Potential role of *Clostridium difficile* as a cause of duodenitis-proximal jejunitis in horses

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Duodenitis-proximal jejunitis (DPJ) is an idiopathic condition in the horse characterized by inflammation and oedema of the duodenum and proximal jejunum. Clinical signs include colic, ileus, depression, fluid accumulation in the small intestine and stomach, and endotoxaemia. The objective of this study was to investigate prospectively the role of *Clostridium difficile* in this idiopathic disease. Nasogastric reflux from 10 consecutive cases with DPJ and 16 consecutive horses with other causes of nasogastric reflux was cultured for *C. difficile*, other *Clostridium* spp., and *Salmonella*. Toxigenic strains of *C. difficile* were isolated from 10/10 (100%) of horses with DPJ and 1/16 controls ($P < 0.0001$). No other known pathogenic clostridia were isolated from either group. Results of this study suggest that *C. difficile* might be an important cause of this syndrome.

INTRODUCTION

Duodenitis-proximal jejunitis (DPJ) is an acute sporadic disease in horses, characterized by inflammation and oedema of the duodenum and proximal jejunum. Clinical signs include colic, depression, fluid accumulation in the small intestine and stomach, ileus, and endotoxaemia (Blackwell & White, 1982; Freeman, 2000). In addition, complications such as cardiac arrhythmias, hepatic injury and laminitis have been reported (Cohen *et al.*, 1994; Cornick & Seahorn, 1990; Davis *et al.*, 2003).

The initiating cause of this disease remains unknown; however, clostridia, *Salmonella* spp. and mycotoxins have been suggested elsewhere (Freeman, 2000; Griffiths *et al.*, 1997; Schumacher *et al.*, 1995). *Salmonella* spp. have been sporadically isolated from the reflux and faeces of horses with clinical signs of DPJ; however, this has been inconsistent and histopathological lesions observed in the small intestine of such cases do not closely resemble those described in salmonellosis (Tyler *et al.*, 1985). Mycotoxins fail to produce clinical disease in experimentally challenged horses, but interestingly it has been reported that the histopathological lesions are similar to those described in DPJ cases (Schumacher *et al.*, 1995). In earlier reports, a clostridial cause was suspected, because a large number of Gram-positive rods were observed on Gram stains of nasogastric reflux samples (White *et al.*, 1987). Additionally, histopathological lesions seen in affected horses are similar to those produced by *Clostridium* spp. exotoxins (Tyler *et al.*, 1985). However, specific clostridial species cannot

be consistently and repeatedly isolated from affected horses (White *et al.*, 1987; Griffiths *et al.*, 1997).

Clostridium difficile is an important cause of colitis in horses (Jones, 2000; Baverud, 2002); however, its role in DPJ has not been evaluated. Jones *et al.* (1988) have described severe lesions to the small intestine mucosae of foals naturally infected or following experimental oral challenge with *C. difficile*. Recently, Braun and co-workers have isolated *C. difficile* strains from horses and ponies affected with various small-intestinal disorders (Braun *et al.*, 2000). In humans, small intestinal enteritis with *C. difficile* is not commonly recognized but does occur (Freiler *et al.*, 2001; Elinav *et al.*, 2004). These findings have led to renewed interest in the role of *C. difficile* as a potential cause of DPJ in horses. The objective of this study was to further investigate the hypothesis that clostridia, particularly *C. difficile*, are involved in the pathogenesis of DPJ.

METHODS

Nasogastric reflux samples. Gastric fluid was collected via nasogastric tube from 10 consecutive clinical cases of DPJ and 16 consecutive horses with other causes of nasogastric reflux admitted to the Ontario Veterinary College-Veterinary Teaching Hospital between January 2004 and August 2005. Horses were diagnosed with DPJ via clinical signs (Freeman, 2000). Control horses were confirmed not to have DPJ via exploratory laparotomy or clinical signs. Samples were collected at the time of admission or within 12 h of hospitalization. All samples were handled in a blinded manner, stored at 4 °C and processed within 24 h of collection.

Culture conditions for *Clostridium* spp. One to two millilitres of reflux was inoculated into 8 ml non-selective enrichment broth (Brain heart infusion; Oxoid), incubated at 37 °C for 10–14 days, and subjected to subculture onto blood agar plates. Plates were

Abbreviation: DPJ, duodenitis-proximal jejunitis.

incubated under anaerobic conditions at 37 °C for 48 h. Clostridia were identified via colony and Gram-stain morphology, and also using a biochemical system (API strip test; bioMérieux Canada).

Selective enrichment culture for *C. difficile* was performed by the inoculation of 1–2 ml of reflux into cycloserine-cefoxitin fructose broth enriched with 0.1% sodium taurocholate, and incubated at 37 °C for 10–14 days. After incubation, 2 ml was transferred into a sterile test tube, mixed with an equal amount of absolute ethanol and left at room temperature for 60 min. Samples were then centrifuged at 4400 g for 10 min, the supernatant was discarded, and the resulting pellet was plated onto blood agar and incubated anaerobically at 37 °C for 48–72 h. *C. difficile* was identified via colony characteristics, Gram-stain morphology and production of L-proline aminopeptidase (ProDisc test; Remel, Carr-Scarborough Microbiologicals).

Salmonella culture conditions. One millilitre of reflux was inoculated into 9 ml buffered peptone water and incubated at 35 °C for 24 h, from which 1 ml was transferred into 9 ml Mueller–Kaufman tetrathionate broth and incubated under the same conditions. A loopful from the enrichment broth was plated onto XLT agar (Oxoid) and incubated as above for up to 48 h. Colonies suspected to be *Salmonella* were tested using the BBL Enterotube II, as per the manufacturer's instructions (BD Canada).

DNA extraction. DNA was extracted from *C. difficile* isolates by using a Chelex resin-based DNA extraction kit (InstaGene Matrix; Bio-Rad), as per the manufacturer's instructions.

PCR ribotyping and screening for *C. difficile* genes encoding toxins A and B, and binary toxin. *C. difficile* isolates were typed by using a PCR ribotyping method, as described elsewhere (Bidet *et al.*, 1999). Isolates were tested for toxigenicity by PCR gene detection of toxin A (*cdtA*) and toxin B (*cdtB*), as well as via testing for the presence of binary toxin (CDT) by amplification of part of the gene encoding the receptor-binding component of this toxin (*cdtB*), as described elsewhere (Kato *et al.*, 1998; Stubbs *et al.*, 2000).

Statistical analysis. Categorical analyses were performed using Fisher's exact test. A *P* value of <0.05 was considered significant.

RESULTS

Toxigenic strains of *C. difficile* were isolated from the nasogastric reflux of 10/10 (100%) horses with DPJ, but from only 1/16 of the controls (*P* < 0.0001). Seven ribotypes were identified among the 10 isolates, and these ribotypes were indistinguishable from types isolated from horses with *C. difficile*-associated enterocolitis at this teaching hospital (data not shown). Eight of 10 (80%) of the isolates possessed genes encoding the production of both main toxins, toxins A and B, while two were variant strains that produced toxin B but not toxin A. Additionally, genes encoding binary toxin (CDT) were present in one isolate that also carried the genes encoding toxins A and B. Three (30%) horses with DPJ were euthanized, while the remaining seven cases recovered fully. Three of the 10 horses with DPJ developed diarrhoea during hospitalization and *C. difficile* was recovered from their faeces. *C. difficile* toxins were detected in faeces via ELISA in one case, but the other two cases were not tested. One horse required surgical intervention, but the rest responded to medical treatment. All cases that were euthanized were colonized by a variant (A^-B^+ and $A^+B^+CDT^+$) strain of *C. difficile* (*P* = 0.008).

Clostridium perfringens was isolated from 4/10 DPJ cases and 12/16 controls (*P* = 0.11). *Salmonella* spp. were isolated from the reflux of one horse in the control group but not from horses with DPJ (*P* = 1.0).

DISCUSSION

The strong association of the presence of toxigenic strains of *C. difficile* in gastric reflux from horses with DPJ is an important finding that suggests an aetiology for this previously idiopathic disease. While DPJ is a condition of the small intestine, it is reasonable to assume that gastric fluid analysis can be useful because of the tendency for excessive fluid production and decreased aboral intestinal motility to result in reflux of small intestinal fluid into the stomach. The low prevalence of *C. difficile* in gastric fluid from horses with other conditions that result in the flow of small intestinal fluid into the stomach (i.e. small intestinal obstructions) suggests that isolation of *C. difficile* from gastric fluid of horses with DPJ does not simply represent backflow of commensal *C. difficile* from the small intestine.

Ileus and fluid accumulation in the small intestine and stomach lumen, prominent characteristics of DPJ, have also been reported in some humans and in laboratory animals with *C. difficile* infection (Perkins *et al.*, 1995; Siemann *et al.*, 2000; Freiler *et al.*, 2001; Elinav *et al.*, 2004). *In vitro* experiments have shown that *C. difficile* toxin B causes electromechanical disturbances to the small intestine smooth muscle (Gilbert *et al.*, 1989). These observations suggest that *C. difficile* toxins may play an important role in the gastrointestinal motility of humans or animals suffering from *C. difficile*-associated enterocolitis. These factors support the implication of *C. difficile* in DPJ.

The timing of sample collection, the diverse geographical origin of the cases and molecular type diversity of the isolates may suggest that the strains were of community origin. However, this observation requires further molecular epidemiological studies. Contrary to the common situation in humans, in which most of the cases are nosocomial in origin (Barbut & Petit, 2001; Riley, 2004), most *C. difficile*-associated infection in horses appears to be community associated (Baverud, 2004). The reasons for development of DPJ versus colitis are unclear and require further investigation; however, the fact that isolates recovered from horses with DPJ were comparable to isolates commonly identified in horses with colitis suggest that specific *C. difficile* DPJ strains are not the reason.

The proportion of toxin variants was surprising, particularly the strains possessing genes for toxin B but not toxin A (A^-B^+). These variant strains are not commonly identified in humans (Brazier *et al.*, 1999), but may be more common in some animals (Rodriguez *et al.*, 2005). One *C. difficile* isolate carried the gene encoding binary toxin and was recovered from a horse with severe clinical signs of DPJ. The clinical significance of the binary toxin (CDT) in humans and animals remains currently unknown, but it is

expected that this toxin constitutes an additional virulence factor (Rupnik *et al.*, 2003; Geric *et al.*, 2004; Barbut *et al.*, 2005).

The isolation of *C. perfringens* from DPJ cases and control horses is in agreement with previous reports (Griffiths *et al.*, 1997), and does not indicate an association between *C. perfringens* and DPJ. Similarly, there was no evidence that *Salmonella* was associated with DPJ, as it was not isolated from the reflux of any DPJ cases, which is in agreement with reports elsewhere (White *et al.*, 1987; Griffiths *et al.*, 1997). In our study, cultures for *Salmonella* were performed on a single reflux sample; therefore, intermittent shedding could not be discounted. However, the strong association of *C. difficile* with DPJ and the results of other studies evaluating *Salmonella* in DPJ make it unlikely that *Salmonella* is an important cause of this disease.

The results of the present study suggest that *C. difficile* has a role as a cause of DPJ in horses. The isolation of *C. difficile* from the reflux of horses with DPJ does not prove causation, but indicates that further investigation of the potential role of this pathogen by means of a larger prospective study is indicated. Further, associated risk factors that ultimately may favour bacterial growth and toxin production also need to be determined. Recently, Cohen and co-workers have found that horses kept on pasture or fed high amounts of concentrate diets appear to be at higher risk of developing this disease (Cohen *et al.*, 2005). This presumably could be associated with disruption of the intestinal microflora, thereby facilitating colonization and overgrowth of *C. difficile*. The reasons for development of DPJ versus colitis also require further study.

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