ORIGINAL ARTICLE

# The Studies on the Aetiology of Diarrhoea in Neonatal Calves and Determination of Virulence Gene Markers of *Escherichia coli* Strains by Multiplex PCR

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## Impacts

- Rotavirus, coronavirus, *Escherichia coli* and *Enterococcu*s ssp. were determined to play a role in the aetiology of diarrhoea in the neonatal calves.
- The multiplex PCR may be useful for determining the characterization of *E. coli* isolated from calves.
- K99, F41, STa, Stx1 and Stx2 were the most common virulence gene markers of *E. coli* strains isolated from calves with diarrhoea.

**Keywords:**
Calf; diarrhoea; gene marker of *E. coli*; PCR

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## Summary

The purpose of this study was to determine aetiological agents of diarrhoea in neonatal calves and to investigate virulence gene markers of *Escherichia coli* strains isolated from calves by multiplex polymerase chain reaction (PCR). Eighty-two diarrhoeic calves and 18 healthy calves were used as subjects. Faeces were taken from the rectums of all the calves and were subjected to bacterial culture. Antigen enzyme-linked immunosorbent assay (ELISA) was performed to detect rotavirus, coronavirus and *E. coli* K99 in faeces of all the calves. A multiplex PCR was used to characterize *E. coli* strains in all the calves. *Escherichia coli* was isolated from 37 faeces samples, *Enterococcus* ssp. was isolated from 22 faeces samples and *Salmonella* was isolated from one faeces sample in diarrhoeic calves. Furthermore, only *E. coli* was isolated from all 18 faeces samples of healthy calves. Of the 37 *E. coli* isolated from diarrhoeic calves, K99 (18.9%), F41 (18.9%), heat-stable enterotoxin a (STa) (18.9%), Shiga toxin 1 (Stx1; 13.5%) and Shiga toxin 2 (Stx2; 5.4%) and intimin (8.1%) genes were identified by multiplex PCR. Of the 18 *E. coli* isolated from healthy calves, K99 (16.6%) and intimin (55.5%) genes were identified by PCR. A total of 15 rotavirus, 11 coronavirus and 11 *E. coli* K99 were detected in diarrhoeic calves by the antigen ELISA. As a result, this study shows that rotavirus, coronavirus, *E. coli* and *Enterococcu*s ssp. were determined to play a role in the aetiology of diarrhoea in the neonatal calves. K99, F41, STa, Stx1 and Stx2 were found as the most common virulence gene markers of *E. coli* strains isolated from calves with diarrhoea. Multiplex PCR may be useful for characterization of *E. coli* isolated from calves.

## Introduction

Diarrhoea is one of the most important diseases of neonatal dairy and beef calves. Substantial economic loss occurs as a result of increased morbidity and mortality, treatment costs and reducing growth rates. Neonatal enteritis in calves is a common problem all over the world (de la Fuente et al., 1998; Fecteau et al., 2001; Bell et al., 2002). The diarrhoeal syndrome has a complex aetiopathogenesis, because various infectious agents,

either alone or in combination, may be associated with field's outbreaks. In addition, environmental, management and nutritional factors influence the severity and outcome of the disease. Rotavirus, coronavirus, *Cryptosporidium parvum* and enterotoxigenic *Escherichia coli* (ETEC) are the four major pathogens associated with neonatal calf diarrhoea worldwide (Reynolds et al., 1986; Klingenberg and Svensson, 1998; de la Fuente et al., 1998; Fecteau et al., 2001; Bell et al., 2002; Jay et al., 2004). In the USA, rotavirus and coronavirus were found together in 35% of the diarrhoea cases in beef calves (Maes et al., 2003). The significance of rotavirus and coronavirus as a major cause of neonatal calf diarrhoea has also been reported all over world (Bellizoni et al., 1990). de la Fuente et al. (1998) reported that neonatal calf diarrhoea accounts for approximately 75% of the mortality of dairy calves under 3 week old. Enterotoxigenic *E. coli* has two groups of virulence factors: (i) fimbriae (pili) and (ii) enterotoxins. K99 and/or F41 fimbriae mediate adherence to the ileum and are found on most calf ETEC (Franck et al., 1998). Calf ETEC produces heat-stable enterotoxin a (STa), which causes hypersecretion in the gut lumen (Butler and Clarke, 1994; Kuhnert et al., 2005). Shiga toxin-producing *E. coli* (STEC) produces two types of Shiga toxins; one is immunogically similar to the Shiga toxin produced by *Shigella dysenteriae* and the other is immunogically distinct from *S. dysenteriae* Shiga toxins (Karmali, 1989). Vero cytotoxin-producing *E. coli* of different serotypes has become a major concern in human and animal diseases in different countries in the last few years (Beutin et al., 1993; Wieler et al., 1998; Beutin, 2006). Several authors (Bouzari et al., 2005; Osek, 2005) described the prevalence of cytolethal distending toxin genes among STEC isolated from humans and animals (Janka et al., 2003; Thot et al., 2003; Bielaszewska et al., 2004; Wagner et al., 2004; Scott et al., 2006).

Bovine rotavirus and coronavirus are important aetiological agents of neonatal calf diarrhoea worldwide. Economic loses because of bovine rotavirus infection in the cattle industry include loss associated with retarded growth, medication cost and animal death (Parwani et al., 1992; Lu et al., 1994; Erdoğan et al., 2003). Klingenberg and Svensson (1998) reported that group A rotavirus was detected by enzyme-linked immunosorbent assay (ELISA) and RNA-polyacrylamide gel electrophoresis 14/33 (43.8%) of the samples from scouring calves and 1/30 (3.7%) of the samples from non-scouring calves.

Several assays to detect rotavirus, coronavirus and *E. coli* have been described. These include polyacrylamide gel electrophoresis, latex agglutination, immune electron microscopy, ELISA and polymerase chain reaction (PCR) (Chinsangram et al., 1995; Hussein et al., 1996). Most of the available antigen detection ELISAs were designed and licenced for use in detecting human rotavirus. A multiplex PCR which detects genes encoding intimin, K99, F41, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) has been developed. In recent years, ELISAs and PCR are being increasingly used to detect enteropathogens in faeces samples from calves (China et al., 1996).

The purpose of this study was to determine aetiological agents of diarrhoea in neonatal calves and to detect virulence gene markers of *E. coli* strains by multiplex PCR.

## Materials and Methods

### Animals

This study was conducted in eight different farms in the province of Konya in Turkey. Eighty-two diarrhoeic (aged between 2 and 30 days) and 18 non-diarrhoeic neonatal calves (aged between 2 and 30 days) were used as subjects in this study. All of experimental and control group calves were Swiss–Holstein. The diarrhoeic calves had fever, anorexia, general weakness, loss of condition, moderate and severe profuse watery diarrhoea and dehydration. There was also diarrhoea with little blood. Non-diarrhoeic calves were examined and judged to be clinically normal. Faeces samples were taken from the rectums of all the diarrhoeic and non-diarrhoeic calves.

### Bacteriological examinations

Faeces samples were cultured on MacConkey agar, blood agar, XLD agar and *Campylobacter* selective agar (supplemented with 7% sheep blood and skirrow selective supplement). MacConkey agar and blood agar plates were incubated for overnight at 37°C, *Campylobacter* selective agar plates were incubated for 3 days at microaeorophilic conditions. Isolated bacteria were identified by biochemical characteristics (Quinn et al., 1998).

### Antigen ELISA

Antigen ELISA (C.E.R. Immunogie, Marloie, Belgium) was performed for the detection of rotavirus, coronavirus and *E. coli* K99 serotypes in faeces of all the calves. ELISAs were performed and evaluated according to the manufacturer's instructions.

### Determination of virulence factors of *Escherichia coli*

A multiplex PCR was performed for the identification of enterotoxigenic (ETEC), attaching and effacing *E. coli* (AEEC) and Shiga toxin (STEC) yielding *E. coli.* Primers for K99, F41, STa, intimin, Stx1 and Stx2 genes determined previously by Franck et al., 1998 were used (Table 1).

 

**Table 1.** Primers used in multiplex PCR for the determination of virulence factors of *Escherichia coli*

| | Virulence factors | Primer sequences 5′–3′ | Band length (bp) |
|---|---|---|---|
| 1. | Stx1 (F) | TTC GCT CTG CAA TAG GTA | 555 |
| | Stx1 (R) | TTC CCC AGT TCA ATG TAA GAT | |
| 2. | Intimin (F) | ATA TCC GTT TTA ATG GCT ATC T | 425 |
| | Intimin (R) | AAT CTT CTG CGT ACT GTG TTC A | |
| 3. | F41 (F) | GCA TCA GCG GCA GTA TCT | 380 |
| | F41 (R) | GTC CCT AGC TCA GTA TTA TCA CCT | |
| 4. | K99 (F) | TAT TAT CTT AGG TGG TAT GG | 314 |
| | K99 (R) | GGT ATC CTT TAG CAG CAG TAT TTC | |
| 5. | STa (F) | GCT AAT GTT GGC AAT TTT TAT TTC TGT A | 190 |
| | STa (R) | AGG ATT ACA ACA AAG TTC ACA GCA GTA A | |
| 6. | Stx2 (F) | GTG CCT GTT ACT GGG TTT TTC TTC | 118 |
| | Stx2 (R) | AGG GGT CGA TAT CTC TGT CC | |

### Processing of samples for PCR

Bacterial DNA was obtained by suspending a colony of bacteria grown overnight on MacConkey agar in 50 $\mu$l of $H_2O$ and boiling at 100°C for 10 min.

### PCR assay

The assay was performed in final reaction volume of 50 $\mu$l mixture containing PCR buffer [60 mM Tris–HCl, (pH 9.0), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$]; 250 mM from each of the deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP) (Promega, Davis, CA, USA), 0.5 $\mu$M concentration of each primer, 1.25 U *Taq* polymerase and 5 $\mu$l template. The amplifications were carried out on a MJ Research thermal cycler in the following order: initial denaturation at 95°C for 30 s, at 50°C for 45 s, at 70°C for 1 min and 30 s for 25 cycles, with final extention at 70°C for 10 min (Franck et al., 1998). The products (7 $\mu$l) were analysed by electrophoresis through a 1.5% agarose gel (Gibco, San Francisco, CA, USA), after which the gel was stained with ethidium bromide (Sigma, St Louis, MO, USA) and photographed under UV light.

## Results

### Bacteriological findings

*Escherichia coli* from 37, *Enterococcus* ssp.from 22 and *Salmonella* from one faeces samples were isolated in diarrhoeic calves (Table 2). Furthermore, only *E. coli* was isolated from all 18 faeces samples of healthy calves (Table 2). No enteropatogen was isolated from faeces of 15 neonatal calves with diarrhoea**.**

### PCR results

Thirty-seven *E. coli* were isolated from faeces samples of diarrhoeic calves and, only 18 *E. coli* were isolated from faeces samples of healthy calves, totally 55 *E. coli* were investigated. Of the 37 *E. coli* isolated from the diarrhoeic calves, K99 (18.9%), F41 (18.9%), STa (18.9%), Stx1 (13.5%), Stx2 (5.4%) and intimin (8.1%) genes were identified by multiplex PCR (Table 2). Of the 18 *E. coli* isolated from the healthy calves, K99 (16.6%) and intimin (55.5%) genes were identified by PCR (Table 3). No STa, Stx1 or Stx2 was detected in the healthy calves. (Fig. 1).

### Antigen ELISA results

A total of 15 rotavirus (18.2%), 11 coronavirus (13.4%) and 11 *E. coli* K99 (13.41%) gene were determined in the diarrhoeic calves by ELISA, both rotavirus and coronavirus (3.6%) were detected in three faeces samples of the diarrhoeic calves (Table 2).

## Discussion

In microbiological surveys of diarrhoeic and healthy calves, mixed infections were much commonly detected in diarrhoeic calves than in healthy calves. Reynolds et al. (1986) suggested that the presence of more than one enteropathogen may be one of the factors determining whether an infection result in a clinical or subclinical presentation. Rotavirus, coronavirus, *cryptosporidium* ssp. and ETEC are the four major pathogens associated with neonatal calf diarrhoea worldwide (Reynolds et al., 1986; Klingenberg and Svensson, 1998; de la Fuente et al., 1998; Bell et al., 2002). In this study, we detected enterogenic *E. coli, Enterococcus* ssp., *Salmonella* ssp., rotavirus and coronavirus in faeces taken from the diarrhoeic calves, and only *E. coli* was isolated in faeces taken from the healthy calves. Mix infections were detected in some diarrhoeic calves in our study. In addition, no bacteria or virus was detected in 18.29% of the diarrhoeic calves ($n$ = 15). This percentage of negative results is similar to the results of earlier reporters (Reynolds et al., 1986; de la

**Table 2.** Results of multiplex PCR and ELISA and bacteriologic isolation of calves with diarrhoea

| | Antigen ELISA results | | | | |
|---|---|---|---|---|---|
| No | Rota-virus | Corona-virus | *Eschericha coli* K99 | Bacteriologic isolation | *E. coli* PCR result |
| 1 | – | – | + | *E. coli* | – |
| 2 | + | + | – | *E. coli* | – |
| 3 | – | – | – | *E. coli* | – |
| 4 | – | – | – | *E. coli* | – |
| 5 | – | – | + | *E. coli* | STa + K99 + F41 |
| 6 | + | – | – | *E. coli* | – |
| 7 | – | – | + | *E. coli* | STa + K99 + F41 |
| 8 | – | – | – | *E. coli* | – |
| 9 | – | – | – | *E. coli* | – |
| 10 | + | – | – | *E. coli* | – |
| 11 | – | – | – | *E. coli* | STa + K99 + F41 |
| 12 | – | – | + | *E. coli* | STa + K99 + F41 |
| 13 | – | + | – | *E. coli* | – |
| 14 | – | – | + | *Enterococcus* ssp. | |
| 15 | – | – | + | – | |
| 16 | + | + | – | *Enterococcus* ssp. | |
| 17 | – | – | + | *Enterococcus* ssp. | |
| 18 | – | – | – | – | |
| 19 | – | – | – | – | |
| 20 | + | – | – | – | |
| 21 | – | – | – | – | |
| 22 | – | + | – | *Enterococcus* ssp. | |
| 23 | – | – | – | – | |
| 24 | + | – | – | – | |
| 25 | – | + | – | – | |
| 26 | – | – | – | *Enterococcus* ssp. | |
| 27 | – | – | + | *Enterococcus* ssp. | |
| 28 | – | – | – | – | |
| 29 | – | – | – | *E. coli* | – |
| 30 | – | – | – | – | |
| 31 | – | – | – | *Enterococcus* ssp. | |
| 32 | – | – | – | *Enterococcus* ssp. | |
| 33 | – | + | – | *Enterococcus* ssp. | |
| 34 | – | + | – | *E. coli* | – |
| 35 | – | – | – | *E. coli* | – |
| 36 | – | – | – | *Enterococcus* ssp. | |
| 37 | – | – | – | *E. coli* | Intimin |
| 38 | + | – | – | *Enterococcus* | |
| 39 | – | – | – | *E. coli* | – |
| 40 | – | – | – | *Enterococcus* ssp. | |
| 41 | + | – | – | *E. coli* | – |
| 42 | + | – | – | *Enterococcus* ssp. | |
| 43 | – | – | – | *E. coli* | Stx1 |
| 44 | – | + | – | *E. coli* | Stx1 |
| 45 | – | – | – | *E. coli* | Stx1 |
| 46 | – | + | – | *Enterococcus* ssp. | |
| 47 | – | – | – | *E. coli* | Intimin |
| 48 | + | – | – | – | |
| 49 | – | – | – | – | |
| 50 | – | – | – | *E. coli* | – |
| 51 | – | – | – | *Salmonella* | |
| 52 | – | – | – | *E. coli* | Stx1 + Stx2 |
| 53 | – | – | – | *E. coli* | Stx1 + Stx2 |
| 54 | + | + | – | *E. coli* | Intimin |
| 55 | – | – | – | – | |

 

**Table 2.** *Continued*

| No | Antigen ELISA results | | | Bacteriologic isolation | *E. coli* PCR result |
|---|---|---|---|---|---|
| | Rota-virus | Corona-virus | *Eschericha coli* K99 | | |
| 56 | – | – | – | *E. coli* | STa + K99 + F41 |
| 57 | – | – | – | – | |
| 58 | – | – | – | *E. coli* | STa + K99 + F41 |
| 59 | – | – | – | – | |
| 60 | – | – | + | *E. coli* | STa + K99 + F41 |
| 61 | – | – | – | *Enterococcus* ssp. | |
| 62 | – | – | – | – | |
| 63 | – | – | – | – | |
| 64 | + | – | – | *Enterococcus* ssp. | |
| 65 | – | + | – | *Enterococcus* ssp. | |
| 66 | – | – | – | *Enterococcus* ssp. | |
| 67 | – | – | + | *Enterococcus* ssp. | |
| 68 | – | – | – | – | |
| 69 | + | – | – | – | |
| 70 | – | – | + | *Enterococcus* ssp. | |
| 71 | + | – | – | *E. coli* | |
| 72 | – | – | – | *E. coli* | |
| 73 | – | – | – | – | |
| 74 | – | – | – | – | |
| 75 | – | – | – | – | |
| 76 | – | – | – | *Enterococcus* ssp. | |
| 77 | + | – | – | *Enterococcus* ssp. | |
| 78 | – | – | – | *E. coli* | – |
| 79 | – | – | – | *E. coli* | – |
| 80 | – | – | – | *E. coli* | – |
| 81 | – | – | – | *E. coli* | – |
| 82 | – | – | – | *E. coli* | – |

Fuente et al., 1998; McDonough et al., 1994). Diarrhoea is not only related to viral or bacterial agents but it may also be related to protozoal, parasiter, nutritional or other management factors.

Diarrhoea in calves is commonly caused by ETEC (Jay et al., 2004). More recently, AEEC and STEC have also been identified as causes of diarrhoea and dysentery in calves (Mainil et al., 1990; Butler and Clarke, 1994; Franck et al., 1998). Mainil et al. (1990) reported that STa, K99 and F41 genes were the most common (67.4%) strain among *E. coli* isolated from calves. Yuste et al. (2006) also reported that the percentages of enteropathogenic *E. coli* strains astA- were positive in 15.6% of healthy cattle. K99 (18.9%), F41 (18.9%), STa (18.9%), Stx1 (13.5%), Stx2 (5.4%) and intimin (8.1%) were common strains among *E. coli* isolated from the diarrhoeic calves detected in this study. Calves are more suspectible to ETEC during the first 3 or 4 days of their lives, and the organisms are often investigated only in calves aged 7 days or less (de la Fuente et al., 1998). The bacteriological diagnosis of neonatal calf diarrhoea because of *E. coli* should focus not only on ETEC strains but also on Stx- and Ae-positive *E. coli* strains (Wieler et al., 1998).

**Table 3.** Results of multiplex PCR and ELISA and bacteriological isolation of healthy calves

| No | Antigen ELISA results | | | Bacteriologic isolation | *E. coli* PCR results |
|---|---|---|---|---|---|
| | Rota-virus | Corona-virus | *Escherichia coli*, K99 | | |
| 1 | – | – | – | *E. coli* | – |
| 2 | – | – | – | *E. coli* | Intimin |
| 3 | – | – | – | *E. coli* | K99+Intimin |
| 4 | – | – | – | *E. coli* | – |
| 5 | – | – | – | *E. coli* | Intimin |
| 6 | – | – | – | *E. coli* | K99 |
| 7 | – | – | – | *E. coli* | – |
| 8 | – | – | – | *E. coli* | – |
| 9 | – | – | – | *E. coli* | – |
| 10 | – | – | – | *E. coli* | K99+Intimin |
| 11 | – | – | – | *E. coli* | Intimin |
| 12 | – | – | – | *E. coli* | – |
| 13 | – | – | – | *E. coli* | Intimin |
| 14 | – | – | – | *E. coli* | Intimin |
| 15 | – | – | – | *E. coli* | Intimin |
| 16 | – | – | – | *E. coli* | – |
| 17 | – | – | + | *E. coli* | Intimin |
| 18 | – | – | – | *E. coli* | Intimin |

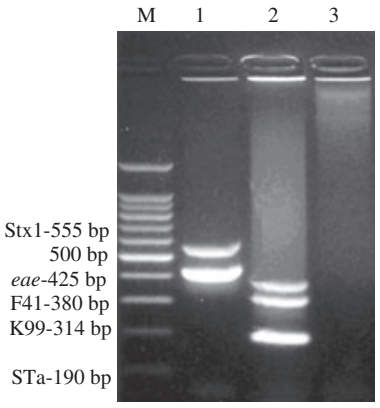


**Fig. 1.** PCR for the gene of K99, F41, STa, intimin (*eae*), Stx1 and Stx2. M: 100 bp DNA ladder; 1: positive control, *Escherichia coli* O157:H7; 2: positive control, *E. coli* (O101:H-:K99+:F41+); 3: negative control, *E. coli* ATCC 25922.

Not only K99, F41, Stx1, Stx2 and STa were generally identified in the calves with diarrhoea between 2- and 7 days old but also intimin was detected in calves with diarrhoea over 10 days old in this study. Virulence factors of intimin and Shiga toxins were isolated commonly from faeces of healthy calves (Orden et al., 2002). Bielaszewska et al. (2004) and Kuhnert et al. (2005) reported that STEC were isolated from faeces of animals. In this study, 18 *E. coli* (100%), K99 (16.6%) and intimin (55.5%) genes from faeces samples of healthy calves were identified by PCR (Table 3). However, no STa, Stx1 or Stx2 was detected in the healthy calves. We were observed that healthy calves are the most important reservoir of *E. coli*. Leomil et al. (2003) indicated that the frequency of carriage of Stx was higher in diarrhoeic calves (20%) than in non-diarrhoeic calves. We found that the frequency of carriage of Stx1 (13.5%) and Stx2 (5.4%) was high in the diarrhoeic calves, but no Stx1 or St2 was detected in non-diarrhoeic calves.

Güneş et al. (2004) reported that *E. coli* O157 was detected in the faeces of 5.8% of the diarrhoeic calves by ELISA. However, de la Fuente et al. (1998) determined that *E. coli* F5 was detected in the faeces of 11.9% of the diarrhoeic calves by antigen ELISA. In this study, *E. coli* K99 was detected as not only 13.41% in the diarrhoeic calves but also 5.55% in the healthy calves by ELISA. Osek (2001) described that *E. coli* F17 was detected as 26.6% in the healthy calves by PCR. In this study, we found that while *E. coli* K99 in 11 faeces samples of diarrhoeic calves were positive by antigen ELISA, *E. coli* K99 in seven faeces samples of diarrhoeic calves were positive by PCR. Out of 11 ELISA positive (for *E. coli*, K99) diarrhoeic calves, only five were positive bacteriologically. However, out of 11 ELISA positive (for *E. coli*, K99) diarrhoeic calves, only four (calves numbered as: 5, 7, 12, 60) were positive in PCR. Both the rate of *E. coli* K99 and F41 was found to be 18.91% in same faeces samples of diarrhoeic calves by PCR. While *E. coli* K99 in three faeces samples of healthy calves was positive by PCR, one faeces sample was positive by ELISA.

Rotavirus and coronavirus infections are well-known causes for acute diarrhoea in neonatal calves (Klingenberg and Svensson, 1998), but subclinical infections also occur frequently (Chinsangram et al., 1995; Erdoğan et al., 2003). Rotaviruses are, among the enteropathogenic agents, more commonly associated with neonatal diarrhoea in calves (Falcone et al., 1999). de la Fuente et al. (1998) found that rotavirus was the most commonly detected agents (42.7%). Erdoğan et al. (2003) reported that the prevalence of rotavirus and coronavirus in neoanatal calves with diarrhoea was determined by ELISA, 26.9% and 1% respectively. However, Ekik and Öztürk (2002) detected rotavirus in the faeces of 16.21% of the diarrhoeic calves by antigen ELISA. In this study, a total of 15 samples for rotavirus (18.2%) and 11 samples for coronavirus (13.4%) were positive in diarrhoeic calves by antigen ELISA. In addition, both of rotavirus and coronavirus were found in three faeces samples in diarrhoeic calves (calves numbered as; 2, 16, 54). There was no rotavirus or coronavirus in positive samples in healthy neonatal calves by antigen ELISA. The results indicated that rotavirus and coronavirus may be important in the aetiology of diarrhoea in neonatal calves that over 7 days old. In this study, this percentage of rotavirus and coronavirus in diarrhoeic calves was similar to the results reported by some researchers (Chinsangram et al., 1995; Falcone et al., 1999; Ekik and Öztürk, 2002; Erdoğan et al., 2003). In contrast, the percentage of rotavirus in this study was lower than the results of de la Fuente et al. (1998). These differences in incidence rate among the studies may be attributed to different diagnostic methods used, farm management practices exercised in different regions and related to ageing of calf, because coronavirus and rotaviruses were detected in calves with diarrhoea aged between 7- and 30 days old in our study. In addition, the most of calves with diarrhoea ($n = 47$) were 7 days old or less.

As a result, findings given in this study show that rotavirus, coronavirus, *E. coli* and *Enterococcus* ssp. play a role in the aetiology agents of diarrhoea in the neonatal calves. Especially, K99, F41, STa and Shiga toxins (Stx1–2) were found as the most common virulence gene markers of *E. coli* strains isolated from diarrhoeic calves. The multiplex PCR may be useful as diagnostic tool in identification and characterization of *E. coli* isolated from calves.

 

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