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A possible role for *Clostridium difficile* in the etiology of calf enteritis

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Abstract

Clostridium difficile was investigated as a possible cause of enteritis in calves. The organism and its toxins (TcdA and TcdB), respectively, were found in 25.3% and 22.9% of stool samples from diarrheic calves. Culture positive samples were more likely than culture negative samples to be toxin positive. However, toxin positive stools were more common among nondiarrheic calves, but diarrheic calves were nearly twice as likely to be culture positive. Ribotype 078 was dominant among isolates. *Salmonella* sp. was isolated from both diarrheic and nondiarrheic calves, but large numbers of *E. coli* were found more commonly in diarrheic calves than in nondiarrheic animals. Prevalence rates for coronavirus and *Cryptosporidium* sp. were substantially higher in nondiarrheic calves than in diarrheic, but rates of detection of rotavirus and *Giardia* sp. were more nearly equal between groups. Lesions in naturally infected calves included superficial mucosal erosion with associated fibrinous exudates. Neutrophils and eosinophils infiltrated lamina propria. Large Gram-positive rods morphologically compatible with *C. difficile* were abundant in the colonic lumen and the organism was isolated by bacteriologic culture. Toxins were found throughout the colon. Purified toxins A and B (individually and conjointly) caused comparable lesions, as well as fluid accumulation, in ligated intestinal loops. Our findings are in substantial agreement with those of others [Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., Weese, J.S., 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12, 1730–1736; Porter, M.C., Reggiardo, C., Bueschel, D.M., Keel, M.K., Songer, J.G., 2002. Association of *Clostridium difficile* with bovine neonatal diarrhea. Proc. 45th Ann. Mtg. Amer. Assoc. Vet. Lab. Diagn., St. Louis, MO, U.S.A.] and add strength to a working hypothesis that *C. difficile* infection and the accompanying intoxication can manifest as diarrhea in calves. It seems clear that calves serve as multiplying hosts for this organism.

Keywords: *Clostridium difficile*; Calves; Enteritis; Toxin A; Toxin B

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1. Introduction

Clostridium difficile causes antibiotic-associated and pseudomembranous colitis in humans (Svenungsson et al., 2001), but *C. difficile*-associated disease (CDAD) also affects swine, horses, and laboratory animals (Keel and Songer, 2006). Clinical signs and lesions may be mild, as in porcine neonatal colitis (Songer and Anderson, 2006; Songer, 2005), but range to elevated leukocyte count, abdominal pain, profuse watery diarrhea, anorexia, lethargy, and death in humans. Collective pathology is comprised of pseudomembrane formation, inflammation, necrosis, and an intercryptal exudate of neutrophils and fibrin (''volcano lesions'') (Keel and Songer, 2006). Toxins A (TcdA) and B (TcdB) are apparently foremost among the virulence factors of *C. difficile* (Keel and Songer, 2006; Songer and Anderson, 2006).

More than 50% of preweaning deaths in intensively raised calves may be due to diarrheal disease (U.S.D.A., 1996), and, in 2002, we initiated work to determine the role of *C. difficile* in bovine neonatal diarrhea. Our findings revealed *C. difficile* toxins in more than 25% of calves, and nearly 40% of these were strongly positive. Incidence peaked at 1–2 weeks of age (Porter et al., 2002). Rodriguez-Palacios et al. (2006) obtained isolates of *C. difficile* from stools of 11.2% of 144 diarrheic (7.6%) and 134 nondiarrheic (14.9) calves. Seven of the eight PCR ribotypes isolated have been found in humans with CDAD, and two (017 and 027) have caused severe human disease. Toxins were detected in feces from 39.6% of diarrheic calves and 20.9% of controls. In both cases, differences were statistically significant ($p \leq 0.0009$). These results (Porter et al., 2002; Rodriguez-Palacios et al., 2006) suggested an association of *C. difficile* with calf diarrhea, and a possible bovine reservoir of infection for humans. Results of the present work strengthen the view that this organism may be associated with calfhood diarrheas.

2. Materials and methods

2.1. Field specimens

Fecal samples ($n = 253$) were collected from diarrheic calves 1 day to 6 weeks of age, originating in dairies in the southwestern U.S.A. (states of California, Utah, Nevada, New Mexico, Texas, and Arizona) and housed in calf ranches in southern and central Arizona. All were removed from the dam before nursing, but were fed up to 4 L of pooled colostrum on the first 2 days after birth. A commercial milk replacer was fed thereafter. Most sampled animals were Holstein bulls and freemartin-heifers ($n = 237$), and the remainder were Brown Swiss ($n = 14$) or crossbred ($n = 2$). Samples were also collected from 53 nondiarrheic Holstein calves in a single dairy. Fecal specimens were collected by rectal swab or in 15 mL conical centrifuge tubes. These were transported on wet ice and processed within 4 h of collection.

Two 23-day-old Holstein calves with high toxin titers were necropsied. Duodenum, jejunum, ileum, cecum, upper colon, mid-colon, and distal colon were examined for gross and microscopic lesions, for toxins, and for *C. difficile*.

2.2. Detection of C. difficile toxins

TcdA and TcdB were detected via a commercial enzyme immunoassay (EIA; Tox A/B, Techlab, Blacksburg, VA, U.S.A.).

2.3. Bacteriologic culture for C. difficile

Rectal swabs were placed in brain heart infusion (BHI) broth (Difco, Sparks, MD, U.S.A.) with 0.05% cysteine and 0.5% yeast extract in stoppered Hungate tubes, heat-shocked (80 °C, 10 min), and then incubated 18–20 h at 37 °C. Subcultures were made onto BHI agar with cysteine and yeast extract, 0.1% taurocholate, and 5% bovine blood, and taurocholate cefoxitin cycloserine fructose agar (TCCFA, Wilson et al., 1982). Plates were incubated for 48 h at 37 °C in an atmosphere of 5% H_2:5% CO_2:90% N_2. *C. difficile* was identified by its colony morphology, lack of hemolysis, yellow color on TCCFA, distinctive horsebarn odor, and production of L-proline aminopeptidase (Remel, Lenexa, KS, U.S.A.) and yellow-green fluorescence under Wood's lamp illumination.

2.4. Detection of other pathogens

Specimens ($n = 253$) were examined for *E. coli* and *Salmonella enterica* by standard enrichment and

subculture protocols. A subset ($n = 60$) was examined for *Cryptosporidium* sp. and *Giardia* sp. (Meridien Diagnostics, Cincinnati, OH, U.S.A.), rotavirus (Murex Diagnostics Ltd., Dartford, U.K.), and coronavirus (Syracuse Bioanalytical, East Syracuse, NY, U.S.A.).

2.5. PCR for tcdA and tcdB

Template was prepared by suspending one or two colonies in 200 μL HPLC-grade water and boiling for 20 min. After centrifugation (15,000 × *g*, 5 min), 10 μL of the supernatant fluid was used as template. The 100 μL reaction mix included 1 mM dNTPs (Fisher Scientific, Phoenix, AZ, U.S.A.), primers (1 μM [*tcdA* Forward GCATGATAAGGCAACTTCAGTGG; *tcdA* Reverse GAGTAAGTTCCTCCTGCTCCATCAA; *tcdB* Forward GGTGGAGCTTCAATTGGAGAG; *tcdB* Reverse GTGTAACCTACTT TCATAACACCAG, Gummerlock et al., 1993; Tang et al., 1994]), *Taq* polymerase (1 unit) in *Taq* buffer A (Promega, Madison, WI, U.S.A.), and HPLC-grade water. The amplification cycle consisted of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Products (15 μL) were separated by electrophoresis (20 min, 120 V) in a 1.5% agarose gel and examined via UV transillumination.

2.6. PCR ribotyping

Ribotyping of 33 randomly selected isolates was performed essentially as described (O'Neill et al., 1996; Keel et al., 2007). Each 100 μL reaction mixture contained 5 U of *Taq* polymerase (Promega), 1.5 mM $MgCl_2$, and 100 pmol of each primer [RiboF (CTGGGGTGAAGTCGTAACAAGG) and RiboR (GCGCCCTTTGTAGCTTGACC)]. Template DNA was prepared by emulsification of an isolated colony in 100 μL of 5% Chelex-100 (Bio-Rad, Hercules, CA, U.S.A.) and boiling for 10 min. Cell debris was removed by centrifugation (10 min, 17,000 × *g*) and 10 μL supernatant fluid added as template to each reaction mixture. PCR products were separated by electrophoresis in 3% NuSieve agarose at 150 mV for 6 h. Ladder (100 bp) was placed at five lane intervals. Gels were examined by UV transillumination and band patterns compared with GelCompar image analysis software (Applied Maths, Kortrijk, Belgium). Ribotype classifications were based on the typing scheme established by the Anaerobe Reference Unit, Cardiff, Wales, UK (O'Neill et al., 1996).

2.7. Antimicrobial susceptibility testing

Susceptibility of 25 randomly selected isolates was determined by the reference agar dilution procedure (National Committee for Clinical Laboratory Standards, 2001) with antimicrobials chosen from among those used to treat diarrheal disease in calves and for comparison to previous susceptibility test results on domestic animal strains of *C. difficile* (Jang et al., 1997; Post and Songer, 2004). Serial twofold dilutions of bacitracin (Alpharma, Fort Union, NJ, U.S.A.), erythromycin (Sigma–Aldrich, St. Louis, MO), tetracycline HCl (U.S. Pharmacopeia, Rockville, MD, U.S.A.), ceftiofur (Pfizer Animal Health, Kalamazoo, MI, U.S.A.), tilmicosin (Eli Lilly, Indianapolis, IN, U.S.A.), tylosin (Sigma–Aldrich, St. Louis, MO, U.S.A.), and virginiamycin (Phibro Animal Health, Fairfield, NJ, U.S.A.) were incorporated into *Brucella* agar (Difco) with vitamin K (1 μg/mL), hemin (5 μg/mL), and laked sheep blood (5%), at final concentrations of 0.125–256 μg/mL.

Prior to antimicrobial susceptibility testing, isolates were subcultured twice on prereduced *Brucella* agar supplemented with bovine blood (5%), and hemin and vitamin K, as above. Colonies were suspended in trypticase soy broth to an optical density equivalent to that of a McFarland 0.5 standard. Plates were inoculated with a modified Steer's replicator (1–2 μL per spot) and incubated for 48 h at 37 °C in an atmosphere of 5% H_2:5% CO_2:90% N_2. The lowest dilution of antimicrobial that affected growth on the test plate (as compared to control plates containing no antimicrobial) constituted the MIC. MIC_{50} and MIC_{90} were the concentrations at which 50% and 90%, respectively, of strains were inhibited. *Bacteroides fragilis* ATCC 25285, *Eggerthella lenta* ATCC 43055, *C. difficile* ATCC 9689, and *Clostridium perfringens* ATCC 13124 were used as quality control organisms.

2.8. Toxin production and purification

An overnight culture of *C. difficile* strain VPI 10463 in BHI broth with yeast extract and cysteine

was added to a loop of dialysis tubing (10 kDa M.W. cutoff) suspended in 4 L of the same BHI broth in stoppered 4 L Erlenmeyer flasks. Incubation was for 7 days at 37 °C in air; eH was not measured and no efforts (other than inclusion of cysteine, stoppering immediately after autoclaving, and avoiding agitation) were made to maintain anaerobiosis. After incubation, the content of the dialysis bag was centrifuged (15,000 × *g*, 4 °C, 20 min) and the clarified supernatant filtered (450 nm pore size). The volume was adjusted to 8 mL and applied to an Amicon ultracentrifugal filter device (Millipore, 100 kDa M.W. cutoff) which was then centrifuged at 4000 × *g* until 200 μL remained in the column. Phosphate-buffered saline (4 mL, 0.01 M, pH 7.4) was added to each column and the centrifugation repeated three times (Sullivan et al., 1982).

One milliliter of concentrated supernatant fluid was loaded onto a DEAE-Sepharose CL-6B column (3 cm × 20 cm) equilibrated with 50 mM Tris–HCl (pH 7.5). After sample loading, the column was washed with 50 mM Tris–HCl (pH 7.5) containing 0.05 M NaCl. The sample was eluted with a linear NaCl gradient (0.05–0.25 M NaCl in 50 mM Tris–HCl), and TcdA was collected in 4.2 mL fractions. A second linear gradient (0.30–0.60 M NaCl) was used to elute TcdB. The flow rate was 55–60 mL/h at 4 °C. Fractions were assayed for cytotoxicity on Chinese hamster ovary (CHO) cell monolayers cultivated in Iscove's modified Dulbecco's medium (IMDM) in 96-well cell culture plates. Cells were inoculated with fractions diluted in IMDM and then incubated at 37 °C for 18 h in an atmosphere of 5% CO_2. The cytotoxin titer was the reciprocal of the highest dilution producing 50% cytopathic effect, and its specificity was confirmed in parallel assays in which toxins were neutralized with goat polyclonal antitoxic antibodies prepared commercially (Antibodies, Inc., Davis, CA, U.S.A.). Fractions with highest titers were pooled, filtered (200 nm pore diameter), and stored at 4 °C (Sullivan et al., 1982). Examination by SDS-polyacrylamide gel electrophoresis (7.5% gels) revealed that purification was to ≥95% homogeneity.

2.9. Calf intestinal loops

Holstein calves (with stools negative for TcdA and TcdB) were anesthetized with isofluorane and xylazine, to effect. Access to the intestines was gained via surgical incision in the right side of the abdomen. Two ligated loops (control and principal, 2–5 cm length) were prepared in duodenum, jejunum, ileum, upper spiral colon, and mid-spiral colon, with 2 cm interloops. Control loops were injected with 1 mL of 50 mM Tris–HCl, while principal loops were inoculated with 1 mL of diluted toxin (8 μg TcdA in 50 mM Tris–HCl, 5 μg TcdB in 50 mM Tris–HCl, or 4 μg TcdA and 2.5 μg TcdB in 50 mM Tris–HCl). One calf received only TcdA, another received only TcdB, and the remaining calf received TcdA and TcdB. After injection, incisions were closed and the animals maintained under anesthesia for 4–6 h, at which time they were euthanized by pentobarbital overdose. Fluid accumulation was determined by comparing weight-to-length ratios of control and principal loops. Gross lesions were noted, and segments fixed in 10% phosphate-buffered formalin were paraffin-embedded, sectioned, stained, and examined for microscopic lesions.

2.10. Statistical analysis

Data were analyzed by chi-square test.

2.11. Institutional approval of animal experiments

All animal experiments were reviewed and approved *a priori* by The University of Arizona Institutional Animal Care and Use Committee.

3. Results and discussion

Fifty-eight (22.9%) stool samples from 253 diarrheic calves contained toxins, 64 (25.3%) yielded isolates of *C. difficile* (Table 1) and 94 (37.2%) were positive by either method (Table 2). Chi-square analysis revealed a significant ($p < 0.05$) effect of bacteriologic culture results on toxin detection results; culture positive samples were more likely to be toxin positive (26 of 44, 40.6%) and culture negative samples were more commonly toxin negative (Table 1). Only 16.9% (32 of 189) of culture negative calves were toxin positive (data not shown). Toxin negativity at any given sampling time is not uncommon in humans with CDAD (Delmée et al.,

Table 1
Comparison of toxin testing and bacteriologic culture in detection of *C. difficile* infection in diarrheic calves

	Toxin positive, *n* (%)	Toxin negative, *n* (%)	Total, *n* (%)
Culture positive	26 (10.3)	38 (15)	64 (25.3)
Culture negative	32 (12.7)	157 (62.1)	189 (74.7)
Total	58 (23)	195 (77.1)	253 (100)

Results of bacteriologic culture are significantly associated with outcome of toxin testing ($p < 0.05$).

Table 2
Detection of TcdA/TcdB and/or *C. difficile* in calves with or without diarrhea

	Nondiarrheic calves, pos/total (%)	Diarrheic calves, pos/total (%)
TcdA/B pos	16/53 (30.2) a	58/253 (22.9) ab
Culture pos	7/53 (13.2) a	64/253 (25.3) b
Either pos	19/53 (35.9) a	94/253 (37.2) a

Numbers with different letters are significantly different ($p < 0.05$).

2005), and these results in calves could reflect a similar situation. We have since developed improved methods for isolation of *C. difficile* from feces (Michael Anderson, Christine Coursodon, Michael Hailey, and J. Glenn Songer, unpublished results), and use of these may bring toxin detection and bacteriologic culture results more into agreement.

Toxin positives were no more common among diarrheic calves than among nondiarrheic animals, but the former were more likely to be culture positive (Table 2). This nearly twofold higher rate of infection in diarrheic calves was not surprising, but the substantial rate of toxin positivity in nondiarrheic calves was unanticipated. Some nondiarrheic animals may have been incubating an infection, although only one diarrheic calf was observed in the source herd for nondiarrheic animals during the 4-month sampling period. Regardless, toxin positivity (accompanied by typhlocolitis) is not uncommon in nondiarrheic piglets (Songer, 2005), and this may apply to calves, as well.

The rate of *C. difficile* isolation from diarrheic calves in this work was higher overall than that reported by Rodriguez-Palacios et al. (2006) (25.3% versus 7.6%). Rates of isolation from nondiarrheic calves were nearly identical (12.7% versus 14.9%). In contrast, the rate of toxin detection by these workers in diarrheic calves was substantially higher than our findings (22.9% versus 39.6%); toxin detection in nondiarrheic calves was more common in the work reported here (30.2% versus 20.9%). The reasons for these discrepancies are not immediately apparent.

tcdA and *tcdB* were present in all isolates ($n = 71$). Ribotype 078 was dominant among a randomly selected group of strains (31/33, 94%), to a greater extent even than in neonatal pig strains (Keel et al., 2007). This ribotype is also isolated from human CDAD, often in the form of community-associated disease, which is apparently experiencing a remarkable exponential rise in incidence (Beaugerie et al., 2003; Hirschhorn et al., 1994; Levy et al., 2000). Cases are typically in young patients (average 26 years) with no exposure to antimicrobial agents for $\geq$3 months before onset (Hirschhorn et al., 1994; Levy et al., 2000; Rexach et al., 2006). Ribotype 002 (1/33, 3%) is uncommon in humans (JS Brazier, personal communication) and in pigs ($\sim$4%; unpublished data), but more common in a small sample ($n = 20$) of equine isolates (15%; unpublished data). Ribotype 033 (1/33, 3%) is also uncommon in humans (JS Brazier, personal communication) and has not been reported in food animals other than calves.

Salmonella sp. was isolated from 18 of 253 (7.1%) samples from diarrheic calves and from 3 of 53 (5.7%) samples from nondiarrheic animals, a difference which was not statistically significant ($p \sim 0.6$; data not shown). The rate of isolation from toxin or culture positive calves was not significantly different from that in negative calves (Table 3). Large populations of *E. coli* were found in significantly more samples from diarrheic calves [59 of 253 (23.3%)] than from nondiarrheic animals [3 of 53 (5.7%); $p < 0.005$]. Most samples yielding large numbers of *E. coli* (43 of 62, 69.4%) were from calves $\leq$2 weeks of age (data

Table 3
Specimens containing *C. difficile* or toxins and positive by culture for *Salmonella* sp. or *E. coli*

Agent	*C. difficile* positive[a], *n* (% of total)	*C. difficile* negative[b], *n* (% of total)	Total (%)
Salmonella sp.	8/94 (8.5) c	11/212 (5.2) c	19/306 (6.2) c
E. coli	8/94 (8.5) c	51/212 (24.1) d	59/306 (19.3) d

Numbers with different letters are significantly different ($p < 0.005$).
[a] *C. difficile* or toxins detected.
[b] Neither *C. difficile* nor toxins detected.

not shown). Routine PCR genotyping failed to reveal genes encoding heat labile or stable toxins, pili (K99, F18, 987P, K88, or F41), or Shiga-like toxin 2e (data not shown). Thus, the significance of these findings is unknown; however, finding large numbers of this organism in pure culture in diarrheic stools suggests that it may have been an etiologic agent. Examination for virulence factor genes was not exhaustive, and if these *E. coli* strains were producing a clinical response, it may have been by virtue of other factors, known or unknown. Only two specimens (one *C. difficile* culture positive and one culture and toxin negative, both from diarrheic calves) yielded both *Salmonella* sp. and *E. coli*. No samples from diarrheic calves and yielding large numbers of *E. coli* in pure culture were also examined for coronavirus, rotavirus, *Cryptosporidium* sp., and *Giardia* sp. (see below). These data on co-infecting agents are limited, and are perhaps best viewed as providing a basis for hypotheses on which to plan future experimental work.

A limited number of specimens ($n = 60$; 23 from diarrheic calves, 37 from nondiarrheic calves) were examined for presence of coronavirus, rotavirus, *Cryptosporidium* sp., and *Giardia* sp. Prevalence rates for coronavirus and *Cryptosporidium* sp. were substantially higher in nondiarrheic calves than in diarrheic (Table 4; $p = 0.001$), but differences in rates of rotavirus and *Giardia* sp. detection were not significant. The impact of these figures is lessened by the small number of observations in some cells (e.g., *Giardia* sp.). Among stools from nondiarrheic calves, those testing positive for toxins were most likely to contain the viral and protozoal pathogens (data not shown). Only two toxin positive samples from diarrheic calves tested positive for any of these four pathogens (one for *Crytptosporidium* sp. and one for *Giardia* sp.) and only five toxin negative samples from diarrheic calves tested positive (one for coronavirus and rotavirus, two for *Cryptosporidium* sp., and one for *Giardia* sp.). *C. difficile* culture positive specimens also containing these additional pathogens were rare (four from nondiarrheic calves, none from diarrheic). Among culture negative samples, additional agents were uncommon in stools from diarrheic calves (1–3 positives in 23 samples), but 30.3% (10/33) and 60.6% (20/33) of samples from nondiarrheic calves were positive for coronavirus and *Cryptosporidium* sp., respectively. *C. difficile*-positive specimens (by culture and/or toxin detection) were no more likely to be positive for any of these pathogens than *C. difficile*-negative specimens. Our results are somewhat different than those in a case: control study (Rodriguez-Palacios et al., 2006) which suggested that frequent demonstration of *Cryptosporidium* sp. in diarrheic calves did not confound a conclusion that *C. difficile* caused the diarrhea. Among specimens tested for all seven pathogens, six (two from diarrheic calves and four from nondiarrheic) were toxin positive and negative for all other pathogens; one specimen (from a

Table 4
Specimens from diarrheic and nondiarrheic calves and positive in assays for viruses and protozoan parasites

Agent	Diarrheic ($n = 23$), pos (%)	Nondiarrheic ($n = 37$), pos (%)	All calves ($n = 60$), pos (%)
Coronavirus	2 (8.7) a	11 (29.7) b	13 (21.77)
Rotavirus	2 (8.7) a	4 (10.8) a	6 (10)
Cryptosporidium sp.	3 (13) a	21 (56.8) c	24 (40)
Giardia sp.	1 (4.4) a	3 (8.1) a	4 (6.7)

Numbers with different letters are significantly different.

Table 5
Frequency of MICs (0.125–256 μg/mL) for 25 calf isolates

Antimicrobial	Concentration (μg/mL)											
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Bacitracin										25		
Ceftiofur										17	6	2
Erythromycin			4	16	5							
Tetracycline	1						22	2				
Tilmicosin	11	1	4	9								
Tylosin	12	13										
Virginiamycin	25											

nondiarrheic calf) was culture positive and negative for the others. Thus, the general trend is toward higher rates of these pathogens (with the possible exception of *Giardia* sp.) in *C. difficile*-positive calves. However, these data are limited in scope and support no firm conclusions, but suggest both the need and a direction for further study.

Minimum inhibitory concentrations (MIC) of antimicrobials for calf isolates were somewhat lower than those for swine isolates (Table 5; Post and Songer, 2004). MICs for control strains were within limits prescribed by the National Committee for Clinical Laboratory Standards (Benning and Mathers, 1999; National Committee for Clinical Laboratory Standards, 2001). Others have reported *in vitro* anti-clostridial activity of tylosin (Dutta et al., 1983), and our findings concur; $MICs_{50}$ for erythromycin, tilmicosin, and tylosin were relatively low (0.25–0.50 μg/mL). Lack of bimodal distribution of susceptibility to erythromycin and tylosin suggests that erythromycin resistance elements encountered in strains from other species (Dzink and Bartlett, 1980; Delmee and Avesani, 1988; Farrow et al., 2001; Post and Songer, 2004) may be rare in this population. Human strains vary widely in susceptibility to tetracycline (Dzink and Bartlett, 1980; Delmee and Avesani, 1988), but with the exception of a single outlier (susceptible at 0.125 μg/mL), these calf strains were susceptible only at 8–16 μg/mL. The low MIC_{90} for virginiamycin (0.125 μg/mL) suggests that these strains may be susceptible *in vivo*, in contrast to strains from pigs, for which the MIC_{90} was 16 μg/mL (Post and Songer, 2004). Bacitracin had little activity against calf strains (MIC_{90} 64 μg/mL), in keeping with results for human, porcine, and equine strains (Jang et al., 1997; Citron et al., 2001; Post and Songer, 2004). High MICs reported for cephalosporins (Dzink and Bartlett, 1980; Chow et al., 1985; Post and Songer, 2004) mirror our results for ceftiofur (MIC_{90} 128 μg/mL). Positive clinical outcomes seem unlikely for agents with an MIC higher than recommended doses.

Microscopic examination of intestines of calves whose diarrheic stools were strongly toxin positive revealed villous degeneration in the jejunum and ileum and superficial mucosal erosion in the colon. Eroded areas had associated fibrinous exudates. Inflammatory infiltrates varied, but neutrophils and eosinophils infiltrated lamina propria in the jejunum, ileum and colon. Distended crypts in the colon contained degenerate epithelial cells and neutrophils, in keeping with findings in other species (Keel and Songer, 2006). Large Gram-positive rods morphologically compatible with *C. difficile* were abundant in both the proximal and the distal colonic lumen, and the organism was isolated by bacteriologic culture. Toxins were found in the ileum and throughout the colon.

Based upon these findings and precedent in other species, bovine neonatal CDAD should affect mainly the cecum and the colon (Keel and Songer, 2006), but we examined effects of TcdA and TcdB on small intestine, as well. Inoculation of a duodenal loop with TcdA led to luminal accumulation of neutrophils, and fibrin associated with moderate numbers of sloughed, necrotic epithelial cells; hemorrhage and edema occurred in the lamina propria. Superficial mucosal erosions and neutrophil infiltration occurred in the jejunal, ileal, and colonic mucosa. Mucus, fibrin, neutrophils, and sloughed epithelial cells accumulated in the lumen of a loop in mid-spiral colon (Table 6). Lesions in loops inoculated with TcdB were somewhat more severe, and included multifocal, ulcerative

Table 6
Fluid accumulation and lesions in calf intestinal loops inoculated with TcdA, TcdB, or both

Location	Injected with	Fluid accumulation (control/toxin)[a]	Lesions[b]
Duodenum	TcdA	1	PMN infiltration, edema, lost epithelium, hemorrhage
	TcdB	1	PMN infiltration, ulceration, necrosis
	TcdA/B	1	Villous degeneration
Jejunum	TcdA	0.5	Villous degeneration, hemorrhage, fibrin accumulation
	TcdB	0.21	PMN infiltration, hemorrhage, necrosis, edema
	TcdA/B	1	Distended lacteals, lymphocyte infiltration
Ileum	TcdA	0.8	PMN infiltration, necrosis
	TcdB	1	Ulcerated villi, edema
	TcdA/B	0.63	Villous necrosis, edema, necrosis, hemorrhage, inflammatory cell infiltration
Proximal colon	TcdA	0.8	PMN infiltration, necrosis, hemorrhage
	TcdB	0.13	PMN infiltration, lymphocyte infiltration into lacteals
	TcdA/B	0.81	Hemorrhage, erosion, mucus, cellular debris in lumen
Mid-colon	TcdA	0.53	PMN infiltration, necrosis, mucus, fibrin in lumen
	TcdB	0.8	PMN infiltration, ulceration
	TcdA/B	0.83	Villous degeneration, inflammatory cell infiltration, mucus, cellular debris in lumen

[a] Ratio of weight:length of control loop vs. weight:length of principle loop; lower numbers indicate greater toxin effect.
[b] Lesions were detected in principal loops only.

enteritis in the duodenum and the jejunum, marked villous necrosis in the ileum, and neutrophil infiltration into the small intestinal mucosa. Epithelium in a loop in upper spiral colon was hemorrhagic and necrotic, and neutrophils and lymphocytes infiltrated lacteals. The mid-colonic loop was hemorrhagic; deep ulceration extended to the muscularis mucosa, with mild infiltration by and exudation of neutrophils near ulcers (Table 6).

Mucosal lesions in loops exposed to both toxins were similar to those observed with individual toxins, but severe villous necrosis occurred in the duodenum and the ileum. Peyer's patch-associated lacteals in the jejunum were distended and contained lymphocytes; fewer neutrophils were noted than with individual toxins. Ileal mucosa was edematous, and inflammatory cells, including eosinophils, were found in the lamina propria. The loop in the upper spiral colon had deep mucosal erosions with hemorrhage and accumulation of mucus and cellular debris. The mucosal surface in the mid-colonic loop was necrotic, with vacuolation and some loss of supporting lamina propria. Mucus and cellular debris accumulated in the intestinal lumen, and inflammatory cells, including neutrophils, infiltrated into the submucosa.

Weight:length relationships were calculated for control and principal loops and these were then compared (control: principal) to determine the ratios presented in Table 6. Gross examination revealed no fluid accumulation in control loops, but TcdA and TcdB caused fluid accumulation in loops in the jejunum, the ileum, and the colon; TcdA and TcdB inoculated together produced similar effects in the ileum and the colon only. Detection of toxins in the colon in field cases and fluid accumulation in response to TcdA and TcdB intoxication in colonic loops suggests that the diarrheagenic mechanism is toxin-based. Further study will be required to elucidate specific events linking intoxication and fluid secretion.

Results of the work reported here, in company with that of Rodriguez-Palacios et al. (2006), support a working hypothesis that *C. difficile* infection and the accompanying intoxication can manifest as diarrhea in calves. *C. difficile* and its toxins were found in both diarrheic and nondiarrheic calves, and toxin positive diarrheic calves had enteric lesions. Purified TcdA and TcdB caused tissue damage, fluid accumulation, and neutrophil infiltration, suggesting that *in vivo* toxin production may lead to development of similar lesions. In

counterpoint, calves inoculated with *C. difficile* (Rodriguez-Palacios et al., 2007) became colonized, but neither toxins nor signs of enteric disease were detected. Further work with experimental inoculation, under various conditions, will be required to conclusively establish *C. difficile* as an etiologic agent. However, it is reasonable to conclude that calves may serve, at the very least, as multiplying hosts for this organism.

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