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Short communication

Natural and experimental infection of neonatal calves with *Clostridium difficile*

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Abstract

Clostridium difficile toxins were associated with calf diarrhea in a recent retrospective study; however, no causal relationship has been prospectively investigated. This infection study tested whether the oral inoculation of neonatal calves with a toxigenic strain of *C. difficile* (PCR-ribotype 077) results in enteric disease.

Fourteen 6–24 h old male colostrums-fed Holstein calves, received either three doses of *C. difficile* ($1.4 \times 10^8 \pm 3.5 \times 10^8$ cfu) ($n = 8$) or sterile culture broth ($n = 6$). Calves were euthanized on day 6 or after the onset of diarrhea, whichever came first. Fecal and intestinal samples were blindly cultured for *C. difficile,* and tested for its toxin A/B (*C. difficile* TOX A/B II ELISA, Techlab). PCR-ribotyping was used to compare inoculated and recovered isolates.

Diarrhea was observed in all control calves and 3/8 of inoculated calves ($p = 0.03$), but it did not occur in calves that tested positive for *C. difficile* toxins. Fecal toxins were identified only from two controls. PCR-ribotyping confirmed the presence of *C. difficile* PCR-ribotype 077 in samples of all inoculated calves, but not from controls. The identification of five other PCR-ribotypes in 3/8 (37.5%) and 2/6 (33.3%) of inoculated and control calves, respectively, indicated early natural infection ($\leq$24 h of age). Five of 14 cecal samples had *C. difficile* ($p = 0.01$). In conclusion, the oral administration of *C. difficile* PCR-ribotype 077 to neonatal calves resulted in fecal/intestinal colonization but not in detection of toxins, or signs of enteric disease. Further studies are required to investigate the clinical relevance of *C. difficile* in calves.

Keywords: Toxins A and B; Experimental infection; PCR-ribotype 077; Intestinal colonization; Zoonosis; Cecum; Duodenum; Carrier; ELISA

1. Introduction

Clostridium difficile is a spore-forming bacterium associated with the development of serious enteric diseases and diarrhea in humans (Pepin et al., 2005).

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Outbreaks of severe *C. difficile*-associated disease (CDAD) in humans have increased in frequency worldwide, due in part to the emergence of epidemic strains PCR-ribotypes 017 and 027 (van den Berg et al., 2004; Warny et al., 2005). Recently, the isolation of these and other strains from feces of dairy calves (Rodriguez-Palacios et al., 2006), and from retail meat products in Canada (Rodriguez-Palacios et al., 2007) has raised epidemiologic interest.

C. difficile is also considered a pathogen for certain animals, including horses, dogs and pigs (Baverud, 2002; Keel and Songer, 2006). In cattle, a recent retrospective study performed in Canadian farms showed a strong association between the presence of fecal *C. difficile* toxins and calf diarrhea (Rodriguez-Palacios et al., 2006). However, *C. difficile* was more frequently isolated from controls (14.9%; 20/134) than from diarrheic calves (7.6%; 11/144) (Rodriguez-Palacios et al., 2006). This finding suggests that a disease-free carrier status might exist.

In humans, asymptomatic carriage of toxigenic strains of *C. difficile* is more common in neonates, whereas disease is more common in elderly (Pepin et al., 2005). In contrast, neonatal foals and piglets are reported to be susceptible to disease (Arroyo et al., 2004; Songer et al., 2000). Despite the recent association between *C. difficile* toxins and calf diarrhea, the role of *C. difficile* as a causal pathogen in calves is unknown. In other species, the histological and functional lesions attributable to *C. difficile* are associated with the local effect of two exotoxins, toxins A and B (Schirmer and Aktories, 2004). A binary toxin (known as CDT) has also been hypothesized to be another virulence cofactor (Geric et al., 2006).

An experimental model of disease is desirable to characterize the pathogenicity of *C. difficile* in calves, and to facilitate studies evaluating therapeutic and preventive measures. Oral infection with *C. difficile* may induce diarrhea in hamsters (Sambol et al., 2001), foals (Arroyo et al., 2004), and piglets (Songer et al., 2000), but it is unknown if experimental infection would induce disease in ruminants. This study investigated whether the oral administration of a toxigenic strain of *C. difficile* to neonatal calves would result in fecal and intestinal colonization, development of CDAD, and production of detectable *C. difficile* toxins in feces and intestinal contents using a commercial ELISA.

2. Materials and methods

2.1. Animals and C. difficile strain

Holstein–Friesian male calves ($\leq$12 h of age), obtained from three farms in fall 2005, were assigned before arrival to two groups. Animals were eligible if calving was normal, and if the calf stood up without assistance within the first 2 h of life. Four litres of colostrum from their dams were fed to calves within 2 h of birth. Calves were transported to an isolation unit and housed in individual pens until the end of the study on day 6. Barrier precautions were used to prevent cross-contamination. Calves were inoculated with *C. difficile* or placebo if they had no diarrhea at the end of a 6 h observation period and if they tested negative on a fecal ELISA test for *C. difficile* toxin A/B on arrival (*C. difficile* TOX A/B II, Techlab, Virginia). If diarrhea occurred before or within 6 h of inoculation, the calf was assigned to a third group for descriptive analysis. Calves were only bottle-fed fresh bovine antibiotic-free whole milk (2 L/12 h).

A *C. difficile* strain (PCR-ribotype 077) which contained genes encoding production of toxin A (*tcdA*$^+$) and toxin B (*tcdB*$^+$), but not CDT (*cdtB*$^-$) or *tcdC* deletions was used. This strain had been isolated from a diarrheic calf (Rodriguez-Palacios et al., 2006) and is a recognized cause of CDAD in humans. Initially, pure colonies of this strain were incubated anaerobically (37 °C for 48 h) in 50 mL of *C. difficile* selective broth supplemented with cysteine hydrochloride, moxalactam and norfloxacin (CDMN, Oxoid, England) (Aspinall and Hutchinson, 1992). This broth was used to inoculate bottles with 400 mL of pre-reduced brain heart infusion (BHI, Oxoid, Canada) broth. After 24–36 h of incubation, and centrifugation at 4000 $\times$ *g* for 10 min, the sediment was re-suspended in 50 mL of sterile BHI broth, which was administered to the calves within 2 h of preparation. Sterile BHI broth (50 mL) was used as placebo for control calves.

Purity and quantification of *C. difficile,* vegetative cells and spores, were determined for each batch of inoculum on 1 mL aliquots sampled before administration to the calves. Spore quantification was performed after mixing 1 mL aliquots with 96% ethanol (v/v, 30 min), followed by quantitative culture on blood agar.

2.2. Experimental infection and follow-up

After initial physical examination, blood samples were obtained for total plasma protein quantification (hand-held refractometer). Fecal samples were collected for *C. difficile* culture and toxin A/B testing. After 6 h of observation, calves were inoculated every 12 h with three doses of placebo or *C. difficile* inoculum. Inocula were fed to the calves with their last quarter of milk. Observation and physical exams were performed every 6–12 h by two blinded observers. Fecal samples were collected from the rectum once a day. Diarrhea was defined as the presence of stools with a fecal score of 4 (watery) in a 4-grade system (Larson et al., 1977).

Calves were euthanized at the end of the study period on day 6, or after 24 h of the onset of diarrhea or systemic disease (marked depression/dehydration), whichever came first. Samples of intestinal content (5 mL) from duodenum (30 cm from pylorus), ileum (30 cm from cecum), and cecal apex were collected during post-mortem examination by transmural aspiration. Samples were stored at −70 °C until processing. Histological examination of specimens preserved in 10% formaldehyde was conducted using light microscopy and haematoxylin and eosin stain. Lesions were blindly scored (0–3) by a pathologist; an overall additive score was given per intestinal segment. The Animal Care Committee of the University of Guelph approved the protocols of this study.

2.3. Laboratory analysis of samples

Testing for *C. difficile* toxin A/B was performed on feces of days 1, 3, 5 and 6, on samples of intestinal contents using *C. difficile* TOX A/B II. Readings were performed visually by agreement between two observers: positive and negative controls were used with every run.

The isolation of *C. difficile* from fecal samples (days 1–6) and from intestinal samples was performed using CDMN broth (with 0.1% sodium taurocholate; Oxoid, Canada) and CDMN agar (with 5% laked horse blood; Sigma–Aldrich Inc., Canada). After incubation of 1 mL of samples in 9 mL of CDMN broth for 5–7 days, this broth was mixed with 96% ethanol (v/v, for 30 min) and centrifuged ($4000 \times g$ for 10 min). Sediments were plated onto CDMN agar and incubated for up to 5 days. Confirmation of suspicious *C. difficile* colonies (swarming, non-hemolytic), extraction of DNA from pure colonies, and PCR-ribotyping analysis were conducted as previously described (Bidet et al., 1999; Rodriguez-Palacios et al., 2006).

Cultures for *Salmonella* spp., *Clostridium perfringens* and *Escherichia coli* was performed on fecal samples of day 2 at the Animal Health Laboratory, University of Guelph. *C. perfringens* toxins were not investigated. Multiplex PCR genotyping of *E. coli* isolates was performed using primers for enterotoxigenic (Sta, K99, F41) and verotoxigenic (stx1, stx2, eaeA, hlyA, VETC) markers (Slavic and Gyles, 2005). Qualitative tests for rotavirus type A (RotaScreen Latex, England), and bovine coronavirus (BIO-X Diagnostics, Belgium) were also performed.

2.4. Statistical analysis

Sample size estimations (Intercooled Stata v9.0, TX,) based on an 88% success rate after oral *C. difficile* infection in foals (Arroyo et al., 2004), and an estimated rate of natural calf diarrhea of 20% (Larson et al., 1998), indicated that two groups $\geq$12 calves would provide adequate study power (73%) if natural diarrhea occurred in $\leq$25% (3/12) of controls, and if infection induced diarrhea in >83% (10/12) of calves. This study would be terminated if partial results led to reduced study power. Continuous characteristic variables were tested using non-parametric two-sample Wilcoxon rank tests (SAS v9.1, NC). Differences in proportions of dichotomous variables were tested using Fisher's exact test. Measure agreement for categorical variables was estimated via Cohen's κ coefficient. Statistical significance was held at $p < 0.05$.

3. Results

This study was terminated after the inclusion of 14 calves as the first 8 inoculated animals did not develop CDAD (diarrhea and concurrent fecal *C. difficile* toxins).

On arrival, 13 of 14 calves were clinically normal and had no signs of diarrhea; the calf with diarrhea was used for descriptive analysis and euthanized on day 2.

One of eight inoculated calves developed diarrhea within 6 h of inoculation; it was used for descriptive analysis, received two more doses of *C. difficile,* and was monitored until day 6 as its diarrhea improved by day 2.

Comparative statistical analysis was conducted with five control and seven inoculated calves. There was no age difference between the groups (mean ± 1S.D.; 17.6 ± 7.4 h, range = 6–28 h, and 12.6 ± 8.1 h, range = 5–24 h, respectively; $p = 0.24$), and all calves had adequate plasma protein concentrations (inoculated calves, 73.3 ± 7.1, range = 64–85 g/L; controls, 63.5 ± 3.7, range = 60–70 g/L; $p = 0.07$) (Rea et al., 1996). The inocula administered to the calves had a concentration of $1.4 \times 10^8 \pm 3.5 \times 10^8$ cfu of *C. difficile* PCR-ribotype 077 organisms (range = 1×10^5, 1×10^9; $n = 15$), of which, $7.3 \times 10^4 \pm 1.5 \times 10^5$ cfu were spores (range = 1×10^2, 4.6×10^5; $n = 10$). By the end of the study, 5/5 control calves and 2/7 inoculated calves developed diarrhea ($p = 0.03$), but the average length of study was not different between groups (inoculated: 5.7 ± 0.3 days; range = 5.5–6 days; controls: 4.4 ± 1.5 days; range 3–6 days; $p = 0.3$). Three of five control calves were euthanized before day 6.

In total, 24 and 43 fecal samples were collected from 6 control and 8 inoculated calves, respectively, for *C. difficile* culture. Of these, 18 and 27 samples, respectively, were processed for *C. difficile* toxins. All 14 calves tested negative with the toxin ELISA the day of arrival. Toxins were detected in one of the fecal samples tested (1/18 (5.5%) versus 0/27 (0%), $p = 0.4$); it was from a control calf with diarrhea on day 3. The ELISA was also positive in one of the intestinal samples tested (1/12 (8.3%) versus 0/16 (0%), $p = 0.43$); the ileum of another control calf euthanized on day 3 due to severe diarrhea.

C. difficile was isolated from 26/43 (60.5%) fecal samples of the 8 inoculated calves but not from 24 samples of the 6 non-inoculated calves ($p < 0.001$). *C. difficile* was also cultured from 6/42 (14.3%) intestinal samples representing six calves; 2/6 ceca of controls, and 3/8 ceca and 1/8 duodenum of inoculated calves. Isolation of *C. difficile* was significantly more frequent from cecum (5/14) than collectively from duodenum (1/14) and ileum (0/14) ($p = 0.01$) (Table 1). A very low measured agreement ($\kappa < 0.2$) was observed when the last fecal culture and the intestinal cultures interpreted in parallel were compared. PCR-ribotyping confirmed that 22/26 (84.6%) fecal and 4/6 (66.6%) intestinal *C. difficile* isolates were the inoculated PCR-ribotype 077 (Table 1). The remaining six isolates, representing five calves (feces of three inoculated, and ceca of two control calves), showed five different PCR-ribotypes (data not shown). Both control calves harbouring *C. difficile* in the cecum were euthanized due to severe watery diarrhea on days 2 and 3, but tested negative with the toxin ELISA. These calves were the only animals that tested positive for enterotoxigenic *E.coli*; and one additionally harboured *Salmonella* spp. *C. perfringens* was present in both groups (3/6 control, 5/8 inoculated; $p = 1.0$). No verotoxigenic *E. coli*, rotavirus or coronavirus were identified in the study.

Table 1
Clostridium difficile culture and ELISA toxin A/B results in six controls and eight inoculated calves

	Fecal samples (day)						Last fecal[a]	Intestinal samples		
	1	2	3	4	5	6		Duodenum	Ileum	Cecum
Controls										
Culture	0/6[b]	0/6	0/5	0/3	0/2	0/2	0/6	0/6	0/6	2/6[c]
ELISA	0	0	1	0	0	0	0	0	1	0
Diarrhea	1/6	4/6	4/5	2/3	1/2	1/2	5/6	–	–	–
Inoculated										
Culture	3/8[c]	7/8[c]	3/8	5/8	6/8	1/3	4/8	1/8	0/8	3/8
ELISA	0	0	0	0	0	0	0	0	0	0
Diarrhea	1/8	2/8	1/8	0/8	0/8	0/3	0/8	–	–	–

[a] Result for last fecal sample obtained the day of post-mortem examination.
[b] Number of positive calves/number of calves remaining in the study.
[c] Two of the *C. difficile* isolates were non-PCR-ribotype 077, the remaining isolates corresponded to the inoculated strain ribotype 077.

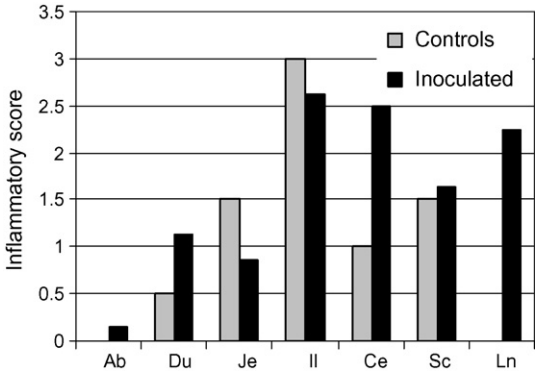

Fig. 1. Average microscopic inflammatory score (AMIS) of intestinal segments and ileo-cecal lymph node of calves naturally ($n = 2$) and experimentally ($n = 8$) infected with *Clostridium difficile*. AMIS represents the average number of lesions/severity identified per segment. Each segment was given cumulative scores (0–3; mild, moderate, severe) for six indicators of inflammation. Inoculated calves did not have diarrhea at necropsy; but controls did. Ab, abomasum; Du, duodenum; Je, jejunum, Il, ileum; Ce, cecum; Sc; spiral colon; Ln; ileo-cecal lymph node.

Three *C. difficile* isolates, one PCR-ribotype 077 and two of other ribotypes, were cultured from feces of three calves with <24 h of age, before inoculation. Similarly, two other PCR-ribotypes, cultured from cecum of two controls at 48 and 72 h of age, and from feces of two inoculated calves at 48 h of age, indicated early natural infection.

Post-mortem analysis revealed mild-to-moderate non-specific macroscopic changes (intestinal mucosa congestion, increased intestinal fluid content, mesenteric edema and edematous lymph nodes) in 4/6 control and 2/8 inoculated calves ($p = 0.28$). Microscopic inflammatory changes were overall mild–moderate, but they were more commonly seen in cecum and ileo-cecal lymph node of inoculated calves (Fig. 1). No intestinal ulceration, edema, or mucosal pseudomembranes were identified in inoculated animals.

4. Discussion

In this study, oral inoculation of toxigenic *C. difficile* PCR-ribotype 077 to colostrum-fed neonatal calves resulted in fecal shedding but no detection of *C. difficile* toxins, or induction of enteric disease for up to 5 days post-inoculation. These finding contrasts previous studies in hamsters, piglets and foals where diarrhea was induced in most animals within 48–72 h of oral infection (Arroyo et al., 2004; Jones et al., 1988; Post et al., 2000; Sambol et al., 2001). It is uncertain if diarrhea would have developed later, if calves had been monitored further. As CDAD in humans occur a few days after discontinuation of antimicrobial therapy (Beaugerie et al., 2003), some models of infection include the concurrent administration of antimicrobials to facilitate the induction of CDAD (Sambol et al., 2002). Antimicrobials were not used in this study as the main objective was to investigate the sole effect of an early ingestion of *C. difficile*. It is unknown if diarrhea had occurred if these calves had received antimicrobials. However, the failure to induce disease in the absence of antimicrobial therapy indicates a difference in susceptibility compared to neonatal foals (Arroyo et al., 2004), and questions the role of *C. difficile* as a primary pathogen in calves. It is important to consider, however, that failure to induce disease experimentally does not rule out the possibility that *C. difficile* is a bovine pathogen. Rather it indicates that simple exposure to *C. difficile* is not adequate to cause disease.

Previous infection studies of adult horses and dogs with toxigenic *C. difficile* also resulted in fecal shedding but not in signs of enteric disease or fecal detection of *C. difficile* toxins (Clooten et al., 2003; Gustafsson et al., 2004). Potential explanations for the outcome in these studies may include natural age-dependent resistance, lack of immune-compromising conditions, presence of an established and non-disrupted intestinal flora, and perhaps unknown yet factors inhibiting *C. difficile* colonization, toxin production, or binding to intestinal cells. Differences in toxin production and virulence across *C. difficile* strains could also be considered (Warny et al., 2005; Sambol et al., 2001).

Although a potential protective effect of milk (impaired adhesion of *C. difficile* to cells, and inhibition of toxin A mediated cytotoxicity) was suggested in previous in vitro studies (Dallas and Rolfe, 1998; Naaber et al., 1996), the roles of passive immunity and bovine whole milk on the transfer of anti-*C. difficile* factors to calves is unknown. Considering the wide distribution of *C. difficile* in the environment (Simango, 2006), the isolation of *C.*

difficile from mature cows (Rodriguez et al., unpublished data), and the common presence of serum titers of anti-*C. difficile* toxin immunoglobulins in humans (~70%) (Johnson, 1997), it is plausible that passive immunity might play a protective role for calves.

C. difficile was isolated from calves under 24 h of age indicating early natural ingestion of spores. The isolation of PCR-ribotype 077, a strain associated with CDAD in humans, from healthy and diarrheic calves in 2004 (Rodriguez-Palacios et al., 2006), and from the calf that arrived naturally infected to this study suggests this ribotype is circulating in the cattle population, and that calves might be healthy carriers.

C. difficile was more frequently isolated from the cecum of calves in both groups. Although there are no comparable studies for *C. difficile* in calves, the severity of the histological lesions seen in the cecum of infected piglets and hamsters (Borriello and Barclay, 1985; Keel and Songer, 2006) suggest that cecum could be a site for residence of *C. difficile* in calves. The frequent presence of mild–moderate microscopic inflammatory changes observed in the cecum of inoculated calves compared to the other intestinal segments and two controls might be consistent with such observation (Fig. 1). However, the clinical significance of this finding is uncertain due to limited sample size and the absence of signs of disease.

The toxin ELISA test used in this study has been adequately validated for diagnostic purposes in humans (Wilkins and Lyerly, 2003). In animals, despite its regular use in hospital settings, validation studies are scarce. In piglets the ELISA performance approached human standards (Post et al., 2002); but in dogs, it was considered poorly sensitive and specific (Chouicha and Marks, 2006). Validation of available ELISAs is needed to better understand the significance of *C. difficile* in cattle.

This study documented fecal/intestinal colonization for up to 5 days following oral inoculation of calves with a strain of *C. difficile*. No induction of enteric disease or detection of fecal *C. difficile* toxins, was observed. Cecum commonly yielded positive *C. difficile* cultures. PCR-ribotyping showed that calves may naturally harbour *C. difficile* by 24 h of age. Validation studies for diagnostic immunoassays for toxins are needed. *C. difficile* should not be ruled out as a potential cause of calf diarrhea.

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