

Comparison of the odds of isolation, genotypes, and in vivo production of major toxins by *Clostridium perfringens* obtained from the gastrointestinal tract of dairy cows with hemorrhagic bowel syndrome or left-displaced abomasum

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Objective—To compare the frequency of isolation, genotypes, and in vivo production of major lethal toxins of *Clostridium perfringens* in adult dairy cows affected with hemorrhagic bowel syndrome (HBS) versus left-displaced abomasum (LDA).

Design—Case-control study.

Animals—10 adult dairy cattle with HBS (cases) and 10 adult dairy cattle with LDA matched with cases by herd of origin (controls).

Procedure—Samples of gastrointestinal contents were obtained from multiple sites during surgery or necropsy examination. Each sample underwent testing for anaerobic bacteria by use of 3 culture methods. The genotype of isolates of *C perfringens* was determined via multiplex polymerase chain reaction assay. Major lethal toxins were detected by use of an ELISA. Data were analyzed with multivariable logistic regression and χ^2 analysis.

Results—*C perfringens* type A and type A with the beta2 gene (A + beta2) were the only genotypes isolated. Isolation of *C perfringens* type A and type A + beta2 was 6.56 and 3.3 times as likely, respectively, to occur in samples from cattle with HBS than in cattle with LDA. Alpha toxin was detected in 7 of 36 samples from cases and in 0 of 32 samples from controls. Beta2 toxin was detected in 9 of 36 samples from cases and 0 of 36 samples from controls.

Conclusions and Clinical Relevance—*C perfringens* type A and type A + beta2 can be isolated from the gastrointestinal tract with significantly greater odds in cattle with HBS than in herdmates with LDA. Alpha and beta2 toxins were detected in samples from cows with HBS but not from cows with LDA. (*J Am Vet Med Assoc* 2005;227:132–138)

Hemorrhagic bowel syndrome (HBS) is a frequently fatal intestinal disease of adult dairy cows characterized by acute, progressive, segmental intraluminal hemorrhage and obstruction in the small intestine.^{1-3,a,b}

Clostridium perfringens type A or type A with the beta2 gene (A + beta2) have been isolated from feces or intestinal lesions of affected cows.^{2,3,a,b} *Clostridium perfringens* is a gram-positive spore-forming anaerobic bacillus that is ubiquitous in the environment and the gastrointestinal tract of most mammals.^{4,9} There are 5 defined types of *C perfringens* (A, B, C, D, and E), distinguished on the basis of production of 4 major lethal exotoxins: alpha, beta, epsilon, and iota.⁸ Designating an isolate as 1 of the 5 types is accomplished by detection of major toxin production or of the genes coding for the major toxins.⁸⁻¹² The enterotoxin gene (*cpe*) may be detected in any of the 5 types of *C perfringens*.⁸⁻¹³ The gene for beta2 toxin (*cpb2*) may be detected in types A, B, C, and E.¹³

The pathogenesis of enteric disease caused by *C perfringens* type A in cattle is poorly defined.⁹ The clinical importance of isolating *C perfringens* type A from intestinal contents or feces of cattle with gastrointestinal tract disease can be questionable because the organism is part of the normal flora of the intestine of livestock, proliferates rapidly in cadavers, and is inconsistently isolated from animals with enterotoxemia.^{4,9} Alpha toxin, the primary lethal toxin produced by type A, is a calcium-dependent phospholipase that is capable of cleaving phosphatidylcholine in eukaryotic cell membranes.^{14,15} Strains of *C perfringens*, including type A, that carry the beta2 toxin gene have been isolated from multiple species of domestic animals, including horses, camelids, cattle, sheep, and swine.^{13,16} Beta2 toxin is also a lethal toxin,¹⁷ and strains of *C perfringens* with the *cpb2* gene produce variable amounts of beta2 toxin in vitro.¹³

The extent of colonization of the gastrointestinal tract by *C perfringens* in cows with HBS remains undefined, and production of the major lethal toxins and beta2 toxin has not been detected in affected cows. The purpose of the study reported here was to compare *C perfringens* isolated from the gastrointestinal tract of cows with HBS and from herdmates with left-displaced abomasum (LDA) on the basis of rates of isolation, genotype, and in vivo major toxin production.

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Materials and Methods

Study overview—A case-control design was used to investigate the relationships among isolation of *C perfringens*, production of major lethal toxins, and occurrence of HBS. Cases were adult cattle with HBS from client-owned dairies. The control cattle were cows in which LDA was diagnosed from the same dairy and at approximately the same time as cows enrolled in the case series. Samples of gastrointestinal contents were obtained from multiple sites in the gastrointestinal tract of study cattle and evaluated via microbial culture for *C perfringens* and testing for alpha and beta2 toxins. Signalment and microbiologic data were analyzed to detect potential associations with the occurrence of HBS.

Cattle—Cattle were enrolled in the study from October 2001 through September 2002 and originated from 3 dairy farms in northeastern Colorado. Cases were identified at the time of exploratory surgery or necropsy examination and were designated as a case if segmental or diffuse hemorrhagic enteritis with intraluminal blood clot formation was present in the small intestine³ but there were no other intestinal or extraintestinal lesions (eg, intussusception or adhesions) that could cause obstructive disease of the small intestine, and no evidence of coagulopathy in the initial physical examination. Samples obtained during necropsy of cattle were taken within 1 hour of death to limit spurious detection of postmortem enteric overgrowth of *C perfringens*. Of the 10 affected cattle, samples were obtained from 7 after admission to the James L. Voss Veterinary Teaching Hospital for surgery; 2 animals were euthanatized and samples were obtained during necropsy at the Colorado State University Veterinary Diagnostic Laboratory; and 1 animal was euthanatized and samples were obtained during necropsy at the dairy of origin. The control series included 10 cows in which LDA was diagnosed; after enrollment of a cow with HBS, the next herdmate cow in which a diagnosis of LDA was made during exploratory laparotomy was enrolled as a control cow. Nine of the control animals were identified during surgeries performed at the teaching hospital, and 1 was identified and enrolled during surgery on the farm of origin. The diagnosis of LDA was confirmed in all control cows via right paralumbar celiotomy and surgical exploration and was corrected via an omentopexy procedure in each instance. Samples of gastrointestinal contents were obtained from control animals at the time of surgery. Approval for animal use for this project was obtained from the Institutional Animal Care and Use Committee at Colorado State University.

Sample collection and microbial cultures—In cows with LDA, 2 swab samples of gastrointestinal contents were obtained from the ventral sac of the rumen, the descending duodenum, mid-jejunum, and distal portion of the jejunum. Rumen contents were sampled via percutaneous ruminocentesis prior to or immediately after surgery with a 16-gauge 1.5- or 3-inch needle and sterile syringe. Enteric contents were obtained during surgery via enterocentesis with a sterile 18-gauge 1- or 1.5-inch needle and sterile syringe. Swab samples were transferred immediately into anaerobic culture transport tubes containing Amies medium without charcoal.^c In cows with HBS, samples were obtained during surgery or necropsy examination by use of identical methods, except that the mid-jejunal sample was taken from an affected segment in the approximate area of the mid-jejunum. All samples were placed into culture transport medium as described.

Swab samples were refrigerated at 4°C and processed for microbial culture 12 to 72 hours after collection. For colony isolation, 1 swab sample from each site was streaked directly onto a 5% sheep blood agar plate (BAP)^d and the BAP was incubated at 37°C for 24 hours in an anaerobic culture system.^e This method of microbial culture (direct culture) was intended to isolate the vegetative (rod) form of *C perfringens* from study samples.¹⁸

After streaking a BAP for direct culture, the same swab sample was inoculated into a 10-mL sterile vial of cooked meat medium.^f The vials were incubated under anaerobic conditions^e at 37°C for 24 hours. One hundred microliters of this broth was transferred to a BAP, streaked for colony isolation, and incubated under anaerobic conditions as described for direct culture. This method of culture (enriched culture) is used to isolate *C perfringens* from samples in which there are low numbers of the vegetative form of the organism.¹⁸

A second swab sample from each site was used to inoculate a separate vial of cooked meat medium, which was placed in an 80°C water bath for 10 minutes and immediately incubated as described for enriched culture. This method of culture (heat shocking plus enrichment) was performed to induce germination of clostridial spores into the vegetative form, enabling detection by microbial culture.¹⁹

A fecal sample was obtained during physical examination for all cows, and 2 swab samples of feces were subjected to microbial culture, as described. For cows admitted to the teaching hospital (7 cows with HBS and 9 cows with LDA), a separate fecal sample was obtained for microbial culture of *Salmonella* spp by use of described methods²⁰ as part of a hospital *Salmonella* surveillance program. A fecal sample for 1 additional cow with LDA was obtained on the dairy and used for culture for *Salmonella* spp by use of identical methods.

Determination of bacterial genotype—After 24 hours of incubation, BAPs were visually inspected for growth of colonies that were morphologically consistent with *C perfringens* and that had a characteristic pattern of double zone (alpha and beta) hemolysis.¹⁸ If no such colonies were present, the sample was classified as negative for *C perfringens*. If characteristic colonies were present, 6 colonies were analyzed for genotype by use of multiplex polymerase chain reaction (mPCR) assays for the *C perfringens* major toxin genes (*cpa*, *cpb*, *etx*, and *iA*), which encode the alpha, beta, epsilon, and iota toxins, respectively; the beta2 toxin gene (*cpb2*); and the enterotoxin gene (*cpe*).^{11,16} Once a colony was genotyped as *C perfringens* type A, B, C, D, or E, results for the sample were considered to be positive for isolation of the organism and the colony was considered a *C perfringens* isolate. After an isolate was typed, it was also designated as positive for the beta2 toxin gene if the beta2 gene was amplified in the mPCR assay. If the gene for enterotoxin was amplified, the isolate was designated as the genotype followed by '+ ent.' Samples with positive results for *C perfringens* were designated by the genotypes identified by use of mPCR assay.

Detection of major lethal toxins—Indirect noncompetitive ELISA assays were used to detect *C perfringens* alpha and beta2 toxins by use of a technique modified from that of Naylor et al.²¹ Approximately 3 mL of gastrointestinal contents was obtained from the rumen, descending duodenum, mid-jejunum, and distal portion of the jejunum via aspiration with a sterile 18-gauge needle attached to a sterile syringe. Feces were not analyzed for toxin because it was considered likely that the toxins would be degraded by bacterial enzymes. After swab samples were obtained for microbial culture, the samples of gastrointestinal contents were added to an equal volume of 0.75% EDTA in phosphate-buffered saline (PBS) solution and stored at -4°C. Samples were transferred within 12 hours to a second freezer and stored at -70°C until analysis. On thawing, samples were kept on ice and diluted 1:40 with sterile distilled water containing sodium carbonate (1.59 g/L), sodium bicarbonate (5.93 g/L), and sodium azide (0.2 g/L) adjusted to a pH of 9.6. Samples were centrifuged at 16,000 × g for 4 minutes. The supernatant was removed and used as sample for toxin analysis via ELISA assay.

Flat-bottom plastic microtiter plates^g (volume, 200 µL) were used as the solid phase for the ELISA assays. Fifty micro-

liters of a 1:40 diluted sample was added to 4 wells of the microtiter plate and serially diluted such that the last wells contained a 1:1,280 dilution of intestinal contents. Two positive controls for toxin^h were added to wells on each plate and were serially diluted from 1:1,000 to 1:32,000 in a solution of 0.75% EDTA in PBS solution. A solution of 0.75% EDTA in PBS solution was used as a negative control in 2 wells for each plate. Plates were incubated at 25°C for 1 hour while being shaken at 100 revolutions/min on an orbital plate shaker.

After incubation, plates were washed 4 times in Tris-buffered saline (0.9% NaCl) solution (pH, 8.0) with Tween 20 (TBST). A block consisting of dried milk in sodium/Tris base/EDTA plus Tween 20 was applied, and samples were incubated at 25°C for 1 hour. After incubation, the plates were emptied and 50 µL of mouse monoclonal antibodyⁱ against *C perfringens* alpha toxin or beta2 toxin diluted 1:200 in TBST was added to the wells and incubated at 25°C for 1 hour. After washing, 50 µL of hydrogen peroxidase-labeled goat anti-mouse antibody conjugate^j diluted 1:3,000 in TBST was added to the plates and incubated at 25°C for 1 hour. Sample and control toxin were detected by addition of 100 µL of the substrate o-phenylenediamine in sodium citrate (substrate buffer; pH, 5.0).^k After 5 minutes, the substrate-enzyme reaction was stopped by the addition of 50 µL of 2M sulfuric acid. Absorbance at 450 nm was measured with a spectrophotometer.^l Optical density values were calculated by determination of mean background absorbance values from negative control wells; this value was subtracted from values from sample wells. Wells containing samples that had absorbance values exceeding the absorbance values for negative controls by > 0.1 were considered positive; all other samples were considered to have negative results. Results were reported as positive or negative for toxin. Each sample was tested for alpha and beta2 toxins on separate plates, with each sample run in duplicate. The ELISA assay detected alpha toxin at concentrations ≥ 11 pg/mL and beta2 toxin at concentrations ≥ 27 pg/mL. Gastrointestinal contents from cases and controls were combined with hydrogen peroxidase-labeled goat anti-mouse antibody conjugate^j in the amounts described and tested for nonspecific binding; the results for all samples were negative.

Data analyses—For purposes of data analyses, *C perfringens* isolates from samples were classified via genetic analysis as type A without genes for beta2 toxin (type A) or type A with genes coding for beta2 toxin (type A + beta2). Samples were similarly classified on the basis of detectable alpha and beta2 toxins by use of the ELISA assay.

Multivariable logistic regression for repeated measures^m was used to analyze differences in likelihood of recovering *C perfringens* from samples. The dependent variable in these analyses was the outcome (yes/no) of microbial culture for type A or type A + beta2 from samples. Parallel analyses were conducted for each of these outcomes in separate models. Independent variables evaluated in models included the study group of cow (HBS or LDA), dairy of origin (dairies A through C), age of cow (years), time elapsed between the admission of each case and control animal from the same dairy (days), and the anatomic site from which samples were obtained (rumen, descending duodenum, mid-jejunum or lesion, distal portion of the jejunum, or feces). Because infection status of cows from the same dairy might be correlated or the culture status of 1 sample might predict the culture status of another sample obtained from the same cow, this repetition of measurements was controlled in all analyses. Specifically, the identity of cows from which samples were obtained (identified as A to T) was nested within the case-control pair (ie, a case animal and the next control animal enrolled from the same dairy of origin; PAIR = 1 to 10) and controlled as a repeated measure in the analyses. Odds ratios

(ORs) and their 95% likelihood ratio-based confidence intervals (CIs) were determined from results of regression analyses. Independent variables were screened singly for association with the outcome variables, and those with detectable bivariable relationships ($P < 0.25$) were included in multivariable analyses. Final multivariable models were determined by means of a backward-stepping approach with a critical α for retention of 0.05. First-order interaction terms were evaluated for all main effects included in final models.

Logistic regression for repeated measures was used to investigate the potential relationships between major lethal toxins in samples (yes/no) and whether samples were obtained from case or control animals.ⁿ The dependent variable in these analyses was the outcome of ELISA assays for alpha and beta2 toxins, and the independent variable was the study group of the cow (HBS or LDA). The repeated nature of data was controlled as described for models related to culture data by use of the identity of cows and their case-control pair designation. Odds ratios and their 95% CIs were determined from results of regression analyses. For the purpose of data analysis and to enable the statistical software to perform logistic regression, a single sample with negative results from a cow of the control series (cow H) was arbitrarily assigned a positive result for both alpha and beta2 toxins. χ^2 Analysis was used to compare the proportion of cows in each study group that had positive results for *Salmonella* spp on microbial culture of feces.

Results

Fifteen samples were obtained from each cow. Samples of gastrointestinal contents were obtained from 5 sites in each cow, and the samples were subjected to bacterial culture by use of 3 separate methods, yielding 150 samples from each group. Because of sampling or laboratory handling error, 7 samples from cows in the HBS group were lost and 3 samples from cows in the LDA group were lost. Thus, 143 samples from cows in the HBS group and 147 samples from cows in the LDA group were analyzed via microbial culture.

All colonies characteristic of *C perfringens* that were tested via mPCR were successfully genotyped. *Clostridium perfringens* types A and A + beta2 were the only genotypes isolated from cows in this study. The enterotoxin gene was not detected in any of the isolates. For each study group, the proportion of samples with positive results for each genotype of *C perfringens* was tabulated (Table 1). *Clostridium perfringens* type A was isolated either alone or with type A + beta2 in 79 of 143 (55.2%) samples from cows with HBS and 28 of 147 (19.0%) samples from cows with LDA. *Clostridium perfringens* type A + beta2 was isolated either alone or with type A in 59 of 143 (41.3%) samples from cows with HBS and 35 of 147 (23.8%)

Table 1—Proportion (percentage) of samples obtained from dairy cattle with hemorrhagic bowel syndrome (HBS) or herd-matched cattle with left-displaced abomasum (LDA) with positive results for growth of *Clostridium perfringens* by genotype.

Variable	HBS (n = 143 samples)	LDA (147)
<i>C perfringens</i> type A	66/125 (52.8)	22/57 (38.6)
<i>C perfringens</i> type A + beta2	46/125 (36.8)	29/57 (50.9)
Both genotypes	13/125 (10.4)	6/57 (10.5)
Total	125	57

samples from cows with LDA. For all culture methods combined, the frequency of isolation of either type of *C perfringens*, *C perfringens* type A, and *C perfringens* type A + beta2 from cows in each study group was categorized by site in the gastrointestinal tract (Figure 1).

Of the 125 samples obtained from all sites in the case series that had positive results of growth for *C perfringens*, 138 isolates were type A or type A + beta2 genotypes. The distribution of these isolates by site in the gastrointestinal tract was tabulated (Table 2). Among these isolates, 79 (57.2%) were type A and 59 (42.8%) were type A + beta2. Of 57 samples obtained from all sites in the control series that had positive results for growth of *C perfringens*, 63 isolates were genotypes A or A + beta2. Among those isolates, 28 (44.4%) were type A and 35 (55.6%) were type A + beta2.

Controlling for the effects of repeated measures, cow age, herd of origin, site sampled, culture

method, and days elapsed between the time of sampling a cow with HBS and a herd-mate with LDA, samples from cows in the case series were 6.56 times as likely to yield growth of *C perfringens* type A as samples from cows in the control series ($P < 0.01$; OR, 6.56; 95% CI, 3.49 to 13.07). Controlling for the same variables, the odds of a sample having positive results for growth of *C perfringens* type A + beta2 were 3.3 times as great for samples from the case series as those from the control series ($P = 0.01$; OR, 3.30; 95% CI, 1.78 to 6.31).

The site sampled did not significantly influence the likelihood of isolation of type A in either group ($P = 0.56$). The odds of a fecal sample from the case series having positive results for growth of the A + beta2 genotype was 3.11 times as great as samples obtained from other sites in those cattle ($P = 0.03$; OR, 3.11; 95% CI, 1.51 to 6.52).

The method of bacterial culture influenced the odds of a sample having positive results for growth of *C perfringens* type A. The direct culture and enriched culture methods increased the odds of isolation of the type A genotype by 1.3 and 3.4 times, respectively, compared with the heat shocking plus enrichment method ($P = 0.05$). Method of culture had no effect on the odds of isolating type A + beta2 ($P = 0.13$).

For every year increase in a cow's age, the odds of a sample being positive for growth of *C perfringens* type A increased by a factor of 1.57 ($P = 0.01$; OR, 1.57; 95% CI, 1.13 to 2.17). There was no effect of age on the odds of isolating type A + beta2 ($P = 0.33$).

The likelihood of recovering type A or type A + beta2 from cows with HBS and cows with LDA was not affected by the number of days that elapsed between sampling pairs of cases and controls ($P = 0.20$ and 0.81 , respectively).

Samples obtained via enterocentesis that were used for toxin detection were available from all intestinal sites for 9 cows with HBS and their herd-matched controls; for the remaining pair of study cattle, the volume of sample obtained from the control cow was insufficient for analysis. Alpha toxin was detected in 7 of 36 (19.4%) samples from 5 of 9 cows in the case series and in 0 of 36 (0%) samples from cows of the control series. Beta2 toxin was detected in 9 of 36 (25%) samples from the case series and 0 of 36 (0%) samples from the control series. Controlling for the repeated nature of sample collection, the likelihood of detecting alpha toxin from cows with HBS was not significantly higher than the likelihood of detection in control animals ($P = 0.08$; OR, 2.03; 95% CI, 0.78 to 74.71). Similarly, the likelihood of detecting beta2 toxin in samples from cows with HBS was not significantly higher than the likelihood of detection of the toxin in samples from control animals ($P = 0.06$; OR, 2.35; 95% CI, 0.95 to 115.23). The genotype of *C perfringens* isolated via bacterial culture from the corresponding site in the intestine was concordant with the toxin type identified by ELISA assay in 11 of 15 toxin-positive samples (Table 3). In 4 samples from 2 cows with HBS (cows A and B), beta2 toxin was detected by the ELISA assay, but *C perfringens* type A was isolated via microbial culture.

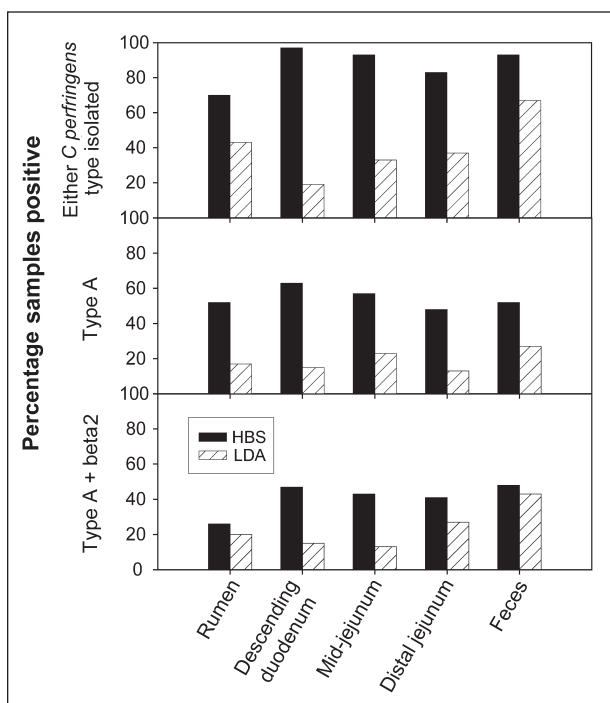


Figure 1—Percentage of samples with positive results for growth of any *Clostridium perfringens* (either type A or type A + beta2), *C perfringens* type A, and *C perfringens* type A + beta2 by site sampled for 3 culture methods combined. Proportions include samples from which both types of *C perfringens* were isolated. Samples were obtained from dairy cattle with hemorrhagic bowel syndrome (HBS) and herd-matched cattle with left-displaced abomasum (LDA).

Table 2—Number (percentage) of *C perfringens* isolates by site from dairy cattle with HBS or LDA.

Site	HBS	LDA
Rumen	21 (15)	11 (17)
Duodenum	33 (24)	8 (13)
Mid-jejunum*	30 (22)	11 (17)
Distal jejunum	26 (19)	12 (19)
Feces	28 (20)	21 (33)
Total	138	63

*Intestinal lesion in cows with HBS.

Table 3—Samples with positive results for *C perfringens* alpha and beta2 toxins by site obtained from dairy cattle with HBS.

Cow	Alpha toxin	Beta2 toxin	<i>C perfringens</i> genotype isolated at site
A	Intestinal lesion	Intestinal lesion	Type A*
		Distal jejunum	Types A and A + beta2
		Duodenum	Type A†
B	Intestinal lesion	Intestinal lesion	Type A†
		Distal jejunum	Type A†
C	Rumen	Rumen	Types A and A + beta2
	Duodenum	Duodenum	Type A + beta2
	Intestinal lesion	Intestinal lesion	Type A + beta2
E	Duodenum	Duodenum	Types A and A + beta2
G	Intestinal lesion		Types A and A + beta2

**C perfringens* A + beta2 isolated from rumen, duodenum, and feces of this cow. †*C perfringens* A + beta2 isolated from feces of this cow.

There was no difference ($P = 0.76$) in the proportion of fecal samples that had positive results of bacterial culture for *Salmonella* spp from the case series (1/7) and the control series (2/10).

Discussion

In this study, *C perfringens* type A and *C perfringens* type A + beta2 were the only genotypes isolated from cows with HBS or LDA. None of the isolates contained the gene for enterotoxin. These findings are similar to those of previous reports^{2,3} of HBS in dairy cows and surveys of *C perfringens* isolates from the gastrointestinal tract of cattle.^{12,13} As was true in other reports, *Salmonella* spp were not recovered from the feces of most cows in the case series in our study.

The proportion (46.8%) of all *C perfringens* isolates that were classified as type A + beta2 by use of the mPCR assay in our study was similar to that reported (53%) in a previous survey^a of *C perfringens* in enteric and fecal samples from cattle with HBS and in a survey¹⁶ of enteric isolates of *C perfringens* from healthy and diseased animals (50%). In another study,²² 29% of *C perfringens* isolates from the gastrointestinal tract of clinically normal calves and 31% of isolates obtained from calves with enterotoxemia were determined to be type A + beta2. Data from our study indicated that *C perfringens* type A + beta2 can be isolated from multiple sites in the gastrointestinal tract and from feces of cows with HBS as well as from herdmate cows with LDA.

The odds of detecting *C perfringens* types A and A + beta2 via microbial culture were significantly higher for samples taken from the gastrointestinal tract of dairy cows with HBS than for those from herdmates with LDA. Compared with cows with LDA, there appeared to be a change throughout the gastrointestinal environment in cows with HBS that enhanced the likelihood of obtaining positive results of microbial culture of *C perfringens* from luminal contents and feces. We were unable to determine whether colonization of the gastrointestinal tract by these organisms was increased in cows with HBS or if HBS induced changes in the organism that enhanced viability and therefore the likelihood of detection via microbial culture.

In ruminants with enterotoxemia, proliferation of *C perfringens* in the gastrointestinal tract is associated with the feeding of concentrates or overeating.⁴ Diets high in concentrate feeds are associated with an increase

in the rate at which *C perfringens* is isolated from the rumen and cecum of healthy cows.⁶ Cows with LDA have reduced appetite and abomasal atony,²³ and the rate of passage of concentrates from the forestomach to the intestines is likely to be slower than in clinically normal cows. A reduced rate of intestinal passage may have reduced the density of growth of *C perfringens* in the cows with LDA in our study, resulting in lower odds of isolation of *C perfringens* relative to normal cows. Therefore, the differences in likelihood of isolating the organism might have been smaller if cows with HBS had been compared with clinically normal cows.

The number of days that elapsed between the time of collection of samples from a cow with HBS and its herdmate control did not affect the odds of obtaining positive results for microbial culture of *C perfringens* type A or type A + beta2. If cows on a given farm were simultaneously exposed to a factor influencing the rate at which *C perfringens* would be isolated from the gastrointestinal tract, then the number of days that elapsed between the time of sample collection from a cow with HBS and its herdmate with LDA would be expected to influence the odds of obtaining positive culture results. The elapsed time was measured once for each cow with HBS and control herdmate. It is possible that there were insufficient numbers of cows enrolled to detect an influence of this variable on the odds of obtaining a positive culture result.

Age was associated with differences in the likelihood of recovering *C perfringens* type A but not type A + beta2. The reason for this finding was not clear, and other studies have not revealed age-related differences in the odds of recovery of these organisms.

For gastrointestinal contents obtained from each site, 3 methods of bacterial culture (ie, direct culture, enriched culture, and heat shocking plus enrichment) were used in parallel to maximize the likelihood of detecting *C perfringens*. Compared with results from the heat shocking plus enrichment technique, the direct culture and enriched culture methods had significantly greater odds of yielding positive results for culture of type A. No significant effect of culture method on the odds of positive results for growth of type A + beta2 was detected. The reason for this discrepant effect of culture method on the odds of isolation of the 2 genotypes of *C perfringens* was not apparent. The heat shocking plus enrichment method is conducted to induce germination of *C perfringens*

spores. It is possible that type A organisms may have produced fewer spores or spores less responsive to heat shocking than type A + beta2 organisms.

There was no significant difference in the proportion of samples with positive results for either alpha or beta2 toxins among the 2 study groups. However, the major lethal toxins (alpha and beta2) could be detected only in the gastrointestinal contents of cows affected with HBS.

Because *C perfringens* types A and A + beta2 can be isolated from the gastrointestinal tract of apparently healthy animals, the diagnostic significance of isolation of these organisms from animals with enteric disease is increased if the corresponding toxins can be detected in gastrointestinal contents or blood.^{4,24} However, detection of the toxins alone does not confirm involvement of *C perfringens* in the pathogenesis of enteric disease.²⁴ A combination of clinical signs, lesions seen at surgery or necropsy examination, and results of microbial culture and assays to detect toxin is necessary to definitively attribute enteric disease to this organism.^{22,24} On the other hand, major lethal toxins may not be detected in affected animals because the toxins are labile, particularly in the intestinal lumen where host and microbial proteases abound.²⁴ The beta2 toxin can be degraded by trypsin into polypeptide fragments with resultant loss of cytotoxic properties.¹⁷ Therefore, the potential for false-negative results of toxin assays is considerable, and for an animal with clinical signs or lesions compatible with HBS, reliance on results of microbial culture and assays for toxin detection may be problematic.

The elapsed time between obtaining the samples and freezing them at -70°C was as long as 12 hours in some instances; this delay could have permitted degradation of labile major toxins in samples. The sensitivity of the assay may have been insufficient to detect the toxin in some samples. The low proportion of samples that contained the major lethal toxins may also be a result of differences in the quantities of major lethal toxins produced among different isolates of *C perfringens*. Production of alpha toxin has been reported to vary among isolates of *C perfringens* type A.^{25,26} In a previous study,¹³ only 50% of isolates of *C perfringens* type A + beta2 derived from the gastrointestinal tract of cattle were found to produce beta2 toxin in vitro. It is possible that this gene was not expressed in some isolates obtained from the study cows. Disease conditions associated with intestinal obstruction may have influenced the proportion of samples from which toxins were detected. In cows with HBS, obstruction created by intraluminal blood clots could have trapped toxin molecules in the gastrointestinal tract at the site of or ad to the obstruction. In 4 cows with HBS from which samples had positive results for toxins (cows B, C, E, and G), toxin was detected in samples at the site of obstruction or orad to it. In cows A and B (case cows), however, toxin was detected at the site of the obstruction as well as aborad to it. In the cattle of this study, therefore, there was no clear association between the location of the gastrointestinal obstruction and the location of samples with positive results for toxin.

In most samples in which toxin was detected, the corresponding bacterial genotype was isolated from the

same sample. In 4 samples from 2 cows, beta2 toxin was detected in the samples but only *C perfringens* type A was isolated from the samples. However, *C perfringens* type A + beta2 was isolated from at least 1 other sample in both of these cows, and movement of toxin within the gastrointestinal tract may account for these results. Alternatively, false-positive results on the ELISA assay could cause the disparity between results of microbial culture and toxin testing data.

There was an apparent association between isolation of these bacteria from the gastrointestinal tract, production of their major lethal toxins in the gastrointestinal tract, and the occurrence of HBS. Although *C perfringens* types A and A + beta2 may be involved in the pathogenesis of HBS, the particulars of the pathogenesis have yet to be fully elucidated. It is unclear whether *C perfringens* types A and A + beta2 act as primary pathogens in cows with HBS or if enhanced enteric growth or survival of the organisms takes place secondary to another disease process. Intraluminal intestinal hemorrhage from another cause could initiate secondary proliferation of *C perfringens* because the organism is considered likely to proliferate when soluble protein or carbohydrate enters the intestine.^{4,6} Such conditions could be created by intraluminal hemorrhage resulting from another disease process.

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- b. Uzal FA, Blanchard P, Songer G, et al. Studies on the so-called "Clostridial enteritis" of cattle (abstr), in *Proceedings*. 43rd Annu Meet Am Assoc Vet Lab Diagn 2000;15.
- c. Co-pan Venturi Transystem 108C, US Amies without charcoal, Co-pan Diagnostics Inc, Corona, Calif.
- d. BD BBL stacker plate, plated media for growth of microorganisms, trypticase soy agar with 5% sheep blood, Becton-Dickinson & Co, Sparks, Md.
- e. BDL Gas Pak anaerobic system envelopes, Becton-Dickinson & Co, Sparks, Md.
- f. BBL cooked meat medium, Becton-Dickinson & Co, Sparks, Md.
- g. F96 Maxisorp, Nalge Nunc International, Rochester, NY.
- h. Recombinant alpha and beta2 toxins supplied by Dr. Glenn Songer, University of Arizona, Tucson, Ariz.
- i. Monoclonal antibody to alpha toxin (9A24B) and monoclonal antibody to beta2 toxin (9E2B) supplied by Dr. William Scheuchenzuber, Pennsylvania State University, University Park, Pa.
- j. Peroxidase-labeled goat anti-mouse conjugate (610-1302), Rockland Inc, Gilbertsville, Pa.
- k. o-phenylenediamine (OPD) (P-9029), Sigma Chemical Co, St Louis, Mo.
- l. Multiskan spectrum microplate spectrophotometer (model 1500), Thermo Electron Corp, Milford, Mass.
- m. PROC GEN MOD, version 8, SAS Statistical Software, Cary, NC.

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