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ORIGINAL ARTICLE

Detection of enterotoxigenic K99 (F5) and F41 from fecal sample of calves by molecular and serological methods

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Abstract Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of neonatal calf diarrhea. Almost all ETEC bacteria are known to adhere to receptors on the small intestinal epithelium via their fimbriae, (F5 (K99) and F41).This study was undertaken to investigate the phenotypic and genotypic screening of virulence genes in *E. coli* K99 and F41. During January 2008 to December 2009, 298 diarrheic neonatal calves at 1–30 days old were studied by multiplex PCR, isolation, and serological grouping. Of the 298 diarrheic samples, 268 *E. coli* were isolated, of which 16 samples (5.3%) were positive for having the F5 (K99) fimbrial gene by PCR while all of the *E. coli* isolates also carried F41 fimbrial genes. Twenty-five percent of the isolates were proven not to be toxigenic as they did not possess the STa enterotoxin gene.

Keywords *E. coli* K99 · F5 · F41 · Cattle · Iran

Introduction

Neonatal calf diarrhea is an important cause of morbidity and mortality worldwide (Younis and El-Naker 2009). Enterotoxigenic *Escherichia coli* (ETEC), rotavirus, coronavirus, and cryptosporidium are the four major pathogens associated with neonatal calf diarrhea (Miraglia et al. 2001). ETEC is an important and global cause of severe, watery diarrhea in the offspring of some animal species such as newborn calves and pigs (Nagy and Fekete 2005.). Almost all ETEC bacteria are known to adhere to receptors on the small intestinal epithelium by their fimbriae without inducing significant morphological changes. Furthermore, they secrete enterotoxins that cause reduced absorption and increase the fluid and electrolyte secretion of the small intestine (Nagy and Fekete 2005). The most commonly observed fimbriae on ETEC from calves with diarrhea are F5, also named K99 and F41 (Nagy and Fekete 1999). Two biological classes of enterotoxins, heat labile (LT) and heat stable (STa and STb), are produced by ETEC. Most bovine ETEC produce STa (Guth 2000).

A number of diagnostic tests are currently available for detecting ETEC, including Double-antibody enzyme-linked immunosorbent assay (Holley et al. 1984), DNA gene probes specific for genes encoding toxins and adhesions of ETEC (Woodward and Wray 1990), multiplex polymerase chain reaction (PCR) for the rapid screening of ETEC toxins (Watterworth et al. 2005) and monoclonal antibody-based coagglutination test. While it may be convenient to focus on the principal infectious causes of calf diarrhea, it must be remembered that it is generally the result of interaction between a number of related risk factors, including management and environmental factors (Crouch et al. 2001; Lundborg et al. 2005). Local studies in Iran focused on the identification of fimbriae and enterotoxins by the use of serological tests and biological assays. These methods can be time consuming, expensive, and the expression of virulence factors depends on synthetic media and, in some cases, requires the euthanasia of the animals. Moreover, the use of conventional tests for the determination of K99 and F41 fimbriae may give false–negative results. To the best of the authors' knowledge, no studies on

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the identification of virulence genes using DNA-based and serological techniques for the detection of *E. co*li K99 and F41 have been published in Iran to date. Virulence genes in diarrheic neonatal calves and associated risk factors have not been described in Iran nor in the Middle East and Western South Asia. Consequently, the aim of the present study was to investigate the phenotypic and genotypic screening of virulence genes in *E. coli* K99 and F41, as well as to study the risk factors associated with these infections.

Materials and method

Collection of data

A total of 298 diarrheic neonatal calves, 1–30 days old, were studied during the period from January 2008 to December 2009.

These calves were raised in 15 farms belonging to six geographic areas in Fars province, Iran. These farms had a recognized scouring problem in neonatal calves and no antibiotic or vaccines were being used for the control of ETEC. The animals' identification, age, number of animals per herd, and geographical area were recorded. The state of Fars is one of the major agricultural and animal husbandry areas in Iran, with nearly 400,000 cattle and 8,000,000 sheep and goats.

Specimen collection isolation and identification procedures

A rectoanal mucosal swab sample was collected from each of the diarrheic calves. The swab samples were put into a tube containing tryptic soy broth (TSB), transported to the laboratory on ice then incubated at 37°C for 24 h. The overnight TSB culture were streaked on MacConkey and EMB (eosin methylene blue) agar plates and incubated at 37°C for 24 h as well. Four colonies with the typical appearance of *E. coli* from each sample were chosen. *E. coli* strain was identified by biochemical tests, including indole production, citrate utilization, glucose and lactose fermentation, hydrogen sulfate production, and urease negative. The isolated bacteria were stored in TSB with 20% glycerol at −70°C until required. The isolates were not subcultured more than twice before being examined for the presence of virulence genes.

DNA extraction and PCR reaction

An overnight swab culture (1 ml) was centrifuged in a desktop centrifuge at maximum speed (15,000×g) for 10 min to pelleted bacteria. Commercial extraction kit (DNA kit Cina-Gene) was used for the extraction of DNA. PCR was used to determine the following genes encoding the virulence factors of *E. coli* K99, F41, and STa. Three sets of primers were applied for the amplification of genes described in (Table 1). PCR was carried out in a 25-μl reaction volume containing 10×PCR buffer (2.5 μl), 25 mM $MgCl_2$ (1.25 μl), dNTP (10 mM, 0.5 μl), primer (1 μl, 20 pmol), DNA template (1 μl, 100 ng), distilled water (18.5 μl), and Taq DNA polymerase (0.25 μl). The PCR protocol was as follows: 94°C for 30 s, 56°C for 35 s, and 70°C for 1 min for 25 cycles, followed by 72°C for the final extension for 10 min. A negative control was included without the addition of a DNA template. The reference (RCCT 86) strains were used as positive controls. The amplified products were visualized by standard gel electrophoresis of 7 μl of the final reaction mixture in 1.5% agarose. DNA ladder 100 bp was used as the molecular size marker (100–1,000 bp). The gels were stained with ethidium bromide for 5 min, washed in distilled water, analyzed under UV light, and photographed with a Kodak camera system (Gel Logic 200).

Serogrouping

E. coli isolates were serogrouped by standard methods. Slide agglutination tests were performed using a set of O rabbit antisera (Difco) representing the most common calves ETET K99 serogroups (O8, O20, O101) as well as F-antiserum (F5 and F41). For the detection of fimbrial antigen, the colonies of tested strains were cultured on Minca agar (Guinee et al. 1976). An *E. coli* isolate was identified as an ETEC or putative ETEC strain if it possessed genes encoding specific colonizing fimbriae (F5, F41) and/or enterotoxin (STa).

Results

Gene detection by multiplex PCR

Multiplex PCR using primers identified two fimbrial genes (F5 and F41) and a STa toxin gene. Of the 298 diarrheic calves examined by PCR, 16 (5.3%) tested positive for F5 fimbrial genes while all isolates also carried the F41 gene (Table 2).

Among the isolates that carried both F5 and F41 fimbriae, four isolates (25%) did not possess the STa enterotoxin gene. Therefore, 25% of isolates were non-toxigenic.

O serogrouping

The distribution of O serogroups among 16 *E. coli* K99 was shown. Of sixteen *E. coli* K99 isolates, ten were placed in

Table 1 Primer used in multiplex PCR

Gene	Primer	Size of product(bp)
F5	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314
F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT	380
STa	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	190

the O101 serogroup and six belonged to O9. Therefore, ETEC K99 of O101 amounted to 62.5% of these strains while ETEC K99 in O9 occurred with a much lower prevalence, 37.5%.

Relationship between ETEC K99 and risk factors

The relationship between ETEC K99, F41, and the risk factors were investigated and the results are shown in Table 2. Thirteen calves with positive isolates were at the first week of age, whereas three were at week 4. Most of the calves (88%) infected with ETEC K99 were found to be raised in unhygienic conditions (Table 3).Twelve calves were isolated from farms in the south of Fars province (Darab city) with a warm climate, in contrast to the cold region in the north (Zarghan city) where only four cases have been detected. Fourteen infected calves were fed colostrum manually, whereas only two cases were naturally fed. The pregnant dams were not vaccinated.

Discussion

Since ETEC infection is the most common type of colibacillosis in young animals such as calves (Nataro and Kaper 1998), detailed studies of the virulence and risk factors of ETEC in calves are needed. Although colibacillosis in calves is a common infection, any molecular screening of virulence factors and the associated risk

Table 2 Gene detection in *E. coli* isolates from calve diarrhea

Gene	Number of strains
F5	16
F41	16
STa	12
F5+F41	16
F5+F41+STa	12
Total	16

Table 3 Risk factors in prevalence of K99 in calves' diarrhea

Calve diarrhea	Management		Age (days)			Colostrums fed	
	Non-industrial	Industrial	1–10	10–20	20–30	Manually	Naturally
F5	2	14	9	5	2	14	2

factors have not been done, to date, in Iran, nor in the south west of Asia and the Middle East.

The prevalence of K99, F41 fimbriae, and STa toxin genes was 5.3%, 5.3%, and 4.02%, respectively. A similar result was reported by (Younis and El-Naker 2009); however, a higher prevalence was reported by Acha et al. (2004) who reported a prevalence rate of 40% (Acha et al. 2004), on the contrary, a lower prevalence (0.57%, 2.3%, and 7.3%) was recorded by (Zhang et al. 2007) and (Salvadori et al. 2003), respectively. Twenty-five percent of K99 positive strains that carried both F5 and F41 fimbriae genes did not possess the STa toxin gene. The late isolates are still shown to attach to calves enterocytes in experimental infection studies. They are useful as future live vaccine candidates. Acha et al. indicated that enterotoxin STa was not detected in any *E. coli* K99 isolates from the diarrheal calves (Acha et al. 2004). The majority of K99 isolates belong to the O serogroups of O8, O9, O20, and O101 (Nagy and Fekete 1999) and, in this work, we found that K99 positive isolates belong to serogroups O101 and O9. This implied that regional difference or other selective advantages would allow these *E. coli* serogroups to proliferate in the intestine of diarrheal calves from the surveyed regions in Iran. Hence, distinct reasons causing this difference are still to be discovered. In the present study, infection with *E. coli* K99 was found to be significantly affected by the animal's age, season and geographic region, colostrum intake, management measure, and vaccination of dam. A high association was observed between age and *E. coli* K99 infection (Table 3). The first week of life is the main age of infection. This finding was supported by (Akam et al. 2004), who found that the susceptibility was higher to *E. coli* K99 during the first week of life (66.6%). Geographical regions were found to have an impact on the prevalence of calf diarrhea with a high outbreak of *E. coli* K99 infection. Also 12 isolates were recorded from the south of Fars province (Darab city) with a warm climate while only four isolates were from the north of this state with cold weather (Zarghan and Beyza city). These results contradict with Younis and El-Naker (2009) who reported a narrow variation in the climatic condition in the examined area. The effect of the geographical region on the prevalence of colibacillosis may be due to the calving period (autumn and summer), as well as the

variation of temperature in the north and south of the province.

We found that colostrum feeding obviously contributed to the passive immunity and resistance of newborn calves against ETEC infection. The data has shown that passive immunity and resistance of newborn calves against ETEC infection was strongly associated to colostrum feeding. Hand-fed calves were more frequently infected with ETEC K99 compared to those receiving colostrum intakes from their dams. This result coincided with that previously recorded by Barrington et al. (2002), who reported that passively acquired immunity through colostrum is the major risk factor related to preventing calf infection and the prevalence of diarrhea. Some management factors such as providing a clean maternity area, cleaning and disinfecting calf feeding equipment between uses and replacing these types of equipment with new ones are crucial factors in achieving the best possible calves life start. This ensures that calving environment is as clean as pathogen-free as possible.

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