Determination of the effect of single abomasal or jejunal inoculation of *Clostridium perfringens* Type A in dairy cows

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Abstract — A randomized study was conducted to determine if inoculation of the abomasum or jejunum with *Clostridium perfringens* Type A would induce jejunal hemorrhage syndrome in healthy cows. Twelve adult nonlactating dairy cows were inoculated with 10 mL of pure culture broth of *C. perfringens* type A (beta2 toxin positive) into the abomasum (n = 6) or jejunum (n = 6). On day 6, the cows were euthanized and samples for culture were taken from the abomasum, jejunum, and feces. No cows developed clinical signs of jejunal hemorrhage syndrome during the course of the study. Five of 6 abomasal samples and 1 of 6 jejunal samples were positive for *C. perfringens* Type A (beta2 negative) prior to inoculation. Eight of 12 abomasal samples, 11 of 12 fecal samples, and 10 of 12 jejunal samples were positive for *C. perfringens* Type A (beta2 negative) after inoculation. Intraluminal inoculation of *C. perfringens* Type A alone at this dose and under these conditions did not induce clinical signs of jejunal hemorrhage syndrome in adult dairy cows. The multifactorial nature of the disease likely contributed to our inability to reproduce the disease in this study.

Résumé — Estimation des effets d'une inoculation unique dans l'abomasum ou le jéjunum de *clostrodium perfringens* de type A chez la vache laitière. Une étude au hasard a été menée afin de déterminer si l'inoculation de l'abomasum ou du jéjunum avec du *Clostrodium perfingens* de type A pouvait provoquer un syndrome hémorragique chez des vaches en santé. Douze vaches laitières taries ont été inoculées avec 20 ml d'un bouillon de culture pure de *C. perfringens* de type A (bêta 2 toxine positif) dans l'abomasum (n = 6) ou le jéjunum (n = 6). Au jour 6, les vaches ont été euthanasiées et des échantillons pour culture ont été prélevées de l'abomasum, du jéjunum et fèces. Aucune vache n'a développé les signes cliniques du syndrome du jéjunum hémorragique au cours de l'étude. Cinq des six échantillons de l'abomasum et un des six échantillons du jéjunum étaient positifs à *C. perfringens* type A (bêta 2 négatif) avant l'inoculation. Huit des 12 échantillons de l'abomasum, 11 des 12 échantillons fécaux et 10 des 12 échantillons du jéjunum étaient positifs à *C. perfringens* type A (beta 2 négatif) après l'inoculation. L'inoculation intraluminale de *C. perfringens* type A seul à ce dosage et dans ces conditions ne provoquait pas de signe clinique du syndrome du jéjunum hémorragique chez les vaches laitières adultes. La nature multifactorielle de la maladie a vraisemblablement contribué à notre échec à reproduire expérimentalement la maladie.

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Introduction

Recently, a syndrome has been recognized in adult dairy cows involving intraluminal and intramural intestinal hemorrhage and necrosis with subsequent clot formation and intestinal obstruction. This syndrome has been referred to as jejunal hemorrhage syndrome (JHS), hemorrhagic bowel syndrome, intraluminal-intramural hematoma, bloody gut, and hemorrhagic enteritis. Clinical signs of this peracute disease include recumbency, dehydration, shock, abdominal distension, anorexia, abdominal pain, and either a lack of feces or the production of tarry feces with blood clots (Herman JA, personal communication; 1–4). In many cases, the cow is found dead. Affected cows are usually in early lactation, producing large quantities of milk, and are fed silage or total mixed ration (TMR) (3,5). The case mortality rate for this syndrome is marked (77%) (7,8). Jejunal hemorrhage syndrome has recently also been reported in beef cows (7), though it is much less common in beef cattle.

In the search for the cause of this disease, much speculation has fallen upon *Clostridium perfringens* Type A (2,3). *Clostridium perfringens* Type A was suspected as a cause or contributing factor in this disease because this organism has been cultured from the intestinal contents

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or feces of a large number of cows affected with JHS (3,5,6). However, clear evidence regarding the pathogenicity of these isolates has not been produced to link *C. perfringens* Type A to development of JHS. *Clostridium perfringens* Type A has been linked to abomasal ulcers and abomasitis in calves (8–12). However, *C. perfringens* Type A has also been found in the gastrointestinal tract of cattle without it causing clinically apparent disease (8,9,13).

At present, it is thought that JHS is a multifactorial disease (2,3–5). Proposed risk factors include feeding of silage or total mixed ration, feeding finely ground corn in the ration, lactation in its early stage, high level of production, and free-choice feeding. These factors may create an environment in which stress, excess gastrointestinal starch, and subclinical rumen acidosis allow overgrowth of *C. perfringens* Type A bacteria in the gastrointestinal tract. We hypothesized that inoculation of the intestinal tract with *C. perfringens* Type A isolated from a cow affected with JHS could induce clinical disease in normal healthy cows, so the objective of this study was to determine if inoculation of *C. perfringens* Type A, isolated from a confirmed case of JHS, into the abomasum or proximal jejunum would alone induce the disease in healthy cows fed a diet of corn silage.

Materials and methods

Prior to the in vivo study, a sample for culture was obtained from a cow affected with JHS. The cow exhibited clinical signs of JHS, and a sample cultured from the intestine at laparotomy was identified and typed as *C. perfringens* Type A, beta2 toxin positive by analysis of polymerase chain reaction (PCR) results. This organism was then maintained in Brewer modified thioglycollate medium under anaerobic conditions at 37ºC for 2 wk. Prior to initiation of the study, the organism was multiplied in fresh culture medium to provide a broth that was estimated by dilution testing to have a concentration of between 10^8 and 10^{10} colony forming units (CFU) per mL at the time of inoculation into the animals. At inoculation, microscopic examination of a stained smear of the broth did not reveal spores or sporulation.

Twelve adult dairy cows were obtained for use in the study, which was approved by the Animal Use Committee and the Infection Prevention Committee at The Ohio State University. Cows (8 Holstein, 4 Jersey) ranged in age from 3 to 8 y. Three cows (1 in group 1, 2 in group 2) were pregnant (gestational ages estimated to be 90, 120, and 210 d). Cows were housed in pairs in box stalls and fed ad libitum corn silage 2 to 3 times daily (slug feeding style). Estimated daily feed intake was determined by measuring for each pair of cows and averaging between them. The cows were acclimated for 2 to 4 d prior to the initiation of the study. Each cow was determined to be healthy, based on the results of a complete physical examination, prior to entry into the study. The cows were examined twice daily and the presence of urine ketones was tested for (Ketostix; Bayer, Elkhart, Indiana, USA), fecal production and consistency were noted, and the abdomen was examined for the presence of auscultable "pings." If diarrhea developed, the feces were tested for fecal occult blood by using a commercial test kit (Hemocult; Beckman Coulter, Palo Alto, California, USA).

Cows were randomly assigned to individual box stalls and treatment groups in a double blind fashion. Group 1 cows ($n = 6$) received *C. perfringens* Type A by inoculation of the abomasum at its greater curvature. Group 2 cows ($n = 6$) received *C. perfringens* Type A by jejunal inoculation at its most proximal accessible site. Blood samples were collected from the coccygeal artery or vein on days 0 and 6 for complete blood (cell) counts (CBC) and differential, and serum biochemical profile.

On day 0, each cow underwent a right flank laparotomy, following proximal paravertebral regional anaesthesia. The abdomen was explored, any abnormalities noted, and the target organ exteriorized at the incisional site. Samples for culture were obtained from the target organ immediately prior to inoculation with the culture broth to determine the presence or absence of *C. perfringens*. Samples of ingesta were aspirated from the jejunum or abomasum by using a 14-gauge needle and 12-mL syringe. Following collection of the samples, 10 mL of culture broth (10^8 to 10^{10} CFU/mL) was inoculated into the abomasum (group 1, $n = 6$) or proximal jejunum (group 2, $n = 6$).

On day 6, cows were euthanized and a complete gross necropsy was performed. Immediately following euthanasia, samples of ingesta were obtained from the rumen, abomasum, and proximal jejunum for measuring the pH. Samples of ingesta were obtained from the abomasum, jejunum, and rectum for microbial culture. All samples of ingesta were cultured anaerobically in trypticase soy agar with 5% sheep blood at 37ºC for 24 to 48 h at The Ohio Department of Agriculture Veterinary Diagnostic Laboratory, Reynoldsburg, Ohio, USA. Growth in the broth was then identified, and any samples positive for *C. perfringens* were typed by PCR analysis at the University of Arizona Clostridial Enteric Disease Unit, University of Arizona, Tuscon, Arizona, USA.

Results

No complications occurred during surgery. One cow (group 1), which had a left displaced abomasum (LDA), had the displacement corrected by deflation and repositioning only during the surgery for the inoculation. One cow (group 1) developed a left-sided ping consistent with an LDA 3 d postinoculation. This auscultable ping was present for 2 d and then resolved.

Heart rate remained within normal ranges throughout the study (group 1 — mean 67 beats/min [bpm] ± 4 bpm; group 2 — mean 62 bpm ± 7 bpm). Respiratory rates remained within normal limits throughout the study (group 1 — mean 50 breaths/min [brpm] ± 7 brpm; group 2 — mean 45 brpm ± 14 brpm). There were 4 cows (group 1) that exhibited elevated rectal temperatures (range 39.3°C to 40.2°C) during the first 2 d postoperatively. After day 2, the mean rectal temperature for all cows was within normal limits (group 1 — mean 38.6°C ± 0.3°C; group 2 — mean 38.4°C ± 0.4°C). Results of CBC and serum biochemical profiles performed on days 0 and 6 were within normal limits. No cows exhibited ketonuria during the study.

Seven cows (3 in group 1, 4 in group 2) developed diarrhea for 1 to 3 d following the inoculation. All cases of diarrhea resolved spontaneously, and all cows had normal feces by day 4. The diarrhea was green-brown, with no evidence of blood on visual examination or fecal occult blood test.

Eleven of 12 cows had reduced or absent ruminations at some time following the inoculation (6 in group 1, 5 in group 2), ranging in duration from 1 to 6 d. The cow that developed an apparent LDA 3 d after inoculation had decreased ruminations beginning 1 d after inoculation and continuing to the end of the study. All other cows had normal ruminations by day 5.

At necropsy, the pH of ruminal, abomasal, and jejunal contents was similar between both groups. The group 1 mean ruminal pH was 6.1 ± 0.6 and the group 2 mean was 6.0 ± 0.3. Mean abomasal pH was 2.6 ± 0.5 (group 1) and 2.2 ± 0.7 (group 2). Mean jejunal pH was 7.3 ± 0.6 (group 1) and 7.0 ± 0.4 (group 2). One cow from each group had abomasal mucosal hyperemia or hemorrhage. No areas of hemorrhage or necrosis were noted in the small or large intestine. Overall, there were very few lesions noted at gross necropsy.

Cultured samples obtained on day 0 were positive for *C. perfringens* Type A in 5 of 6 abomasal samples (group 1) and in 1 of 6 jejunal samples (group 2). All cultures were beta2 toxin negative. Culture of corn silage was negative for *C. perfringens* Type A. At necropsy, 6 of 6 abomasal samples, 6 of 6 jejunal samples, and 6 of 6 fecal samples were positive for *C. perfringens* Type A in group 1. Group 2 had 2 of 6 abomasal samples, 4 of 6 jejunal samples, and 5 of 6 fecal samples positive for *C. perfringens* Type A. All samples were beta2 toxin negative by PCR analysis.

Discussion

None of the cows in this study developed clinical signs of JHS following inoculation with live pure culture of nonsporulated *C. perfringens* Type A (beta2 toxin positive). In previous studies in calves, clinical disease was induced by direct inoculation of the rumen (11) with pure cultures of *C. perfringens* Type A and produced enterotoxemia, and abomasal ulceration, abomasitis, or both. Spontaneous cases of hemorrhagic abomasitis caused by *Clostridium* spp. have also been reported in calves (10,12). Jejunal hemorrhage and enteritis in a goat was also recently reported to be associated with beta2 toxin positive *C. perfringens* (14). However, inoculation of *C. perfringens* Type A into the duodenum of live calves or ligated intestinal loops produced no clinical signs of enterotoxemia or enteritis (15,16). Jelinski et al (10) found no difference in the prevalence of *C. perfringens* Type A in calves with and without abomasal ulcer/abomasitis syndrome.

Nonlactating cows were used in this study as the biosecurity review by the Infection Prevention Committee classified *C. perfringens* as a level 2 biohazard, so the cows had to be housed in a biosecurity facility where there was no equipment to milk the cows. We hypothesized that if it were possible to produce the disease in nonlactating cows, it should be easier to produce it in stressed lactating cows. The acclimatization period varied between 2 and 4 d depending on the day of the week that the cows were obtained, since we were unable to obtain all the cows on the same day. As stress was desirable for this study, we did not feel that holding the animals any longer prior to the initiation of the study would be beneficial.

We elected to inoculate 2 different sites (abomasum and proximal jejunum) because we wanted to compare locations to maximize chances to induce disease, since previous inoculation studies had used various methods and locations of inoculation with varying results, and it is unclear where the clostridial overgrowth may begin with this syndrome. One hypothesis is that overgrowth in the jejunum is caused by overflow of finely-ground carbohydrates from the forestomachs (2,3). We hypothesized that by depositing bacteria in the jejunum, we would provide an ideal environment for the bacteria to proliferate. Although, theoretically, *C. perfringens* would be killed by the low pH of the abomasum, abomasal inoculation mimics clostridial infusion from the rumen during periods of acidosis.

In the present study, 5 of 6 (83%) of abomasal samples and 1 of 6 (16%) of jejunal samples yielded *C. perfringens* Type A (beta2 negative) prior to inoculation. The high number of positive abomasal samples was surprising, considering the low abomasal pH, which would be expected to kill any bacteria present. Prevalence of normal cattle with *C. perfringens* Type A in the intestine has been reported to be 36.8% to 78.6% (10,15,17). Most studies have utilized samples obtained at necropsy. Thus, the high prevalence of *Clostridium* spp. may be an overestimation associated with rapid postmortem overgrowth. *Clostridium perfringens* Type A grows rapidly in the intestine following death, so the prevalence of live animals with the bacteria may be lower than that reported (8). In fact, Amtsberg et al (15) reported *C. perfringens* in the feces of only 33.9% of live calves. In our study, the time between death and collection of samples was less than 20 min.

Enteritis occurred in some cows following inoculation, as evidenced by transient diarrhea, reduced ruminations, and low grade fever. We presume that the diarrhea was caused by the inoculation, as cattle are not expected to develop diarrhea after exploratory laparotomy and there was no feed change during the study to cause digestive disturbance. There was no control group within the study that was subjected to laparotomy alone, but comparison was made with the 200+ animals presented to the teaching hospital for laparotomy during that year; in these animals, diarrhea and fever were rare complications, unless peritonitis was present. A control group within the study would have been ideal, but financial constraints prevented us from including further animals.

During the brief period of diarrhea, 4 cows had a transient fever, but normal heart and respiratory rates. The disease induced was mild and appeared to resolve rapidly and spontaneously. Feces from cows with post-inoculation diarrhea were not cultured at the time of diarrhea. In retrospect, doing this would have enabled us to differentiate enteritis caused by inoculation with *Clostridium* spp. from enteritis caused by stress. However, we did not expect that the short-time period

following inoculation would allow for recovery of the organism from the feces.

Eighteen of 24 abomasal and intestinal samples obtained at necropsy (75%) were culture positive, compared with 6 of 12 preinoculation samples (50%). This may indicate an increase in the presence of *C. perfringens* Type A, as our samples were collected within minutes of euthanasia and it is unlikely that significant postmortem overgrowth occurred. Presumably, the bacteria cultured were not elaborating toxin in sufficient quantity to induce severe disease in the cows. *Clostridium perfringens* Type A produces a-toxin, and some may also produce a beta2 toxin (8,9,18). Although the inoculant bacteria was typed by PCR analysis as a beta2 toxin positive strain, it may not have been expressing this toxin after in vitro culture or after inoculation. Currently, the type of toxin that may be involved in the pathogenesis of JHS is not known, but a seemingly large percentage of cultures from cows with JHS are beta2 toxin positive. Differences in bacterial cultures or toxin expression may explain some of the variation in the results in this experimental study.

The fact that all isolates collected after death were beta2 toxin negative, whereas the inoculant was beta2 toxin positive, may indicate that the inoculated bacteria a) stopped producing beta2 toxin during passage through the animal, b) were outcompeted so that their growth was inhibited, or c) died. They may have stopped producing the toxin because there was insufficient stress through feeding practices or animal handling to cause activation and toxin expression as is seen in clinical cases. A future study could radiolabel the inoculum to confirm recovery at fecal culture.

The pH of the gut contents tested were consistent with expected values for dairy cows fed a total mixed ration, so the silage probably did not provide enough carbohydrate bypass to lower the pH sufficiently, or sufficient carbohydrate substrate to induce disease. In retrospect, adding finely ground corn to the ration might have helped in this regard. Excess grain or starch in the small intestine is more likely to lead to sudden bacterial overgrowth (2,3,8,9), so passage of grain particles into the small intestine may be a factor in the pathogenesis of JHS in dairy cows fed high grain TMR. Excess mixing of TMR causing finely powdered corn was suspected to be related to an outbreak of clinical cases of JHS on one farm (3).

The level of bacteria inoculated was chosen based on the inoculant volume and concentration reported by Roeder et al (11). In that study, 1×10^9 to 2×10^{10} CFU of *C. perfringens* Type A, inoculated into the rumen of calves, was sufficient to induce clinical cases of abomasitis and abomasal ulceration. In future studies, a higher dose of bacteria, repeated doses of bacteria, inoculation of the sporulating phase of *C. perfringens* Type A, inoculation of the rumen, or inoculation of the toxin itself could be investigated.

Based on the results of this study, we conclude that enteral inoculation of *C. perfringens* Type A alone at this dose is not sufficient to induce JHS in adult dairy cows. Other factors must be involved in the development of the hemorrhage and the disease syndrome. Further investigation is warranted to evaluate the effects of management, including nutrition, stress, and lactation.

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