The relationship between the presence of *Helicobacter pylori*, *Clostridium perfringens* type A, *Campylobacter* spp, or fungi and fatal abomasal ulcers in unweaned beef calves

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Abstract

A case-control study involving 30 unweaned beef calves was conducted to determine whether specific species of bacteria or fungi were associated with fatal abomasal ulcer formation. Special microbiological and histological techniques were used to detect *Clostridium perfringens* type A, *Helicobacter pylori*, or *Campylobacter* spp. It has been speculated that these bacteria are potential ulcerogenic agents of unweaned beef calves. Calves were recruited for the study at necropsy, with those dying of either a perforating or a hemorrhagic ulcer representing the cases, and calves of a similar age dying of a disease unrelated to the abomasum representing the controls. *Helicobacter pylori* was not visualized in or cultured from any of the abomasal tissue samples. *Clostridium perfringens* type A was isolated from 78.6% of the cases and 75% of the controls. These isolates were further dichotomized into "heavy" and "light" growth; no significant association was found between ulcers and the amount of growth. A light growth of *Campylobacter* spp. was recovered from 3 cases and 3 controls. There was no compelling evidence to suggest that *Clostridium perfringens* type A, *Helicobacter pylori*, or *Campylobacter* spp. were involved in ulcer formation.

Résumé

Le lien entre la présence de *Helicobacter pylori*, de *Clostridium perfringens* type A, de *Campylobacter* spp ou de champignons et l'ulcère fatal de l'abomasum chez les veaux non sevrés

Cette étude de cas a été effectuée sur 30 veaux non sevrés afin de déterminer si la présence de certaines bactéries ou de champignons serait associée à la formation d'ulcère fatal de l'abomasum. Une évaluation histologique et des épreuves microbiologiques spéciales ont été utilisées afin déceler la présence de *Clostridium perfringens* type A, *Helicobacter pylori* et *Campylobacter* spp. L'hypothèse que ces bactéries sont potentiellement des agents ulcérogéniques chez des veaux non sevrés avait été émise. Les animaux ont été sélectionnés à l'autopsie et répartis en deux groupes. Les veaux morts à la suite d'un ulcère perforant ou hémorragique ont été considérés dans l'étude de cas, alors que ceux, d'âge comparable, morts à la suite d'une cause non reliée à l'abomasum, représentent le groupe témoin. *Helicobacter pylori* n'a pas été visualisé ou isolé à partir des échantillons tissulaires de l'abomasum. *Clostridium perfringens* type A a été isolé dans 78,6 % des cas de l'étude et dans 75 % du groupe témoin. Les isolats ont été par la suite subdivisés en deux catégories, ceux à croissance « marquée » et ceux à croissance « faible ». Aucun lien n'a pu être démontré entre les ulcères et le taux de croissance. *Campylobacter* spp a été isolé avec un faible taux de croissance chez trois sujets dans l'étude de cas et chez trois sujets du groupe témoin. Les auteurs concluent qu'il n'y a pas d'évidence pouvant suggérer que le *Clostridium perfringens* type A, l'*Helicobacter pylori* ou le *Campylobacter* spp seraient responsables de la formation des ulcères.

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Introduction

Historically, the medical community has classified peptic ulcers as a noninfectious disease. However, in 1982, the bacterium *Helicobacter pylori* was successfully cultured from human gastric ulcers (1), leading researchers to believe that there is an infectious component to gastric ulcer formation. Subsequent research involving animal models (2), therapeutic trials (3,4), and volunteer (5) and epidemiological studies (6) have substantiated *H. pylori* as an important etiological agent of gastritis, and a probable cause of gastroduodenal ulceration, dyspepsia (7–9), and gastric carcinoma (7).

The isolation of *H. pylori* has led to the discovery of 7 other species of *Helicobacter: H. mustelae* (10), *H. felis* (11), *H. acinonyx* (12), *H. nemestrinae* (13), *H. muridarum* (14), and *H. cinaedi* and *H. fennelliae* (15). The isolation of *Helicobacter* spp. from different host species affected with gastritis and gastric ulceration has led to speculation that the Campylobacter-like organisms (CLOs) observed in histological sections of ulcerated bovine abomasum may be *Helicobacter* spp. (16,17).

Although there is no direct evidence to support a link between *Helicobacter* spp. and abomasal lesion formation, there is some evidence supporting a relationship between *Clostridium perfringens* type A and abomasal ulcers (18,19). A variety of other bacteria, including *Campylobacter* spp. (19) and *Streptococcus* spp. (20), and fungi (21) have also been linked to abomasal lesion formation.

The objectives of our study were twofold. The primary objective was to determine if either ulcerated or healthy

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abomasal tissue harbors *H. pylori*. The 2nd objective was to determine if a specific species of bacterium or fungi was related to "fatal" abomasal ulcer formation in unweaned beef calves, with special attention being given to *Clostridium perfringens* type A, *H. pylori*, and *Campylobacter* spp.

Materials and methods

Sample collection and testing

Abomasal tissues were collected from 30 unweaned beef calves submitted to the diagnostic pathology laboratory of the Western College of Veterinary Medicine in spring 1993. These calves originated from beef cow-calf operations located within a 50 km radius of Saskatoon and were considered to be representative of beef calves reared in western Canada. Calves dying of either a perforating or a lethal hemorrhagic abomasal ulcer were designated as cases. Each case was matched with 1 and, if possible, 2 controls. The control calves had to be beef calves less than 6 mo of age, with the primary cause of death being unrelated to the abomasum and having no history of oral antibiotic therapy.

When a case was identified, the entire ulcer and a 3 to 4 cm border of adjacent tissue were excised en bloc. Within 1 h, half of the tissue was fixed in 10% neutral buffered formalin, while the other half was submitted directly for microbiological testing. In addition, the size and location of the lesion was recorded, so that a similar sample of tissue could be harvested from the matched control(s). Two to 4 representative samples of tissue were obtained from each block of fixed tissue, embedded in paraffin, serially sectioned at 6 μm, and orientated onto 2 slides. One series of slides was stained with hematoxylin and eosin (H & E) and the other with Warthin–Faulkner's (W–F) silver stain. Generally, an H & E stain is adequate for identifying *H. pylori* in all but the most lightly colonized tissues. In these cases, the silver stains are more appropriate, since they tend to enlarge the appearance of the *H. pylori*, thereby increasing the rate of detection (22,23).

A direct wet mount was made for each tissue and examined for fungal hyphae; if the wet mount was positive for hyphae, the tissue was swabbed and inoculated onto Sabouraud's medium for culturing. Two impression smears were taken from each tissue and stained with Gram's and Victoria blue (24) stains. Results from the Gram's stain were reported for both gram-positive and gram-negative rods and cocci on a scale of 0 to 4+. Smears stained with Victoria blue were specifically examined for the presence of *H. pylori* and Campylobacter-like organisms (CLOs), with the results being reported as positive or negative. Fluorescent antibody tests were performed using fluorescent antibody conjugates (Coopers Animal Health, Kansas City, Missouri, USA) for *C. chauvoei*, *C. novyi*, and *C. septicum* (25).

Tissues were processed in a routine manner for the isolation of aerobic bacteria. Isolation procedures for *H. pylori* involved inoculating the tissue onto a modified chocolate (blood product)-based medium (GC Medium, Becton Dickinson, Cockeysville, Maryland, USA). The inclusion of phenol red, urea, and sodium hydroxide allowed us to monitor urease production, a biochemical reaction characteristic of *H. pylori* and most other *Helicobacter* spp. (26). The cultures were incubated at 36°C in a microaerophilic environment (5% 0_2, 10% CO_2, 85% N_2) for 7 d, and checked twice daily for urease activity and colony formation. A reference strain of *H. pylori* (American Type Culture Collection #43504, Rockville, Maryland, USA) served as a control.

An anaerobic culture system (Gas Pak 100 Anaerobic System, Becton Dickinson) was used for the isolation of *C. perfringens* type A. Cultures tentatively identified as *C. perfringens* were then inoculated into a cooked meat medium and stored under anaerobic conditions for further testing. The litmus milk test (acid, peptonization, reduced, clot, gas [APRCG] reaction), Nagler's reaction, and biochemical profiling (API 20 A System, Analytab Products Incorporated, bio Merieux Canada, Quebec) were used to confirm that the cultures were *C. perfringens*. Typing of the *C. perfringens* was completed using the mouse serum neutralization procedure (27).

Data analysis

Eight genera of bacteria were cultured and identified; however, only the 4 most common isolates were analyzed separately. These isolates were *C. perfringens* type A, nonhemolytic *Escherichia coli*, *Campylobacter jejuni*, and *Streptococcus* spp. A 5th bacterial category, "other," represented the remaining isolates (*Campylobacter* spp., *Proteus* spp., *Lactobacillus* spp., *Enterobacter* spp., *Bacillus* spp.) and were treated as 1 bacterium.

The culture and Gram's staining scores for the 5 bacterial categories were cross-tabulated and transformed into a dichotomous variable. Scores of 0 (no growth), 1, and 2 were designated as "light" growth, while scores of 3 and 4 represented "heavy" growth. A chi-square test of independence (28) analyzed for differences in "light" and "heavy" bacterial growth between cases and controls, for each of the 5 bacterial categories. To test for differences in overall bacterial growth between the cases and controls, all the dichotomous bacterial results were grouped together and analyzed using the chi-square test of independence.

The Gram's stain scores were cross-tabulated, dichotomized, and analyzed as per the culture results.

All data were entered and cross-tabulated in a spreadsheet (Quattro Pro, version 5.0, Borland International, Scotts Valley, California, USA) and analyzed in Statistix, version 4.0, (Analytical Software, St. Paul, Minnesota, USA).

Results

Abomasal tissue was harvested from a total of 30 calves, 14 cases and 16 controls. Of the 14 fatal ulcers, 12 died of a singular perforating ulcer, while 2 calves died of multiple bleeding ulcers. Two controls were to have been chosen for each case; however, only 16 calves satisfied the criteria needed for a control. Of these 16 controls, 10 died of enteritis and 4 of navel ill, and 2 were intestinal accidents. All 30 calves were less than 3 mo of age, with 28 being less than 2 mo old.

Microbiology results

Table 1 shows the number and percent of bacterial isolates recovered from the cases and controls. There were no statistically significant ($P > 0.05$) differences in the

Table 1. Number (percent) of bacterial isolates obtained from the abomasum of calves dying of ulcers (cases) versus all other diseases (controls)

	Clostridium perfringens type A	*Campylobacter jejuni*	*Escherichia coli*	*Streptococcus* spp.	Other[a]
Cases (n = 14)	11 (78.6)	3 (21.4)	12 (85.7)	4 (28.6)	6 (42.9)
Controls (n = 16)	12 (75.0)	3 (18.8)	13 (81.3)	6 (37.5)	8 (50.0)

[a]All other isolates combined

bacterial culture scores, or the staining scores, between the cases and controls.

No *H. pylori* was cultured and all culture plates tested negative for urease activity. The Gram's and Victoria blue staining techniques were also negative for *Helicobacter* organisms. A "light" growth of *C. jejuni* was isolated from 3 cases and 3 controls.

Clostridium perfringens was isolated from 11 (78.6%) cases and 12 (75.0%) controls. Typing by mouse inoculation identified 6 of the 23 bacterial cultures as toxigenic *C. perfringens* type A; 4 from calves with ulcers and 2 from control calves. The remaining 17 were classified as nontoxigenic *C. perfringens* type A (29). A heavy growth of *C. perfringens* type A was obtained from 6 ulcers and only 2 controls, although this was not statistically significant ($P = 0.061$). Only 2 calves tested positive to the clostridial fluorescent antibody tests (FAT). A calf dying of a hemorrhagic ulcer tested positive for *C. septicum* and *C. chauvoei*, while a calf dying of necrotic enteritis (control) tested positive for *C. septicum*. All other fluorescent antibody tests were negative.

Two genera of fungi, *Mucor* spp. and *Rhizopus* spp., were isolated from 2 cases and 3 controls.

Histology results

No *Helicobacter* spp. were noted on any of the histological sections; however, CLOs were occasionally visualized. These CLOs were deemed to be morphologically distinct from *Helicobacter* spp. In addition, none of the sections showed evidence of a typical *Helicobacter* spp.-induced chronic gastritis, characterized by focal or diffuse inflammatory infiltrates (30,31).

Discussion

Although "no *H. pylori* — no ulcer" (31) may become the new dictum for chronic (recurrent) duodenal ulcers in man, it is unlikely to apply to fatal ulcer formation in beef calves. A combination of histological and microbiological testing procedures failed to detect the presence of *H. pylori* in any of the abomasal tissue examined. We believe that these negative findings can probably be extended to include all *Helicobacter* spp., since no Helicobacter-like bacteria were visualized and the culture methods we employed were similar to those used in the isolation of other *Helicobacter* spp. (23,26,30).

While *Helicobacter* spp. have been recovered from a wide variety of hosts, the lack of an association between this bacterium and fatal abomasal ulcers was not unexpected. Many *Helicobacter* spp. cause a chronic gastritis (8,12), and in humans, this gastritis either precedes or exists concurrently with chronic gastroduodenal ulcers (31). This pathology is very different from the fatal perforating and bleeding ulcers found in beef calves. Furthermore, even in humans, where *H. pylori* is a recognized cause of chronic duodenal ulceration, no association has been found between this bacterium and perforating duodenal ulcers (32). This finding, coupled with the lesions described in other host species, suggests that *Helicobacter* spp. cause gastritis and chronic ulceration, and not perforations. Therefore, *Helicobacter* spp. are likely not involved in the pathogenesis of perforating abomasal ulcers of unweaned beef calves. If a bacterial agent is responsible for fatal abomasal ulcers, previous research suggests that *C. perfringens* type A is a more likely candidate.

Historically, *C. perfringens* type A or its toxins have been recovered from a wide variety of cattle diseases; however, a causal relationship has not always been demonstrated (33). More recently, researchers did induce abomasal ulcers in young calves by way of an intraruminal inoculation of a 20-hour culture of *C. perfringens* type A (18). These calves developed various degrees of depression, diarrhea, abdominal bloat, abomasitis, and abomasal ulceration. However, the ulcers were frequently multiple, diffuse, never perforating, and associated with ecchymotic and petechial hemorrhage and mucosal edema. These ulcerative lesions are very different from the perforating ulcers typically encountered in beef calves, where the lesions are singular and localized to a discrete region of the abomasum (34). The diffuse pattern of lesion formation produced by experimental inoculation (18) suggests that the bacteria did not colonize or penetrate the tissue, but rather that preformed toxins contained within the broth may have caused widespread nonspecific cellular damage. Therefore, although this work provides evidence that *C. perfringens* type A or its toxins may be capable of producing abomasitis, nonperforating abomasal ulcers, and abdominal tympany, it is debatable whether this bacterium is capable of producing a focal perforation, either experimentally or under natural conditions.

The fact that *C. perfringens* type A was frequently isolated from both cases and controls suggests that this bacterium can be found in the abomasum of most dead calves. Whether these frequent isolations are due to antemortem colonization or postmortem invasion is uncertain; however, previous research suggests that the latter is more likely (35). To partially correct for this contamination, the culture results were transformed into a

dichotomous outcome, "light" and "heavy" growth. Presumably, if *C. perfringens* type A was involved in ulcer formation, "heavy" growth would more likely be associated with the site of the lesion, whereas contamination would generally result in a lighter growth of bacteria. We found that heavy growth of *C. perfringens* type A was produced in only 6 of the 14 fatal ulcers, suggesting to us that *C. perfringens* type A is likely not a necessary factor in the formation of fatal ulcers. Rather, it is more likely that the bacterium is a common postmortem contaminant, and that it probably thrives in the devitalized ulcerated tissue.

The lack of histological or microbiological evidence to suggest that a specific bacterium or fungus was necessary for fatal ulcer formation leads us to believe that any role that these agents have in ulcer formation is indirect. Further research into the etiology and pathogenesis of fatal abomasal ulcers is required.

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