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# Proportional morbidity rates of enteropathogens among diarrheic dairy calves in central Spain

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**Abstract**

Faecal samples from 218 diarrheic dairy calves in 65 dairy herds, selected by convenience, were screened for the presence of rotavirus, coronavirus, *Cryptosporidium* spp., $F5^+$ *Escherichia coli* and *Salmonella* spp. Animals surveyed were from 1 to 30 days old. *Cryptosporidium* and rotavirus were the most commonly detected agents (52.3% and 42.7% of the samples positive, respectively). $F5^+$ *E. coli* was detected in the faeces of 11.9% of the calves and bovine coronavirus was detected in the faeces of 7.3% of the calves. *Salmonella* spp. was only found in the faeces of two calves (0.9%). Mixed infections with two or more agents occurred in 28% of the calves. Concurrent infection of rotavirus and *Cryptosporidium* was found in 21.6% of the calves. Two tests were used for the detection of rotavirus (a commercial ELISA and PAGE), $F5^+$ *E. coli* (ELISA and bacterial culture) and *Cryptosporidium* (ELISA and microscopy). The validity of the commercial ELISA for the detection of rotavirus, $F5^+$ *E. coli* and *Cryptosporidium* in faeces from diarrheic calves was evaluated using PAGE, bacterial culture and microscopy as gold standard, respectively. The ELISA showed a very low sensitivity (28.6%) for the detection of $F5^+$ *E. coli* compared to bacterial culture.


*Keywords*: Cattle-microbiological diseases; Neonatal calf diarrhea; Rotavirus; Coronavirus; *Escherichia coli*; *Cryptosporidium* spp.; *Salmonella* spp.

## 1. Introduction

Diarrhea of neonatal calves causes major economic loss directly through mortality and the need for treatment, and indirectly from poor growth after clinical disease. It has been estimated that neonatal calf diarrhea accounts for approximately 75% of the mortality of

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dairy calves under 3-weeks of age (Radostits et al., 1994). Moreover, the possible long-term effects of neonatal diarrhea on the health and performance of calves that survive clinical episodes might constitute an even greater loss (Waltner-Toews et al., 1986b; Warnick et al., 1995).

The diarrheal syndrome has a complex etiopathogenesis, because various infectious agents, either alone or in combination, may be associated with field outbreaks. In addition, environmental, management, and nutritional factors influence the severity and outcome of the disease. Rotavirus, coronavirus, Enterotoxigenic *E. coli* (ETEC), and *Cryptosporidium parvum* are the four major pathogens associated with neonatal calf diarrhea worldwide. These organisms are responsible for the vast majority (75–95%) of enteric infections in neonatal calves worldwide (Tzipori, 1985). Moreover, *Salmonella* spp. may be particularly important in dairy calves (Bulgin et al., 1982; Reynolds et al., 1986; Waltner-Toews et al., 1986a).

Commercial ELISAs are being used increasingly to detect enterophathogens in faeces samples from calves. They have the advantage of not requiring special equipment or expertise and, therefore, they are suitable for small laboratories.

This study reports the proportional morbility rates of five major enteropathogens in a convenience sample of diarrheic dairy calves in central Spain. Moreover, the accuracy of a commercial ELISA for the detection of rotavirus, $F5^+$ *E. coli* and *Cryptosporidium* in faeces from diarrheic calves was evaluated using PAGE, bacterial culture and microscopy as gold standard, respectively.

## 2. Materials and methods

### 2.1. Sampling procedure

In the autumn of 1993, a letter was sent to 33 veterinarians working in the dairy industry in central Spain and members of the National Association of Specialists in Bovine Medicine (ANEMBE), asking for their collaboration for sampling natural cases of neonatal calf diarrhea. The diagnosis of diarrhea was made by veterinarians. Faecal samples were collected directly from the rectum in sterile plastic bottles and submitted on the day of sampling to the laboratory by express mail. Submitted samples were accompanied by a record sheet containing information on the size of herd, number of scouring calves and number of calves less than 30 days old in the herd at sampling time, and age of each sampled calf. Only faecal samples obtained within 48 h of onset of clinical signs from non-treated calves up to 30 days of age were included in this study. Samples were processed within 24 h of reception.

### 2.2. ELISA test

All the faecal samples were tested for the presence of rotavirus, coronavirus, *Cryptosporidium* and $F5^+$ *E. coli* by a commercial ELISA kit (Tetravalent, Vétoquinol, Magny-Vernois, France). The ELISA was performed according to the manufacturer's instructions.

### 2.3. Detection of rotavirus by PAGE

Faeces samples were tested for the presence of rotaviruses by polyacrylamide gel electrophoresis (PAGE). The extraction of viral RNA, its resolution and staining was carried out as described by Herring et al. (1982) with minor modifications. Briefly, faecal specimens were diluted 1:4 by weight with extraction buffer containing 1% sodium dodecyl sulfate, an equal volume of 3:2 phenol–chloroform was added and the mixture was vortexed and centrifuged for 10 min at $1200 \times g$. The aqueous phase was removed. For electrophoresis, 40 μl of the clear supernatant were mixed with 10 μl of the blue marker (25% sucrose containing 0.1% bromophenol blue) and loaded onto a discontinuous polyacrylamide gel (3% concentration for the stacking gel and 7.5% for the running gel). The gels were assembled using a mini-Protean II cell (BioRad) and run with a 50 mA running current and constant voltage for 1.5 h using a 200/2.0 model power supply (BioRad). After that, gels were fixed, developed and silver-stained. A positive rotavirus A control sample was included in each gel for comparison of the segmented viral RNA migration pattern.

### 2.4. Isolation of E. coli strains

The faeces samples were plated on MacConkey agar. After overnight incubation, four colonies with the typical appearance of *E. coli* from each sample were chosen. *E. coli* strains were identified by biochemical tests, including hydrogen sulphide, citrate, urease and indole. They were stored at room temperature in a nutrient broth (Difco, USA) with 0.75% agar. The strains were tested for F5 fimbriae by the slide-agglutination method with live bacteria grown on Minca-Isovitalex solid media. The production of absorbed antiserum used to detect this fimbrial antigen has been described (Morris et al., 1983; Contrepois et al., 1985). The presence of F5 fimbrial antigen was taken as evidence of enterotoxigenicity of *E. coli* isolates.

### 2.5. Isolation of Salmonella spp.

*Salmonella* species were isolated by enrichment of faeces in selective broths. Approximately 1 g of faeces was added both to 9 ml of tetrathionate broth and to 7.5 ml of selenite broth. After 24 h of incubation at 37°C, samples were plated out on brilliant green agar, and incubated for 24 h at 37°C. Lactose-negative colonies were tested on triple sugar iron, urea, and *o*-nitrophenyl-β-D-galactopyranoside media. Those that had *Salmonella*-type reactions were tested for agglutination, using commercial polyvalent O-antisera (*Salmonella* antiserum, Difco). *Salmonella* serotypes were determined by the Centro Nacional de Microbiología (Majadahonda, Madrid, Spain).

### 2.6. Detection of Cryptosporidium oocysts microscopically

The microscopic search for *Cryptosporium* oocysts was carried out using extemporaneous fuchsin staining method (Heine, 1982). A modified Ziehl–Neelsen's acid-fast method (Casemore et al., 1985) was also used to confirm Heine-stain negative results.

Each test was performed by a different investigator and interpreted blinded to all other tests.

### 2.7. Statistical analysis

Calves were grouped according to their age as follows: 1–7, 8–14, 15–21 and 22–30 days. Detection rates of $F5^+$ *E. coli* in the different age-groups were compared by $\chi^2$-test.

The validity of the commercial ELISA for the detection of rotavirus, $F5^+$ *E. coli* and *Cryptosporidium* was evaluated using PAGE, bacterial culture and microscopy as gold standard, respectively.

## 3. Results

From November 1993 to September 1995, 18 among the 33 veterinarians submitted samples from at least two herds (range from 2 to 7). Altogether, 218 faecal samples from diarrheic dairy calves in 65 herds were submitted. Herd sizes ranged from 12 to 650 cows (median 49 cows). Sixty-four of the calves included in this study were 1–7 days old, and 57, 68 and 29 calves were 8–14, 15–21 and 22–30 days old, respectively. The median age of the studied calves was 12 days.

In the animals studied, *Cryptosporidium* was the most-commonly detected agent (52.3% of the samples positive) followed by rotavirus (42.7%) (Table 1). $F5^+$ *E. coli* was isolated from 12.5%, 10.5%, 14.7% and 6.9% of the calves in the age-groups 1–7, 8–14, 15–21 and 22–30 days, respectively. The two *Salmonella* spp. isolates were identified as *S. typhimurium*.

Concurrent infection with two agents occurred in 47 calves (21.6%), with three agents

Table 1
Detection of enteropathogen(s) in the diarrheic dairy calves studied

| Enteropathogen(s) detected | Calves ($n$=218) | |
|---|---|---|
| | Number | % |
| None | 43 | 19.7 |
| *Cryptosporidium* only | 60 | 27.5 |
| Rotavirus only | 39 | 17.9 |
| Coronavirus only | 5 | 2.3 |
| *E. coli* F5 only | 9 | 4.1 |
| *Salmonella* spp. only | 1 | 0.5 |
| *Cryptosporidium*+rotavirus | 33 | 15.1 |
| *Cryptosporidium*+*E. coli* F5 | 7 | 3.2 |
| Rotavirus+coronavirus | 5 | 2.3 |
| Rotavirus+*E. coli* F5 | 2 | 0.9 |
| *Cryptosporidium*+rotavirus+*E. coli* F5 | 7 | 3.2 |
| *Cryptosporidium*+rotavirus+*Salmonella* | 1 | 0.5 |
| *Cryptosporidium*+rotavirus+coronavirus | 5 | 2.3 |
| *Cryptosporidium*+rotavirus+coronavirus+ *E. coli* F5 | 1 | 0.5 |

Table 2
Sentitivity and specificity of ELISA for the detection of rotavirus, F5$^+$ *E. coli* and *Cryptosporidium* using PAGE (*n* positive=83 out of 218), bacterial culture (*n* positive=21 out of 215[a]) and microscopy (*n* positive=113 out of 218) as gold standard, respectively

| ELISA for | Sensitivity | | Specificity | |
|---|---|---|---|---|
| | % | 95% CI[b] | % | 95% CI |
| Rotavirus | 96.4 | 92.4–100.0 | 92.6 | 88.2–97.0 |
| *E. coli* F5 | 28.6 | 9.3–47.9 | 97.4 | 95.2–99.7 |
| *Cryptosporidium* | 84.1 | 77.3–90.8 | 99.0 | 97.2–100.0 |

[a]Three calves excluded because of autoagglutination.
[b]CI, confidence interval.

in 13 calves (6%) and with four agents in one calf (0.5%) (Table 1). The mixed infection most-commonly found was rotavirus–*Cryptosporidium*.

The sensitivity and specificity of the commercial ELISA for the detection of rotavirus, F5$^+$ *E. coli* and *Cryptosporidium*, using PAGE, bacterial culture and microscopy as standard reference methods, are shown in Table 2. All four of the *E. coli* strains isolated from three of the samples showed autoagglutination activity. Therefore, these three samples were not included in the evaluation of ELISA in comparison with bacterial culture. The ELISA test showed a very low sensitivity (28.6%) for the detection of F5$^+$ *E. coli* (Table 2).

## 4. Discussion

This study reports the proportional morbility rates of five recognized enteropathogens in a convenience sample of diarrheic dairy calves in central Spain. Given the sampling procedure, the submission of samples was probably biased, since veterinarians may have tended to select severe cases of diarrhea for sampling, and, thus, mild cases may be underrepresented in this study. In fact, liquid faeces were much more-frequently submitted than semiliquid or pasty faeces.

In microbiological surveys of diarrheic and healthy calves, mixed infections were much more commonly detected in diarrheic than in healthy calves (Morin et al., 1980; Reynolds et al., 1986). Reynolds et al. (1986) suggested that the presence of more than one enteropathogen may be one of the factors determining whether an infection results in a clinical or subclinical presentation. On the other hand, mixed infections may be associated with more severe disease (Morin et al., 1980). The detection rate of mixed infections with two or more enteropathogens found by us (28%) is higher than that reported by others in surveys involving diarrheic calves carried out in different countries (range from 5 to 20%) (Bulgin et al., 1982; Sherwood et al., 1983; Reynolds et al., 1986; Snodgrass et al., 1986; Bellinzoni et al., 1990; Zrelli et al., 1990). The high detection rate of mixed infections in this study may be due to the biased submission of severe diarrheas. In this study, the most common mixed infection was rotavirus–*Cryptosporidium*: more than 20% of the calves excreted rotavirus at the same time as *Cryptosporidium*. This is in agreement with the results published in most surveys all over the world.

All the five enteropathogens included in this study may also be found in a percentage of healthy calves (Sherwood et al., 1983; Reynolds et al., 1986; Snodgrass et al., 1986; Waltner-Toews et al., 1986a). Thus, the detection of these organisms cannot be interpreted by itself as a proof of cause.

Calves are most susceptible to ETEC during the first 3 or 4 days of life, and the organisms are often investigated only in calves aged 7 days or less. However, there is evidence that concurrent infection with enteric viruses and cryptosporidia extend the period of susceptibility (Runnels et al., 1986; McDonough et al., 1994). In this study, no significant differences in the detection rate of $F5^+$ *E. coli* were found among calves on the different age-groups. Although the infection of diarrheic calves older than 1 week with $F5^+$ *E. coli* may not be associated with the disease, the epidemiological importance of these carriers in the spread of the microorganism should be emphasized.

No enteropathogens were detected in 19.7% of the calves. This percentage of negative results is similar to or lower than that reported in other surveys involving diarrheic calves (Reynolds et al., 1986; Snodgrass et al., 1986; Zrelli et al., 1990; Bellinzoni et al., 1990; McDonough et al., 1994). Negative results may be explained because some cases of diarrhea might not be associated with infectious agents (but due instead to nutritional or other management factors), because the detection methods missed some positive stools (especially when low or intermittent shedding of pathogens occurred), or because other non-investigated pathogens were involved. Many other enteric bacteria (such as enteropathogenic *E. coli* that cause attaching and effacing lesions in the intestines of calves) and viruses (especially calicivirus and Breda virus) have also been associated with the disease, but their role as causative agents of calf diarrhea (or their relative importance) is not well defined.

To our knowledge, the commercial ELISA used in this study has not previously been evaluated. The sensitivity and specificity of the commercial ELISA for the detection of rotavirus, $F5^+$ *E. coli* and *Cryptosporidium* were estimated using samples from $\leq$30 days old sick calves, probably with severe diarrhea. Thus, these estimates, particularly specificities, may not be valid in other situations.

Most discrepant results between ELISA and PAGE were positive by ELISA while negative by PAGE (10 samples). Since PAGE depends on the identification of the characteristic rotavirus genomic segments, false-positive results are unlikely. However, PAGE may miss some rotavirus positive stools towards the end of infection due to a lack of RNA in the viruses (Martin and Follet, 1987). That could at least partially explain the ELISA false-positive results. However, it is also possible that these results are true false-positive or even that the ELISA sensitivity was higher than that of PAGE. Only three of the 83 PAGE-positive samples were negative by the ELISA; however, two of these samples showed a B electrophoretype by PAGE and, thus, could not be detected by ELISA since the test detects only group-A rotavirus. The sensitivity and specificity of the commercial ELISA are broadly similar to those reported in several commercial and non-commercial tests.

The performance of the commercial ELISA for the detection of $F5^+$ *E. coli* is clearly unacceptable (Table 2). Bacterial culture may render false-negative but not false-positive results. Thus, the 15 samples negative by ELISA while positive by bacterial culture are true- false-negatives.

Using microscopy as the standard for the detection of *Cryptosporidium*, the commercial ELISA showed a sensitivity of 84.1% (with 18 false-negative reactions) and a specificity of 99.0% (with one false-positive reaction). Our group has estimated the threshold value of the commercial ELISA and staining methods for the detection of *Cryptosporidium* oocysts in faeces to be $1.5\times10^6$ oocysts/g for ELISA and $2\times10^5$ oocysts/g for both Heine and modified Ziehl–Neelsen's method (unpublished data). That could explain the ELISA false-negative results, since oocysts excretion in some animals may be too low to be detected by ELISA.

According to our results, the commercial ELISA used in this study can be effectively employed for the routine detection of rotavirus and *Cryptosporidium*, but not for the detection of $F5^+$ *E. coli*.

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