

Detection of Enterotoxigenic *Escherichia coli* Isolated from Calves' Diarrhoea Samples by Molecular and Serological Methods

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Abstract

This study was performed to estimate the prevalence of Enterotoxigenic *Escherichia coli* (ETEC) in calves' diarrhoea samples by molecular and serological methods. Rectal swabs from 127 diarrhoeaic calves were collected from 10 different farms of Tehran province during March to September 2014. ETEC was detected by both mPCR and ELISA. According to the results, ETEC was detected in 14 (11%) and 5 (3.9%) diarrhoea samples by mPCR and ELISA methods respectively. All 14 positive samples were detected by mPCR method had three virulence genes including K99 (F5), F41 and STa. Although 11 (78.5%) and 3 (21.4%) positive cases were identified from traditional and semi-industrial farms respectively, no isolates were identified related to the industrial farms. The results represented that detection assays such as ELISA which is only able to detect fimbriae (F5), will follow false-positive cases. For this reason mPCR assay has been developed and recognized as effective, rapid and reliable tools for detection of ETEC.

Keywords: calves, comparison, diarrhea, ELISA, ETEC, mPCR

Introduction

Diarrhoea is commonly reported disease in young calves due to several adverse effects including mortality, economic loss, treatment costs, and decrease of growth rate [6]. According to National Animal Health Monitoring System (NAHMS) report in 2007, 57% of weaning calf mortality was due to diarrhoea that most of them were less than one month old [13]. Furthermore, the economic loss related to mortality of diarrheic calves in Norway was estimated about 10 million US dollars in 2006 [10]. Among variety of diarrheagenic agents in calves including viruses, protozoa, intestinal parasites and bacteria, [1,2], ETEC is an important and global cause of sever watery diarrhoea in first week of calving [7]. Adhering of this pathogen to intestinal mucosa through its unique colonization factors as well as producing either heat-labile enterotoxins (LT-I and LT-II), heat-stable enterotoxins (STa and STb), or both have been recognized as the main virulence factors of ETEC [3]. Several investigations reported the high prevalence of calf diarrhoea associated with ETEC around the world [11, 14]. Considering to the importance of detection of ETEC in calves due to reducing mortality and economic loss in dairy farms, this study aimed to estimate the prevalence of ETEC in calves' diarrhoea samples by molecular and serological methods.

Materials and Methods

Sample collection

A total of 127 rectal swab samples were collected from diarrheic calves in 10 different farms of Tehran province including industrial, semi-industrial and traditional farms during March to September 2014. These farms had recognized scouring problems in neonatal calves and some antibiotics such as tetracycline were used for the control of diarrhea. All the diarrheic calves were aged 1–30 days old and the samples were collected within 36 h after diarrhoea. Two swabs were taken from each calf with diarrhoea. The swabs were placed in sterile tubes and transferred to the laboratory in a cool box, within 3 h of collection. Rectal swabs were transferred into separate tubes containing 2 ml of nutrient broth and cultured at 37 °C for 24 h. After that, swabs were streaked onto MacConkey agar and Eosin Methylene Blue (EMB) agar plates and incubated at 37 °C for 24 h.

DNA extraction and PCR

Total DNA extraction by a boiling method was used as a DNA template. Overnight, cultures in 2 ml nutrient broth were centrifuged for 5 min at 5,000 rpm. The bacterial pellet was resuspended in 200 µl of distilled water and boiled for 10 min. Tubes were centrifuged again, and the supernatant was used as template DNA. To detect ETEC, three sets of primers previously described by Frank et al (1998) were used in PCR assays [5]. All the reagents were purchased

from Cinna gene Co. The PCR mixture contained 2.5 µl of 10x PCR buffer (Mg²⁺ plus), 0.5 IU of *Taq* polymerase, 250 µM dNTPs, 1 µM of each primer, 2 µl of DNA template, and deionized water to a final volume of 25 µl. The condition for amplification consisted of 94°C for 5 min for initial denaturation and 35 cycles of 94°C for 1 min (denaturation), 53°C for 1 min (annealing) and 72°C for 1 min 30 s (extension), followed by 72°C for 10 min (final extension). PCR products were separated by 1% agarose gel electrophoresis along with 100 bp Fermentas DNA markers and visualized after staining with ethidium bromide on a UV transilluminator.

Elisa

Detection of ETEC was performed by using Rota-Corona K99 / ELISA Trikit (Pourquier, France). The test proposed in this kit enables the differential diagnosis between three agents: Rotavirus, Coronavirus and *E. coli* (by detecting its attachment factor K99), by detecting the antigens by the ELISA method. Undiluted Overnight culture of Nutrient broth was used as samples. ELISA was done following the test protocol. Optical density was measured using ELISA reader Biotech ELX 800. Sample to positive (S/P) percentage for each sample was calculated. Samples with S/P percentage greater than or equal to 7% were considered positive.

Results

This study was performed to detect ETEC from diarrhoeic calves at the first month of age. Rectal swabs from 127 diarrhoeic calves were collected from 10 different farms of Tehran province during March to September 2014. ETEC was detected in 14 (11%) and 5 (3.9%) diarrhoea samples by mPCR and ELISA methods respectively (Table 2). All 14 positive samples were detected by mPCR method had three virulence genes including K99 (F5), F41 and STa with the sizes 314bp, 380bp and 190bp respectively (Table 1, Figure 1). Also the results indicated that all of the calves with positive isolates were male and at the first week of age, which 71.4% of them were less than four day old (Table 2). Furthermore, it was found that, among 14 positive cases 9 (64.2%) of them had been received antimicrobial agents before sampling. Although 11 (78.5%) and 3 (21.4%) positive cases were identified from traditional and semi-industrial farms respectively, no isolates were identified related to the industrial farms (Table 2).

Table 1. Primer used in multiplex PCR

Gene	Primer	Size of product (bp)
F5	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314
F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT	380
STa	GCTAATGTTGGCAATTTTATTTCTG TA AGGATTACAACAAAGTTCACAGCAG TAA	190

Table 2. Comparison of two different assays for identification of ETEC in calves diarrhea samples

Detection Method	Number of samples		Distribution of positive cases in different ages No (%)		Distribution of positive cases in different farms No (%)		
	Examined	Positive (%)	>4 days	>7 days	Traditional	Semi-industrial	Industrial
mPCR	127	14(11%)	10(71.4%)	14(100%)	11(78.5%)	3(21.4%)	0(0%)
ELISA	127	5(3.9%)	5(100%)	0(0%)	4(80%)	1(20%)	0(0%)

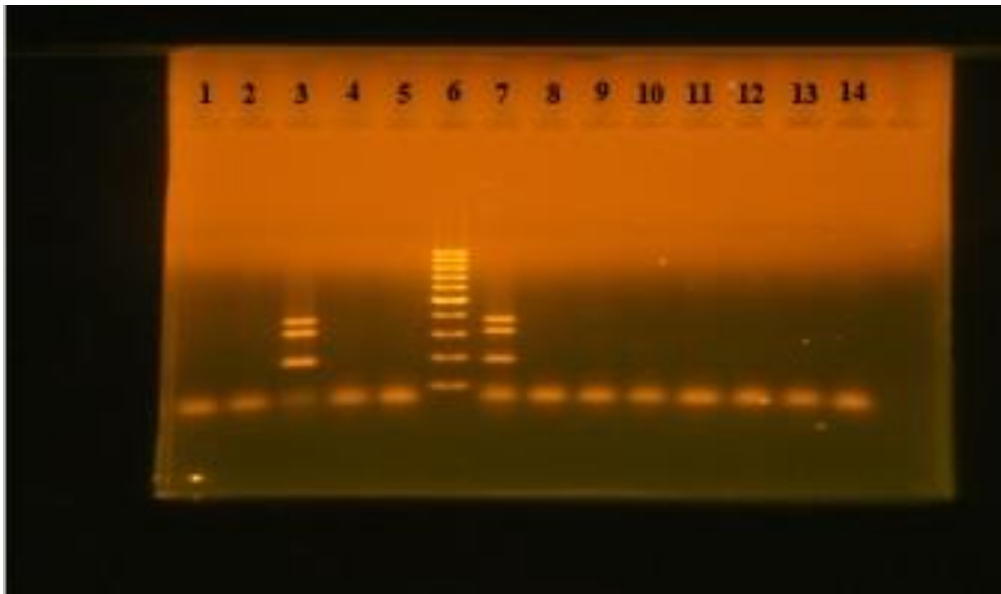


Figure 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products (190, 314 and 380bp) for detection STa, F5 and F41 of ETEC samples. Lane 6: DNA ladder (100 bp Ladders, Fermentas, Germany); lanes 3 positive sample; lanes 1, 2, 4, 5, 8, 9, 10, 11, 12, 13 and 14: negative samples and negative control. And lanes 7: positive control.

Discussion

Diarrhoea syndrome remains as one of the most significant diseases at the first month of calving. In addition to variety of pathogens as the main causative agents of diarrhea, environmental, management, and nutritional factors can influence the severity and outcome of the disease [1]. *Escherichia coli* is recognized as a member of the normal gut flora in both humans and animals and its presence may have nutritional significance [12]. Among the six pathogroups of *E. coli*, ETEC is the most common cause of neonatal calves' diarrhoea especially during first 4 days after birth [4, 8]. Following ingestion, this pathogen can infect the gut epithelium and colonize in the distal portion of the small intestine due to the low pH (less than 6.5) of this region [2]. Villous atrophy and lamina propria damage have been observed as consequences of ETEC colonization which leads to diarrhoea due to enterotoxin productions induced by ETEC [2]. Therefore, both of virulence factors consist of fimbriae and enterotoxins should be present for ETEC pathogenicity [7]. This reflects the fact that identification of ETEC bacteria causing diarrhoea in calves, if only based on the detection of fimbrial genes including K99 (F5) and F41 may not be pathogenic and not lead to diarrhoea due to disability in identification of enterotoxin genes. Considering to the presence ne-

cessity of enterotoxin genes for ETEC pathogenicity, detection assays such as ELISA which is only able to detect fimbriae (F5), may follow false-positive cases. According to the results, ETEC was detected in 14 (11%) and 5 (3.9%) diarrhoea samples by mPCR and ELISA methods respectively. All positive samples that detected as ETEC with ELISA were detected with mPCR too, indicating that ELISA had false negative cases. For this reason mPCR assays have been developed and recognized as effective, rapid and reliable tools for screening of ETEC [14]. In the present study the prevalence of three virulence factors including K99 (F5), F41 and STa was similar to each other (11%) which was approximately in agreement with the result reported by Younis et al. (10.36 %) [14]. Lower prevalence was reported by Shams et al. [11], although higher result (14.1%) was found by Pourtaghi et al. in previous investigation in another province of Iran [9], which indicates variety distribution of ETEC in different geographical areas. Despite to these differences in the prevalence of ETEC, all three virulence factor including K99, F41 and Sta were found in the both investigations. Furthermore both studies represented that 100% of positive cases were at the first week of age, although 71.4% of them in the present investigation possessed less than 4 day old which was lower than the result reported by Pourtaghi et al. in previous study in Iran (95.4%) [9]. Moreover our findings indicated that although all positive cases were male, no significant correlation was identified between sex and the prevalence of diarrhoea caused by ETEC. However higher frequency of ETEC in this sex can be due to higher sample collection from male calves.

Conclusion

Identification of ETEC from the calves that received antibiotics before sampling indicated antimicrobial resistance to this pathogen which leads to veterinary public health hazards. Therefore the results represented that selection of appropriate diagnostic methods for detection of ETEC seems necessary.

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