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## Prevalence of Rotavirus, Escherichia coli K99 and O157:H7 in Healthy Dairy Cattle Herds in Van, Turkey

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## Prevalence of Rotavirus, *Escherichia coli* K99 and O157:H7 in Healthy Dairy Cattle Herds in Van, Turkey

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**Abstract:** A survey was conducted to determine the prevalence of rotavirus, *Escherichia coli* K99 and O157:H7 in healthy dairy cattle herds in Van and environs, Turkey. Some of the cattle herds had a history of recurrent neonatal diarrhoea for previous years, and even while sampling was being done, some newborn calves were diarrhoeic. Three hundred and twelve faecal specimens from dairy cattle farms free of clinically apparent diarrhoea were tested both for the presence of rotaviruses by PAGE silver staining and for *Escherichia coli* K99 and O157:H7 by latex agglutination using specific antisera after the growth of each colony. The enteropathogenic characteristic of K99 and O157:H7 isolates was determined by rabbit ileal loop test. No rotavirus was detected from clinically normal cows, heifers or calves. Only 1 of 9 diarrhoeic calves was found to be positive for rotavirus. Out of the 235 *E. coli* isolates from non-diarrhoeic animals, 28 were positive for K99 and 4 for O157:H7. While 14 of 28 K99 isolates were found to be positive for enteropathogenic *Escherichia coli* (EPEC), 2 of 4 O157:H7 isolates were also positive for EPEC. An in vitro antibiotic sensitivity test of K99 and O157:H7 isolates indicated that the most sensitive antibiotics were enrofloxacin and danofloxacin.

**Key Words:** Rotavirus, *Escherichia coli* K99 and O157:H7, cattle

### Van Yöresinde Sağlıklı Görülen Süt Sığırcılığı İşletmelerinde Rotavirus, *Escherichia coli* K99 ve O157:H7'nin Varlığı Üzerine Araştırmalar

**Özet:** Van yöresinde, sağlıklı görülen süt sığırcılığı işletmelerindeki hayvanların dışkılarında rotavirus, *Escherichia coli* K99 ve O157:H7'nin varlığı üzerine bir araştırma yapıldı. Araştırmanın yapıldığı bazı işletmelerdeki yenidoğan buzağılarda önceki yıllarda zaman zaman ishal olgularının görüldüğü bildirildi. Örneklemenin yapıldığı sırada da bazı buzağılarda ishal olguları tespit edildi. Toplam 312 dışkı örneğinde rotavirusların varlığını belirlemek için PAGE tekniği, *Escherichia coli* K99 ve O157:H7'leri tespit etmek amacıyla da Latex agglutination testi uygulandı. *E. coli* K99 ve O157:H7 izolatlarının enteropatogenik özelliği tavşan lup testi ile ortaya kondu. Sağlıklı görülen hayvanların dışkılarında rotavirus saptanmazken, 9 ishalleri buzağının yalnızca 1 tanesinin dışkısında rotavirus tespit edildi. Toplam 312 dışkı örneğinden 235 *E. coli* izole edildi. İzole edilen 235 *E. coli* suşundan 28'inin K99, 4'ünün de O157:H7 olduğu saptandı. Tavşan ince barsak lup testi ile, *E. coli* K99 suşlarından 14'ünün ve O157:H7 suşlarının da 2'sinin enteropatogenik özellikte olduğu belirlendi. Ayrıca, izole ve identifiye edilen tüm *E. coli* K99 ve O157:H7 suşlarının yapılan antibiyogram testinde en çok enrofloxacin ve danofloxacin'e duyarlı oldukları tespit edildi.

**Anahtar Sözcükler:** Rotavirus, *Escherichia coli* K99 ve O157:H7, sığır

### Introduction

Neonatal diarrhoea is one of the major disease syndromes affecting calves in different countries and a cause of important economic losses (1,2). However, the aetiology of such infections is multifactorial, and calf diarrhoea can be attributed to infection with a single agent or multiple agents. Among its proposed causes are bacteria, viruses, protozoa, and nutritional and environmental factors, as well as management practices (1-5). The enteropathogens most commonly found and extensively studied are rotaviruses, enteropathogenic *Escherichia coli* (EPEC) K99 and O157:H7 (3,4,6-8).

Rotaviruses, which have been recovered from various mammals, have been associated with diarrhoea in young domestic animals. In experimental infections in calves, rotavirus-associated diarrhoea is restricted to the first week of life, but under field conditions, rotaviruses are often isolated from diarrhoeic calves up to 8 weeks old (9). Large numbers of rotavirus particles are excreted in faeces of diarrhoeic calves and are highly resistant to inactivation (10, 11). Thus, the main source of rotavirus in an outbreak is environmental contamination, and the spread of infection can temporarily be broken by thorough cleaning and disinfection of the premises (11).

However, subclinically infected older calves and adult cows may play a role in the spread of infection to young calves as well as in the perpetuation of infection within a herd (12).

EPEC strains possess a number of virulence attributes that distinguish them from non-pathogenic strains. Adherence to the mucosal cells of the gut and production of toxins are the two main factors essential for enteropathogenicity (9,13). EPEC overcomes the peristalsis of the small intestine by sticking to the enterocytes with the adhesin antigen K99, an adherence antigen common in calf strains. Enterotoxins are produced near the mucosal receptor sites, which induce leakage of fluid and electrolytes into the lumen, causing diarrhoea (13,14). EPEC strains are widespread and are detected in faeces from diarrhoeic calves and sometimes also in apparently healthy cows and calves (3,7,15).

The main purpose of the present study was to determine the prevalence of rotaviruses, *E.coli* K99 and O157:H7 in the faeces of cows, heifers and calves in dairy herds free of clinically apparent diarrhoeal disease in Van and environs.

## Materials and Methods

### Sample collection

The survey was carried out on faecal samples of 312 cattle from various herds located in Van and environs (Table 1). The number of cows, heifers and calves from healthy cattle herds are given in Table 2. Some of the herds had a history of calfhood diarrhoea in previous years, and even while sampling was being done, some newborn calves were diarrhoeic. Faeces were taken from the rectum of all the animals. In most cases, laboratory examination for *E.coli* was initiated within 24 hours of the collection of the samples. For rotavirus examination, no pre-treatment of the faeces was required, and the samples were stored at -30°C until used.

### Examination of faeces for rotaviruses

All faecal samples were tested for the presence of rotaviruses by polyacrylamide gel electrophoresis (PAGE). For this purpose, the extraction of viral RNA, its resolution and staining was performed as described previously by Herring et al. (16) with slight modification.

Herd no	Number of samples	Rotavirus	K99		O157:H7	
			EPEC	Non-EPEC	EPEC	Non-EPEC
H1	153	1	11	11	-	1
H2	39	-	2	-	-	1
H3	43	-	-	2	-	-
H4	34	-	1	-	-	-
H5	43	-	-	1	2	-
Total	312	1	14	14	2	2

Table 1. Detection of rotavirus, *E. coli* K99 and O157:H7 in dairy cattle herds.

Animals	Number of samples	Rotavirus	K99		O157:H7	
			EPEC	Non-EPEC	EPEC	Non-EPEC
Cows	177	-	12	8	1	1
Heifers	76	-	2	5	1	1
Calves						
Non-diarrhoeic	50	-	-	1	-	-
Diarrhoeic	9	1	-	-	-	-
Total	312	1	14	14	2	2

Table 2. Presence of rotavirus, *E. coli* K99 and O157:H7 in faecal samples of cows, heifers and calves.

**Extraction of rotavirus RNA:** Faecal samples were diluted 1:4 (w/v) with TNE buffer (Tris 0.01, NaCl 0.1M, EDTA, pH 7.0) containing 1% sodium dodecyl sulfate (SDS) (Merck). An equal volume of 3:2 (v/v) phenol-chloroform mixture was added to the faecal suspensions and the samples were mixed for 1 minute. Then, emulsified mixtures were centrifuged for 20 minutes at 4000 rpm. The aqueous layer was removed and stored. Each sample was then prepared for electrophoresis by the addition of 10 µl of blue marker to 40 µl of the clear supernatant.

**Electrophoresis of rotavirus RNA:** The 50 µl samples were loaded onto 7.5% continuous polyacrylamide gel (Merck) polymerized. Electrophoresis was performed at room temperature for 16 h at 20 mA current (Hoefer Scientific Instruments, USA). A positive control sample was always included in each gel for comparison of the sequenced viral RNA migration pattern.

**Silver staining:** Each gel was placed in a plastic staining dish with 10% ethanol - 0.5% acetic acid for 30 minutes. The gels were then soaked in silver nitrate (AgNO<sub>3</sub>) (Merck) for 20 minutes. After the reduction step was applied until the bands were clearly visible with a solution of sodium hydroxide containing formaldehyde, the gels were placed in fresh carbonate solution in order to stop the reaction.

#### Examination of faeces for *Escherichia coli* K99 and O157:H7

Standard strains: *E.coli* K99 and O157:H7 strains found in the culture stocks of the Microbiology Department of the Veterinary Faculty, Yüzüncü Yıl University, were used as positive control strains in latex agglutination and rabbit ileal loop tests.

**Isolation and identification:** A loop of faecal sample was inoculated on 5% sheep blood agar and Eosin Methylene Blue agar (Oxoid). After overnight incubation at 37°C, 3-10 representative lactose-fermenting colonies were selected and subcultured on Minca-Isovilatex agar as described by Guinee et al. (17). After overnight incubation at 37°C, the growth from each colony was tested for K99 antigen by slide agglutination with anti-K99 serum and control serum (Fimbrex K99 latex agglutination test kit 0261/01, Central Veterinary Laboratory, UK).

For determining *E.coli* O157:H7 strains, a loop of faecal specimen was plated on Sorbitol MacConkey agar (Oxoid). After overnight incubation at 37°C, the Sorbitol negative colony was tested for O157:H7 antigen by slide agglutination with anti-O157:H7 serum and control serum (O157:H7 latex test-DR 620 M test kit bought from Oxoid Company, USA, was used as indicated in the instructions).

**Enteropathogenicity test:** The enteropathogenic characteristic of the detected *E.coli* K99 and O157:H7 strains was determined by rabbit ileal loop test as reported by Sedlock and Deibel (18). Anaesthesia of rabbits was provided by xylazine and ketalar. Then, bacterial suspensions prepared from *E. coli* K99 and O157:H7 isolates were inoculated into each loop made in the mid-gut of rabbits. A loop was used for each isolate, and after 7 to 8 hours, a segment width equal to or greater than 1.0 cm was considered a positive response.

**Antibiotic sensitivity test:** Twenty-eight K99 and four O157:H7 *E. coli* isolates were tested for their in vitro sensitivities to five antimicrobial agents using the disc diffusion method described by Bauer et al. (19). The antibiotics were spectinomycin, ampicillin, danofloxacin, enrofloxacin and cefoperazone. Antibiotic sensitivities were evaluated from the diameter of the zone of inhibition of growth around the discs.

## Results

The results of the survey for rotaviruses, *E. coli* K99 and O157:H7 are given in Table 1. Rotavirus was not detected in any of the faecal samples from apparently healthy animals. However, rotavirus was detected in only one sample of faeces from diarrhoeic calves, and it was characteristic of group A rotaviruses. Of 312 faecal samples, 235 *E.coli* strains were isolated and identified. A total of 235 *Escherichia coli* isolates were obtained from non-diarrhoeic animals. Out of 235 isolates, 28 isolates were found to be positive for K99 and 4 isolates were also positive for O157:H7. In rabbit ileal loop assay, 14 of 28 K99 isolates were found to be enteropathogenic while 2 of 4 O157:H7 isolates were enteropathogenic (Table 2). Sensitivities of *E.coli* K99 and O157:H7 isolates from cattle faecal samples to five antibiotics are given in Table 3.

Antibiotics	K99 (28 isolates)		O157:H7 (4 isolates)	
	EPEC (14 isolates)	Non-EPEC (14 isolates)	EPEC (2 isolates)	Non-EPEC (2 isolates)
Spectinomycin	8 / 14	9 / 14	0/2	0/2
Cefoperazone	9 / 14	8 / 14	2/2	2/2
Enrofloxacin	11 / 14	13 / 14	2/2	2/2
Danofloxacin	14 / 14	13 / 14	2/2	2/2
Ampicillin	0 / 14	5 / 14	1/2	0/2

Table 3. Antibiotic sensitivity of *E. coli* K99 and O157:H7 isolated from the faecal samples.

## Discussion

The presence of some of the potential enteropathogens in faeces of apparently healthy adult animals has been reported by several researchers. The enteropathogens most commonly found and extensively studied are rotaviruses, enteropathogenic *E. coli* K99 and O157:H7 (3,20-22).

Although the correlation between rotavirus infection and clinical diarrhoea in calves is poor during the first 8 weeks of life, the severest of watery diarrhoea is limited to the first three weeks of life, when rotavirus excretion peaks (11,12,23). McNulty et al. (11) reported an onset of rotavirus excretion in 6.1-day-old epidemically infected dairy herds on average. In a similar survey, Burgu et al. (8) revealed that rotavirus excretion was found in 12.7-day-old diarrhoeic calves on average. In this survey, we detected the presence of rotavirus in only 1 faecal samples from nine 2-week-old newborn diarrhoeic calves.

The role that adult animals may play as a source of rotavirus infection for calves has been a matter of controversy. In the present study, rotaviruses were not detected in faecal samples taken from healthy cows, heifers or even healthy calves. This result agrees with those of several authors who also failed to detect rotaviruses in faeces of adult animals (3,24,25). Emre and Fidancı (24) reported that rotavirus was not detected in either diarrhoeic or non-diarrhoeic adult cattle. Garcia-Sanchez et al. (25) reported that rotavirus excretion was not detected in faeces from cows around parturition. However, all of their calves shed rotaviruses during the observation period (25). Sihvonen and Miettinen (3) also reported that, although rotavirus excretion was not detected in faeces from cows, rotavirus was detected in only 1 sample of normal faeces from calves.

In contrast, there have been several reports describing the excretion of rotavirus in faeces of a small number of animals with or without diarrhoea and usually over a short period around parturition (11,23). Recently, Kodituwakku and Harbour (26) reported an intermittent excretion of rotavirus in 10 asymptomatic cows throughout pregnancy although rotavirus excretion and diarrhoea were detected in only 1 of their calves. Goto et al. (12) have indicated that the perpetuation of rotavirus infection within a herd may result from persistent or chronic infections in older calves and adult animals. However, it is generally accepted that once an outbreak is underway, young calves act as amplifiers of the infection and that environmental contamination is the major source of infection for newborn calves, whereas maternal infection is of minor significance (11,12).

Diagnosis of EPEC infections in animals may be based on the detection of K99 antigen. Good correlation has been shown between possession of K99 antigen, production of heat stable enterotoxin, and ability to dilate intestinal loops (13,14,27). Emre and Fidancı (24) reported that *E. coli* K99 was isolated from faeces of 35 (32.1%) diarrhoeic and 23 (25.5%) healthy cattle. Sherwood et al. (27) also reported that ETEC was isolated from 23 of 306 diarrhoeic calves, but not from clinically normal calves. Sihvonen and Miettinen (3) have indicated that enterotoxigenic *E. coli* K99 was found in about half of the diarrhoeic faeces throughout the calving seasons, and in 5-10% of normal faeces. In this present survey of closed dairy herds, out of the 235 *E. coli* isolated from non-diarrhoeic animals, 28 isolates were found to be positive for K99. Of 28 *E. coli* K99 isolates, 14 isolates were also enteropathogenic.

Buncic and Avery (15) demonstrated that *E. coli* O157:H7 was present in the faeces of only 2 animals

from 371 healthy cows. Similar findings were observed in the present study. In our study, out of the 235 *E.coli* isolates from non-diarrhoeic animals, 4 were found to be positive for O157:H7. Of 4 *E.coli* O157:H7 isolates, 2 were also enteropathogenic. Enteropathogenic *E.coli* K99 and O157:H7 isolates were detected from faeces of healthy cows and heifers, but not in diarrhoeic or non-diarrhoeic calves. However, the non-EPEC was found in only 1 of 50 healthy calves.

The comparison of enteropathogenic-nonenteropathogenic *E.coli* K99 and O157:H7 isolates for their sensitivity to five antibiotics showed that the most sensitive antibiotics were enrofloxacin and danofloxacin. In contrast, the higher rates of these isolates were found to be resistant to ampicillin. These results were similar to those of Emre and Fidancı (24) and Sherwood et al. (27).

Mixed infections in calves have been recognized in combination with agents such as rotavirus and *E.coli* K99 or other agents (2,4,6). Snodgrass et al. (1) reported that concurrent infection with two or more microorganisms occurred in 15% of diarrhoeic calves. Although significant interactions have been demonstrated between rotavirus and enterotoxigenic *E.coli* (2,6), this combination was not detected in any calf by Snodgrass et al. (1). In this study, mixed infections were not detected in any of the animals sampled. However, both rotavirus and EPEC were detected in different animals of only herd H1.

The role of maternal antibodies is essential for prevention of diarrhoea in calves. The use of combined rotavirus-*E. coli* vaccine to vaccinate pregnant dams has been shown to increase colostral and milk antibody to rotavirus-*E.coli*, and to decrease the incidence of diarrhoea in calves (28-30). Cornaglia et al. (29) reported that rotavirus was detected in 78% (106/136) and enterotoxigenic *E.coli* in 1.5% (2/136) of the samples obtained from an outbreak of neonatal diarrhoea among beef calves. In comparison, rotavirus was identified in only 1.6% (1/63) of samples from clinically healthy calves. After the dams of this herds were administered a combined rotavirus-*E.coli* inactivated vaccine during the last third of the gestation period by the same researchers, the results suggested that the immunization of cows with the maternal vaccine enhanced the passive immunity levels in calves against rotavirus and *E.coli* (29).

The results obtained in this study indicate that both rotavirus and enteropathogenic *E.coli* could be involved in causing diarrhoea in some closed dairy herds. Therefore, pregnant dairy cattle should be vaccinated against rotavirus and enteropathogenic *E.coli* for the transfer of protective antibodies to newborn calves by colostrum. Exact uptake of colostrum as well as cleaning and disinfection of the calf house would contribute to the rarer occurrence of diarrhoea and mild disease in calves.

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