Studies on Calf Diarrhoea in Mozambique: Prevalence of Bacterial Pathogens

By *S. J. Achá[1,2], I. Kühn[2], P. Jonsson[3], G. Mbazima[1], M. Katouli[4]* and *R. Möllby[2]*

[1]National Veterinary Research Institute (INIVE), Maputo, Mozambique, [2]MTC, Karolinska Institutet, Stockholm and [3]Länsstyrelsen, Nyköping, Sweden, and [4]Faculty of Science, University of the Sunshine Coast, Maroochydore, Australia.

Achá SJ, Kühn I, Jonsson P, Mbazima G, Katouli M, Möllby R: Studies on calf diarrhoea in Mozambique: Prevalence of bacterial pathogens. Acta vet. scand. 2004, 45, 27-36. – The prevalence of diarrhoea in calves was investigated in 8 dairy farms in Mozambique at 4 occasions during 2 consecutive years. A total of 1241 calves up to 6 months of age were reared in the farms, and 63 (5%) of them had signs of diarrhoea. Two farms had an overall higher prevalence (13% and 21%) of diarrhoea. Faecal samples were collected from all diarrhoeal calves (n=63) and from 330 healthy calves and analysed for *Salmonella* species, *Campylobacter jejuni* and enterotoxigenic *Escherichia coli* (ETEC). *Salmonella spp.* was isolated in only 2% of all calves. *Campylobacter* was isolated in 11% of all calves, irrespective of health condition, and was more frequent (25%) in one of the 2 diarrhoeal farms (p=0.001). 80% of the isolates were identified as *C. jejuni.* No ETEC strains were detected among the 55 tested strains from diarrhoeal calves, but 22/55 (40%) strains from diarrhoeal calves and 14/88 (16%) strains from healthy calves carried the K99 adhesin (p= 0.001). 6,757 *E. coli* isolates were typed with a biochemical fingerprinting method (the PhenePlate™) giving the same *E. coli* diversity in healthy and diarrhoeal calves. Thus it was concluded: i) the overall prevalence of diarrhoea was low, but 2 farms had a higher prevalence that could be due to an outbreak situation, ii) Salmonella did not seem to be associated with diarrhoea, iii) *Campylobacter jejuni* was common at one of the 2 diarrhoeal farms and iv) ETEC strains were not found, but K99 antigen was more prevalent in *E. coli* strains from diarrhoeal calves than from healthy, as well as more prevalent in one diarrhoeal farm.

bacteria; calf; diarrhoea; E. coli; Campylobacter; Salmonella, prevalence; dairy; ETEC; K99.

Introduction

Cattle rearing is a tradition in Mozambique. It plays an important role to the country's economy and social welfare. Because of the presence of Tse-Tse fly in the central and northern parts of the country the cattle population is mainly concentrated to southern provinces. Among the factors, which have been hindering cattle production in Mozambique, mortality of calves is one that causes major concern. Presence of infectious agents, poor management and poor nutrition are some of the factors which can be pointed out as causes of calf disease and mortality. However, there is a lack of data on the role of infectious disease in calf morbidity and mortality in Mozambique. The common conditions affecting calves are merely described as diarrhoea and/or pneumonia without identification of their aetiology. The number of cases of diarrhoea is normally higher during the rainy seasons, from October/November to March than during the dry seasons, from March to October.

Diarrhoea in calves can be caused by a variety of pathogens including bacteria, viruses, protozoa and intestinal parasites. Among bacteria, enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella* are known to be the most common and economically important agents (*House* 1978), but other bacteria, e.g. *Campylobacter spp.* have also been identified as cause of enteric disease and diarrhoea in calves (*Firehammer & Myers* 1981, *Prescott & Munroe* 1982, *Myers et al.* 1984). The 2 latter groups also contain important human pathogens that may cause outbreaks of food-borne diseases (*De Rycke et al.* 1986) and thus are of high public health importance. In acute neonatal diarrhoea, an important disease of calves, 4 micro-organisms in particular, are of widespread occurrence and proven enteropathogenicity: rotavirus, coronavirus, cryptosporidia and enterotoxigenic *E. coli* (ETEC) (*Acres et al.* 1975, *Morin et al.* 1976, *Moon et al.* 1978).

Two of the more prominent virulence factors identified for ETEC strains are (i) expression of fimbrial (pili) antigens that enables the bacteria to adhere to and to colonise the luminal surface of the small bowel and (ii) elaboration of one or more enterotoxins that influence intestinal secretion of fluids (*Holland* 1990). The most common observed fimbriae on ETEC from calves with diarrhoea are F5, also named K99 and F41, but strains with F165 fimbriae have also been isolated (*Contrepois et al.* 1989). K99 antigen is a fimbrial adhesin distinct from the capsular polysaccharide K antigens (*Orskov et al.* 1975). Two biological classes of enterotoxins are produced by ETEC: heat labile (LT) and heat stable (STa and STb) (*Gaastra & de Graaf* 1982, *Gross & Rowe* 1985, *Holmgren* 1985, *Scotland et al.* 1985). Most bovine ETEC produce STa enterotoxin and K99 fimbriae (*Moon et al.* 1976, *Kaeckenbeeck* 1981).

The aim of this study was to investigate the prevalence of diarrhoea in dairy farms in Mozambique and the prevalence of *Salmonella, Campylobacter jejuni* and ETEC in diarrhoeic and healthy calves. We were also interested to investigate the prevalence of fimbrial antigen K99 among the *E. coli* isolates.

Materials and methods
Herds studied and sampling protocol
Eight dairy farms (F1 to F8) were chosen for this study, 5 of them located in 2 southern provinces: Maputo (F1 to F3) and Gaza (F4 and F5); 2 in central provinces: Sofala (F6) and Manica (F7) and one (F8) in the northern province of Nampula. The selected farms have a level of organisation which allow gathering of data and collecting samples of reasonable quality for research purposes and they are at easy reach to the laboratory. For some of the farms we also had data produced from a previous survey on bovine virus diarrhoea virus (BVDV), *rotavirus* and *coronavirus* in calf diarrhoea (*Baule & Banze* 1994, *Baule et al.* 1995).

The sampling was carried out on 4 occasions: during rainy and dry seasons in 1994 (S1 and S2) and during rainy and dry seasons in 1995 (S3 and S4). Management of the calves in the farms with variations depending on the conditions of the farm was in general as follows: calves were left to suckle their dams up to 3 days after birth. They were then housed in individual boxes and fed with milk and wheat barn. Hay and water were offered ad libitum after removal from the dam. At one month of age they were moved to a common pen where they were kept up to the age of 4 to 6 months, and milk was gradually replaced by forage and mixtures of cereal by-products. The age of the calves on sampling occasion varied from 1 week to 6 months, and their breed was a mixture of Holstein Friesian and local "Landim" breed . Diarrhoea was considered if faeces were semi-liquid to liquid, with or without other abnormal characteristics such as presence of blood or mucous.

Any calf with faeces whithout these characteristics was considered non-diarrhoeic or healthy. All samples were collected by the same veterinarian who also decided whether the calf was diarrhoeic or healthy upon stool examination. On each sampling occasion, all diarrhoeic calves and about 30% of the healthy calves were sampled from each farm. Faecal samples were collected directly from the rectum of the calf with a plastic glove. The samples were cultured on the same day or stored at 4ºC and cultured within 3 days.

Cultures and bacterial isolation

For isolation of *Salmonella* strains, a small portion of the faecal samples was inoculated into Selenite-F and Tetrat.ionate broths and streaked out on MacConkey and brilliant green agar after overnight incubation at 37°C. Suspected colonies were subjected to biochemical testing according to *Cowan & Steel* (1965). Slide agglutination test was used for identification of serovars according to the Kauffmann-White Schema (*Kauffmann* 1972).

For isolation of *Campylobacter,* a small portion of faecal samples was suspended in 0.85% saline, filtered through 0.45mm Milipore filter papers. Filters were then cultured in Preston broth (Oxoid) and incubated overnight at 37°C. Cultures were then inoculated onto Preston agar plates and incubated for 48 h in an atmosphere of 5% oxygen, 10% CO_2 and 85% nitrogen. Suspected colonies were identified based on their motility, hydrolysis of sodium hippurate and sensitivity to cefalotin and nalidixic acid.

For isolation of *E. coli* strains, faecal samples were inoculated onto MacConkey agar plates which were incubated at 37°C for 18-24 h. Lactose positive colonies were confirmed as *E. coli* using the standard biochemical tests recommended by *Cowan & Steel* (1965). Each faecal sample was also cultured on 5% sheep blood

agar, incubated at 37°C for 24 h and inspected for the presence of other bacterial pathogens, e.g. *Bacillus spp., Corynebacterium spp., Pseudomonas aeruginosa.*

Analysis of E. coli

Typing of *E. coli* isolates. Twenty-four *E. coli* like colonies from each faecal sample were phenotyped with the PhenePlate™ rapid screening system (*Kühn & Möllby* 1993). Each Phene Plate (the PhP-RE plates, PhPlate AB, Sweden, www.phplate.se) contains 8 rows of 12 dehydrated reagents, selected to yield a high discrimination within *E. coli.* In the first column of wells, 300 μl of growth media containing 1% (w/v) proteose peptone and 0.11% (w/v) bromothymol blue were inoculated. In the remaining wells 150 μl of the medium were inoculated. Bacterial isolates were inoculated into the first well of each row, mixed and 25 μl of bacterial suspension were inoculated into the remaining wells of the same row. Plates were incubated at 37°C and the absorbance at 620 nm was measured after 16, 40, and 64 h. The results were automatically read by a microplate reader. Storing of data, calculations of diversity and cluster analysis were performed by the PhenePlate™ software (PhPlate AB). According to data from biochemical fingerprinting, the isolates could be subdivided into different phenotypes. PhP-types with more than one isolate were called common (C) and those with only one isolate were called single (S) PhP-types.

Testing of ETEC. Isolates representing common PhP types present in the diarrhoeal and healthy calves were selected and tested for K99 antigen. *E. coli* isolates were streaked on minimal glucose agar for expression of K99 antigen. Plates were then incubated at 37°C for 24 h, and a single isolated colony was used for slide agglutination using K99 antiserum, and agglutination was observed under light micro-

scope. Detection of STa and LT on the common PhP types from diarrhoeal calves was performed by PCR (*Woodward et al.* 1992) . Positive and negative controls were included in both tests which were performed at the National Veterinary Institute (SVA), Uppsala, Sweden.

Statistical Analysis

The Chi-square test was used with Yate´s correction when applicable. Calculations were perfomed with Statgraphics, version 2.6, Statistical Graphics Corporation, STSC, USA.

Results

A total of 1,241 calves were reared in the 8 farms during the sampling period. Of these, 63 (5%) had signs of diarrhoea, almost all cases occurring during seasons S1 and S2 (Fig. 1). The prevalence of affected animals in the different farms was up to 21% (Table 1) and the incidence varied between farms and between sampling occasions from 0% to 39% (Fig. 1). Fifty-four out of the 63 diarrhoeal calves (86%) were found in farms F3 (n=31) and F6 (n=23)

(Table 1).These farms were thus considered as high prevalence farms. The incidence of diarrhoea appeared to be higher during the rainy seasons. In 1994, more diarrhoeal cases were observed in the rainy season and, in 1995 all diarrhoeal cases were found during the rainy season (Fig.1).

A total of 393 faecal samples were collected from healthy (n=330) and diarrhoeal (n=63) calves. *Salmonella* (n=8) was found in 3 farms in both healthy (n=5) and diarrhoeal (n= 3) calves. According to serotyping they belonged to 5 different serovars: *S. Ohio, S. Newport* and *S. Uganda* in diarrhoeal calves and *S. Arhus, S. Newport, S. Typhimurium* and *S. Uganda* in healthy calves.

Campylobacter was isolated from 44 samples - 7 from cases of diarrhoea and 37 from healthy calves. In farm F3 a significant difference was observed in the rate of *Campylobacter* in all calves (p= 0.001) compared to the remaining farms (Table 2). Out of 40 isolates subject to species identification, 32 (80%) belonged to *C. jejuni* and 8 (20%) to *C. coli.* The former
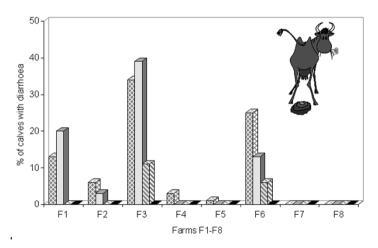

Fig. 1. Incidence of calves with diarrhoea in 8 dairy farms (F1-F8) in Mozambique. Bars indicate 4 different sampling occasions during 2 consecutive years. Grey crossed bars: S1 = rainy season year 1; White bars: S2 = dry season year 1; Grey hatched bars: S3 = rainy season year 2; Black b ars (tops). S4 = dry season year 2.

Table 1. Prevalence of calves with diarrhoea and no. of samples from each farm.

Province	Farm	Total no. of calves in the herd	No. of calves with diarrhoea (%)	Number of samples		
				H[1]	D[2]	T[3]
Maputo	F1	30	2 (7)	11	2	13
	F2	139	3 (2)	41	3	44
	F3	147	31 (21)	45	31	76
Gaza	F4	280	3 (1)	68	3	71
	F5	222	1 (0.4)	44	1	45
Sofala	F6	182	23 (13)	55	23	78
Manica	F7	126	0	29	0	29
Nampula	F8	115	0	37	0	37
Total	8	1241	63 (5)	330	63	393

[1]H = Healthy calves, [2]D = Diarrhoeal calves, [3]T = Total

Table 2. Prevalence of *Salmonella spp.* and *Campylobacter spp.* in calves.

Farm	No. of calves with diarrhoea (%)	No. of samples			No. of samples with Salmonella (%)			No. of samples with Campylobacter (%)		
		H[1]	D[2]	T[3]	H	D	T	H	D	T
F3	31 (21)	45	31	76	0	0	0	14 (31)	5 (16)	19 (25)***
F6	23 (13)	55	23	78	1 (2)	1 (4)	2 (3)	0	0	0
Others	9 (0.9)	230	9	239	4 (2)	2 (22)	6 (3)	23 (10)	2 (22)	25 (10)***
Total	63 (5)	330	63	393	5 (2)	3 (5)	8 (2)	37 (11)	7 (11)	4 (11)

[1]H = Healthy calves, [2]D = Diarrhoeal calves, [3]T = Total

species was more often found in farm F3 (18/32) while the latter was relatively more common in the other farms (7/8) (p=0.05).

E. coli was found in 76% of the calves, and no significant difference between prevalence in healthy and diarrhoeal calves was observed.

A total number of 6,757 isolates from 252 healthy (5,670 isolates) and from 47 diarrhoeal calves (1,087 isolates) were subject to typing with the PhenePlate™ system. Most faecal samples showed the presence of one dominating PhP-type and a few single types. The diver-sity among *E. coli* isolates in diarrhoeal calves was similar to that of healthy calves (0.949 and 0.958 respectively).

Fiftyfive representative strains from diarrhoeal calves and 88 from healthy calves were tested for the presence of K99 antigen (Table 3). The K99 antigen was more prevalent in diarrhoeal calves 22/55 (40%) than in healthy calves 14/88 (16%) (p=0.001). Furthermore, the K99 anti-gen was more prevalent in the diarrhoeal farms than in the other farms (p= 0.009).

The presence of genes for enterotoxins STa and

Table 3. *Escherichia col*i strains tested for K99 antigen.

Farm	No. of strains tested			No. of K99 positive strains (%)		
	H[1]	D[2]	T[3]	H	D	T
F3	26	26	52	5 (19)	15 (58)	20 (38)**[4]
F6	24	20	44	6 (25)	5 (25)	11 (25)**[4]
Others	38	9	47	3 (8)	2 (22)	5 (11)**[4]
Total	88	55	143	14 (16)***	22 (40)***	36 (25)

[1]H = Healthy calves, [2]D = Diarrhoeal calves, [3]T = Total, [4]F3 + F6 versus other farms. p = 0.009

LT was investigated by PCR on the same selected 55 strains from diarrhoeal calves and, since all the results were negative, the isolates from healthy calves were not further assayed for STa and LT genes.

Discussion

The prevalence of diarrhoea among all calves in this study was 5% (Table 1). Similar prevalences have been found by *Olsson et al.* (1993) and *Viring et al.* (1993) in Swedish herds. Results from studies in other countries show higher prevalences of diarrhoea (*Pohjola et al.* 1986, *Roy* 1990, *McDonough et al.* 1994). In Mozambique, *Baule et al.* (1995) reported an overall prevalence of diarrhoeic calves as high as 36% but this percentage includes values of prevalences of diarrhoea from other farms not included in the present study. In our study, diarrhoea in calves was observed in 6 of the 8 farms studied. Farms F3 and F6 were the farms with the highest mean prevalences (Table 1), and almost 90% of the cases occurring in seasons S1 and S2 of the study (Fig. 1). This might indicate an outbreak situation during that period. Possibly the relatively big size of these 2 farms, reared in an intensive system with unhygienic calving accommodation, makes them more prone to outbreaks of infectious diseases. The lower incidence of diarrhoea in these farms dur-

ing 1995 could thus reflect a more "normal" situation with no outbreaks of infection. Also, diarrhoeal outbreaks in calves seem to be more common in the rainy season, and the rainfall in 1994 was more intense than in 1995. Farms F7 and F8 were the farms with no diarrhoea which may have been due to the semi-intensive rearing system in those farms (animals are left grazing at daytime and kept in a kraal at night).

The diarrhoeal 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* and *Crytosporidium parvum* are the 4 major pathogens associated with neonatal calf diarrhoea 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.* 1986). The ETEC strains are often associated with diarrhoea in 2 to 3-day-old calves (*Gyles* 1986).

None of the diarrhoeal pathogens investigated here could be clearly associated with diarrhoea in the calves. The involvement of infectious

agents other than those investigated is also possible. *Baule* (1994) reported the presence of serum antibodies to Bovine virus diarrhoea virus (BVDV) in dairy and beef calves in Mozambique. The higher prevalences in their study, 92%, 87% and 86%, were found in farms F1, F2 and F3 of our study. In another study by *Baule* (1995) in the same farms, a significant statistical association of diarrhoea and the presence of group A *rotavirus* antigen in faecal samples from calves was shown and bovine *coronavirus* infections were found to be common. *Abraham et al*. (1992) found bovine enteric *coronavirus* as the major infectious cause of neonatal calf diarrhoea in some Ethiopian dairy herds. In a survey on faecal samples from 218 diarrhoeic dairy calves by *De la Fuente et al*. (1998) *Cryptosporidium* and *Rotavirus* were the most commonly detected agents. Since our study was aimed at investigating bacterial pathogens, these kinds of infectious agents were not searched for. Most of the samples (87%) without Enterobacteria and *Campylobacter* came from farms F3 and F6 , the 2 farms with high prevalences of diarrhoea. This strengthens our previous suggestion that other pathogens than the ones studied here had caused the diarrhoea. However, the fact that the bacterial pathogens investigated were not found in those samples may also have been due to other factors, e.g. shedding of the agent did not coincide with the sampling occasion, failure to detect the causative agent, some cases of diarrhoea might not be associated with infectious agents but, instead, due to management or to nutritional factors.

Salmonella was only isolated from 2% of the 393 animals studied, and it was not possible to associate the finding with the occurrence of diarrhoea. In some European countries *Salmonella* has been identified as a widespread diarrhoeal agent in dairy calves (*Reynolds et al.* 1986, *Anou.* 1997) and the importance for human health of animal reservoirs of *Salmonella* species has long been recognised (*WHO* 1980). In Africa, *Abraham et al.* (1992) could not detect *Salmonella* excretion on any of 108 diarrhoeic dairy calves in Ethiopia, although earlier studies in Addis Abeba had reported *S. Dublin* and *S. Typhimurium* as causes of disease in calves (*Pegram et al.* 1981) . *C. jejuni* was isolated in 11% of both diarrhoeic and healthy calves. An equal occurrence of *Campylobacter spp.* in diarrhoeic and normal calves has also been observed in England and Scotland (*Snodgrass et al.* 1982, *Snodgrass et al.* 1986), which supports suggestions that the association of *Campylobacter* with enteritis in cattle remains circumstantial as they are common in both healthy and diarrhoeic calves (*Allsup & Hunter* 1973, *Prescott & Bruin-Mosch* 1981). In our investigation, however, we found a high percentage (25%) of *Campylobacter* in one of the 2 considered as high prevalence farms (Table 2, farm F3), all of which but one were identified as *C. jejuni.* This might indicate an association of *C. jejuni* with an earlier outbreak of calf diarrhoea in this particular farm. Our study thus indicates that the bovine reservoirs may be a potential source of *C. jejuni* food borne disease in humans. Outbreaks of *C. jejuni* enteritis in persons have been associated with bovine faecal contamination of unpasteurized milk (*Robinson et al.* 1979).

E. coli was excreted by more than half of the diarrhoeic calves, but since this organism is regarded as a normal member of the intestinal flora of warm blooded animals, the finding of *E. coli* as such was regarded as indicative of a normal flora. Enterotoxin producing *E. coli* is a common cause of diarrhoea in animals as well as in humans (*Tzipori* 1981), *Wadström & Baloda* 1986, (*Levin* 1987, *Holland* 1990). The diversities of *E. coli* isolated in healthy and diarrhoeal calves were roughly the same. This fact speaks against that diarrhoea in several

calves was caused by single pathogenic strains of *E. coli,* like ETEC, since this should have resulted in lowered diversities in these calves. A close correlation between enterotoxigenicity and the presence of the K99 antigen has been confirmed by some authors (*Larivière et al.* 1979, *Sherwood et al.* 1983), but (*Moon et al.* (1976) have reported non-enterotoxigenic *E. coli* possessing the K99 antigen. In the present study, enterotoxins STa and LT were not detected in any *E. coli* isolates from the diarrhoeal calves, however, 40% of these isolates were K99 positive. Although we did find a higher prevalence of K99 positive in isolates from diarrhoeal calves, it is difficult to draw conclusions as to an etiological role of K99 from these findings. Furthermore, *Myers et al.* (1984) found that LT⁻ ST⁻ K99+ strains may exist in healthy calves.

In conclusion: the overall prevalence of diarrhoea was low (5.1%) but 2 farms had high prevalence (13% and 21%); *Salmonella* was rare and did not seem to be associated with diarrhoea; *C. jejuni* was more common, and had a high prevalence at one diarrhoeal farm; and STa and LT producing *E. coli* (ETEC) were not found but K99 antigen was more prevalent in *E. coli* strains from diarrhoeal than from healthy calves and was furthermore associated with one diarrhoeal farm.

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Sammanfattning

Studier av kalve med diarré i Mozambique: Prevalens av bakterie patogener.

Prevalensen av diarré hos kalvar undersöktes på åtta mjölkproducerande gårdar i Mozambique vid fyra tillfällen under 2 konsekutiva år. Totalt uppföddes 1 241 kalvar upp till 6 månaders ålder på gårdarna och 63 (5%) av dessa hade tecken på diarré. Två gårdar uppvisade en hög prevalens (13% och 21%) av diarré. Fekala prover insamlades från alla kalvar med diarré (n = 63) och från 330 friska kalvar. Proverna analyserades med avseende på förekomst av *Salmonella spp., Campylobacter jejuni* och enterotoxinbildande *E. coli* (ETEC). *Salmonella spp.* isolerades hos bara 2% av alla kalvar. Campylobacter isolerades i 11% av alla kalvar, oberoende av hälsotillstånd och påvisades oftare (25%) i en av de två gårdarna med ökad diarréförekomst (p=0.001). 80% av isolaten identifierades som *C. jejuni.* Inga ETEC stammar påvisades bland de 55 testade *E. coli* stammarna från kalvar med diarré, men 22/55 (40%) stammar från kalvar med diarré och 14/88 (16%) stammar från friska kalvar uppvisade K99 adhesin (p=0.001). Vidare typades 6 757 isolat av *E. coli* med hjälp av en biokemisk fingerprinting metod (PhenePlate™). Samma diversitet erhölls bland kalvar med och utan diarré.

Det konkluderas att i) den totala frekvensen av diarré var låg men 2 gårdar uppvisade högre frekvenser, vilket kunde tyda på lokala utbrott; ii) Salmonella tycktes inte vara associerad med diarré; iii) Campylobacter var vanlig på en av de 2 gårdarna med diarréproblem; och iv) ETEC påvisades ej men K99 antigen påvisades oftare hos *E. coli* stammar isolerade från kalvar med diarré än från friska kalvar, liksom oftare på en av gårdarna med ökad diarréförekomst.