identified 11 ETEC isolates (18.33%) out of 60 rectal swab samples of diarrheic calves based on a multiplex PCR assay which targeted F5, F41, and STa.²³ Although our examination determined a prevalence of 7.5% for ETEC in the studied dairy farms which is in the range of the reported levels of contamination in the previous studies, it seems that the prevalence of ETEC is relatively different in various regions of Iran.

Table 2. Detection Frequency of Each of the Virulence Factor Genes in *E. coli* Isolates

Virulence Factor Gene	Number of Positive Isolates (%)
F5	9 (7.5%)
F41	21 (17.5%)
Sta	10 (8.33%)
F5 + F41	1 (0.83%)
F5 + STa	1 (0.83%)
F41 + STa	1 (0.83%)
F5 + F41 + STa	7 (5.83%)

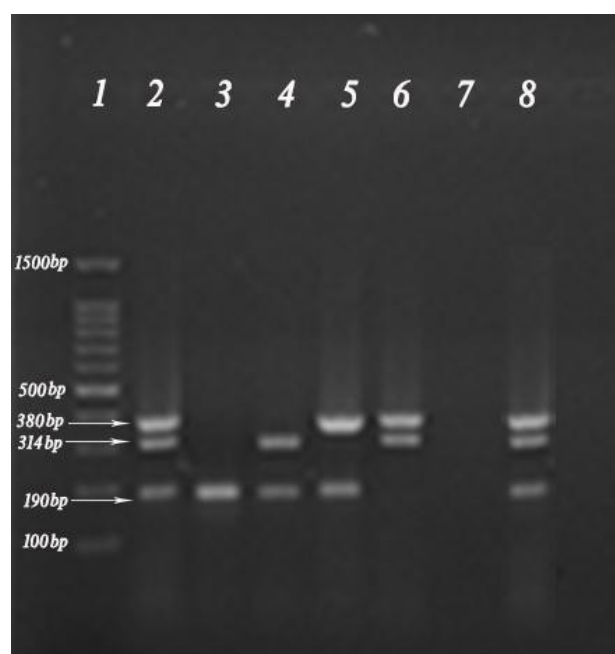


Figure 1. Agarose Gel Electrophoresis of the Multiplex PCR Products for Detection of STa, F5 and F41 in *E. coli* Isolates. Lane 1: A 100 bp DNA ladder, lane 2: *E. coli* ATCC 35218 as the positive control which possessed all of the investigated genes, lanes 3-6: Isolates that were positive for 1 or more virulence factor genes, lane 7: Negative control, lane 8: An ETEC isolate which was positive for three genes.

Table 3. The Results of Antibiotic Susceptibility Test for the 9 ETEC Isolates

Antibiotic	Sensitive	Semi-sensitive	Resistant	<i>E. coli</i> ATCC 35218
Trimethoprim sulfamethoxazole	9 (100%)	0	0	S
Cefazolin	1 (11.11%)	8 (88.88%)	0	I
Tetracycline	1 (11.11%)	7 (77.77%)	1 (11.11%)	I
Ciprofloxacin	9 (100%)	0	0	S
Amoxicillin–Clavulanic acid	0	0	9 (100%)	R
Gentamycin	0	9 (100%)	0	R
Enrofloxacin	9 (100%)	0	0	S
Streptomycin	1 (11.11%)	6 (66.66%)	2 (22.22%)	R
Ceftriaxone	6 (66.66%)	3 (33.33%)	0	I
Chloramphenicol	6 (66.66%)	3 (33.33%)	0	I
Polymyxin B	0	0	9 (100%)	R

Barrington et al conducted a study on 200 healthy and diarrheic calves in North America in which the prevalence of ETEC was reported to be 8%.²⁴ Moreover, Salvadori et al, tested 250 samples of healthy and diarrheic calves in Brazil, and reported that the prevalence of STa in diarrheic calves was 3.9% and the prevalence rates of F5 and F17 were 7.3% and 4.8%, respectively.²⁵ Additionally, Nagy and Fekete carried out a study on 350 healthy and diarrheic calves in 2005 that showed a prevalence of 4% for the enterotoxigenic *E. coli* strains.³ Moreover, in a study done in India on 404 calves (286 cases of diarrhea and 118 of healthy ones), 23 ETEC isolates were identified.²⁶

In addition to its veterinary importance, ETEC strain is associated with traveler's diarrhea in humans and transmission of the bacterium through contaminated water and food products can lead to acute, self-limited, secretory diarrhea in humans which typically lasts 3 to 5 days. Although, the clinical signs may persist up to 20 days without complications or sequelae, the disease does not need antimicrobial treatment.²⁷

The frequency of enterotoxigenic *E. coli* in different parts of the world, as well as different regions of a country, can be varied due to reasons such as immunization or non-immunization of pregnant cows against enterotoxigenic *E. coli* cells before pregnancy, adherence to health principles in dairy animals in addition to feeding the right amount of colostrum to calves at the right time.²⁴ In addition to the geography of the area, the application of different investigating methods, the sampling condition, and concurrent intestinal tract infections may play significant roles, but the definitive reason for this is not widely known in many studies.²⁵

In the present study, 9 ETEC isolates were identified which showed a prevalence of 7.5% among all 120 stool samples of healthy and diarrheic calves and 15% among diarrheic calves (9 out of 60). However, all ETEC strains were considerably isolated from the diarrheic calves,

implying that the issue is more severe in the farms with scours. Furthermore, the results of this study showed that most of the identified ETEC isolates (7 out of 9 isolates) possessed all 3 genes (STa, F5, and F41), suggesting that ETEC strains basically carry all three genes together. However, two ETEC isolates contained only STa and one of the F5 and/or F41 fimbriae encoding genes.

Regarding that antibiotic resistance is a major problem in the treatment of bacterial infections, determination of antibiotic resistance profiles is of particular importance, especially in the case of intestinal bacteria such as *Escherichia coli*, which is a very common bacterium. Antibiotic sensitivity studies on *E. coli* isolates have shown that usually a high number of these bacterial cells are resistant against antibiotics commonly used to treat diarrhea.²⁴ While sensitivity to a specific antibiotic, such as gentamicin, has been shown in some studies,^{17,24} findings of other studies revealed extensive resistance to antibiotics in *E. coli* cells.¹⁹ Therefore, antibiotic susceptibility of the 9 ETEC isolates was also evaluated. The results of disk diffusion test revealed that all of the isolates were susceptible to trimethoprim-sulfamethoxazole, ciprofloxacin, and enrofloxacin. In contrast, amoxicillin-clavulanic acid and polymyxin B had no effect on the isolates and all of them were resistant against these antibiotics.

In a study carried out by Shams et al, the investigation of antibiotic susceptibility of 13 ETEC isolates showed that the highest susceptibility belonged to enrofloxacin.²² Besides, in the study performed by Behzadian Nejad et al on the 39 ETEC *Escherichia coli* isolates from cattle fecal samples, a sensitivity rate of 95% was recorded for Gentamicin.²⁸ Taghi-AKhi et al evaluated 200 diarrheic calves and reported that all of the 20 ETEC isolates were sensitive to gentamicin and ciprofloxacin.²⁹

Additionally, Khurana et al conducted a study on 37 ETEC strains isolated from cows and calves in 2006.

The result of the antibiotic susceptibility test using disc diffusion method showed that the highest sensitivity percentage was observed for ciprofloxacin (100%), norfloxacin (94.5%), and gentamicin (83%), whereas tetracycline was the most ineffective antibiotic.³⁰

Totally, antibiotic susceptibility profiles determined in various studies relatively indicate some variations which might be due to the vast scattering of dynamic genetic elements such as plasmids and integrins since the transition of antibiotic resistance, especially in intestinal bacteria such as *E. coli* is quite common.²⁸ In addition, the consumption of antibiotics in veterinary medicine and other animal breeding systems for treatment and prevention purposes can be one of the reasons for the high level of antibiotic resistance.

Our findings indicated that ETEC bacteria are present in the region and the isolates also showed resistance against certain antibiotics. Therefore, it is suggested that more stricter hygiene measures should be taken in dairy farms to prevent possible transmission of these ETEC strains among animals. Besides, an antibiotic susceptibility test is necessary for the appropriate and effective treatment of diarrhea caused by these ETEC strains.

Authors' Contributions

QG: sampling, methodology, and writing-original draft; PM: conceptualization, project administration, supervision, and editing the manuscript; AB: sampling, advising, and editing the manuscript.

Ethical Approval

The experiments were conducted according to the protocol approved by the Faculty of Veterinary Science, Bu-Ali Sina University, Hamedan.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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