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Prevalence of β2-Toxigenic *Clostridium perfringens* in Horses with Intestinal Disorders

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The incidence of a new, yet unassigned toxin type of *Clostridium perfringens* containing the genes for the α-toxin and the recently described β2-toxin in horses with intestinal disorders is reported. The study included 18 horses suffering from typical typhlocolitis, 7 horses with atypical typhlocolitis, 16 horses with other intestinal disorders, and 58 horses without intestinal disease. In total, 20 samples of ingesta of the small and large intestines, five biopsy specimens of the intestinal wall, and 74 fecal samples were analyzed bacteriologically. *C. perfringens* isolates were typed for the presence of the α-, β-, β2-, and ε-toxin and enterotoxin genes by PCR, including a newly developed PCR for the detection of the β2-toxin gene *cpb2*. β2-Toxigenic *C. perfringens* was detected in samples from 13 of 25 (52%) horses with typical or atypical typhlocolitis, with a particularly high incidence in specimens of ingesta and biopsy specimens (75%), whereas only 6 of 16 specimens from horses with other intestinal diseases yielded β2-toxigenic *C. perfringens*. No β2-toxigenic *C. perfringens* was found in the samples from the 58 control horses, of which only one fecal sample contained *C. perfringens* type A. Among the samples from the 15 horses with fatal cases of typical and atypical typhlocolitis 9 (60%) were positive for β2-toxigenic *C. perfringens*, whereas samples from only 4 of the 10 (40%) animals with nonfatal cases of infection were positive. We found an interesting correlation between the antibiotic-treated horses which were positive for β2-toxigenic *C. perfringens* and lethal progression of the disease. No *C. perfringens* strains isolated in this study contained genes for the β- and ε-toxins and enterotoxin. The high incidence of β2-toxigenic *C. perfringens* in samples of ingesta, biopsy specimens of the intestinal wall, and feces from horses suffering or dying from typhlocolitis together with the absence of this organism in healthy horses provides strong evidence that β2-toxigenic *C. perfringens* play an important role in the pathogenesis of typhlocolitis.

Different *Clostridium* species are frequently isolated from horses with typhlocolitis (14, 17) and are generally suspected to be involved in the disease (1, 5, 13, 15). However, it is not clear which *Clostridium* species and, in particular, which subtypes or toxinotypes are the main cause of typhlocolitis and other toxic intestinal diseases in horses. While *Clostridium difficile* was reported to play an important role in the disease (1), other reports showed a high rate of isolation of *Clostridium perfringens* from horses with intestinal diseases (2). *C. perfringens*, which causes various diseases in different animals, is differentiated into five subtypes (subtypes A to E) according to the major toxins produced by each subtype (11). Type A is subdivided further into enterotoxigenic and nonenterotoxigenic strains (16). It has been reported that acute colic signs and hemorrhagic gastroenterocolitis could be induced experimentally in Shetland ponies after intravenous injection of enterotoxin obtained from enterotoxigenic *C. perfringens* type A (12). However, other studies showed that enterotoxigenic *C. perfringens* isolates do not play a role in intestinal disorders of horses (2).

In addition to the *C. perfringens* types mentioned above, an unassigned type of *C. perfringens* that produces α-toxin and the newly discovered β2-toxin was recently described (9). It was isolated from piglets with necrotic enterocolitis and was also found in horses with enterocolitis (9). Since the α-toxin, which is produced by all types of *C. perfringens* including nonpathogenic type A strains, is not considered a primary cause of digestive lesions (10), it was suggested that the β2-toxin, which is present in this new type of *C. perfringens*, plays a role in causing the digestive diseases (9).

This report describes the incidence of this novel β2-toxigenic *C. perfringens* type in horses with typical symptoms of typhlocolitis, horses with atypical typhlocolitis, and horses suffering from other intestinal disorders. The results obtained with fecal samples, specimens of ingesta from the small and large intestines, as well as biopsy specimens of the intestinal walls of diseased and control horses were compared. All samples were also analyzed for the occurrence of *C. difficile*, since its role as a nosocomial pathogen in horses with typhlocolitis, especially in association with antibiotics, has been described (1, 3, 5, 13).

MATERIALS AND METHODS

Animals. Ninety-nine horses of different breeds, ages, and sexes were included in the study and were subdivided into four groups. Group I contained 18 horses suffering from typical typhlocolitis symptoms and presenting with all four cardinal symptoms of the disease: hemorrhagic, profuse, watery diarrhea, subfebrile body temperature (38 to 38.5°C), severe leukopenia with less than 3×10^9 cells/liter due to neutropenia, and hypoproteinemia (less than 5×10^9 cells/liter). Of the 18 horses, 10 developed typhlocolitis during hospitalization between 2 and 5 days after surgery for colic, 5 suffered from typhlocolitis but were hospitalized for other reasons, and 3 with typhlocolitis were referred to the animal hospital from their home stable. Ten of the horses with typhlocolitis were treated with antibiotics (gentamicin and penicillin) in combination with the nonsteroidal anti-inflammatory drug (NSAID) flunixinum (Finadyne; Biokema, Crissier, Switzerland) 2 to 5 days prior to the acute onset of typhlocolitis and 7 were treated with the NSAID only. All horses developed a significant leukocyte drop due to

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TABLE 1. Specific oligonucleotide primers for PCR amplification of the genes for *C. perfringens* toxins α, β, β2, and ε and enterotoxin

Toxin/gene	Primer	Oligonucleotide sequence	Fragment length (bp)	Annealing temp (°C)	Ramping time (s)[a]	Reference or source
α/*cpa*	CPALPHATOX1-L	5′-AAG ATT TGT AAG GCG CTT-3′	1,167	46	60	3
	CPALPHATOX1-R	5′-ATT TCC TGA AAT CCA CTC-3′				
β-*cpb*	CPBETATOX1-L	5′-AGG AGG TTT TTT TAT GAA G-3′	1,025	39	90	3
	CPBETATOX1-R	5′-TCT AAA TAG CTG TTA CTT TGT G-3′				
β2/*cpb2*	P319BETA2	5′-GAA AGG TAA TGG AGA ATT ATC TTA ATG C-3′	573	48		This work
	P320BETA2	5′-GCA GAA TCA GGA TTT TGA CCA TAT ACC-3′				
ε/*etx*	CPETOXIN1-L	5′-AAG TTT AGC AAT CGC ATC-3′	961	46	60	3
	CPETOXIN1-R	5′-TAT TCC TGG TGC CTT AAT-3′				
Enterotoxin/*cpe*	CPENT1-L	5′-TAACAATTTAAATCCAATGG-3′	933	46	60	3
	CPENT1-R	5′-ATTGAATAAGGGTAATTTCC-3′				

[a] Ramping time from the annealing temperature to an extension temperature of 72°C.

neutropenia to less than 3×10^9 cells/liter prior to the onset of profuse, watery diarrhea. Apart from the four cardinal symptoms, the horses suffered from an important hypovolemic iliocolitis syndrome and anorexia. Group II contained seven horses suffering from atypical typhlocolitis symptoms but not showing all four symptoms characteristic of typical typhlocolitis. Five of those seven horses developed fever (40 to 41°C) and hemorrhagic or profuse, watery diarrhea. Two horses showed profuse, watery diarrhea, hypoproteinemia, and subfebrile body temperatures, but the typical, severe leukocyte drop prior to the onset of diarrhea was not present. All horses in group II were referred to the clinic with diarrhea as the primary concern. Prior to the onset of diarrhea four horses were treated with the antibiotics gentamicin and penicillin in combination with the NSAID, and three were treated with the NSAID only. Group III contained 16 horses suffering from intestinal disorders other than typhlocolitis. Of those, six untreated horses were referred to the clinic with a history of chronic indigestion. Three horses suffered from gastroduodenojejunitis and had been treated with the NSAID during hospitalization. Seven horses had surgery for colic (four large-colon and one cecal torsion and two renosplenic entrapments) and were treated with gentamicin and penicillin in combination with the NSAID prior to and 3 days after laparotomy. None of these 16 horses developed acute, watery diarrhea or a leukocyte drop to less than 3×10^9 cells/liter. Group IV contained 58 control horses without intestinal disease. Of these horses, 35 were hospitalized or euthanized in the clinic for reasons other than intestinal disorders, and 23 were apparently healthy competition horses from a private riding stable. None of these horses had received any medication in at least the 3 weeks prior to the study.

Sampling. Seventy-four fecal samples from 21 horses with intestinal disorders and from 53 control horses were taken from the rectum and packed in plastic bags, and excess air was eliminated for transport of the samples to the bacteriology laboratory. Twenty-five specimens were taken from ingesta of the small and/or large intestine or biopsy specimens of the intestinal wall during laparotomy or immediately after euthanasia from the proximal jejunum, pelvic flexure of the large colon, and/or apex of the cecum from 20 horses with intestinal diseases and from 5 horses that were euthanized. Specimens of ingesta were cultivated within 12 h. Biopsy samples were frozen in liquid nitrogen until analysis. Each of the 99 horses provided just one sample, whether it was feces, ingesta, or a biopsy specimen.

Bacteriological examinations. The samples were cultured on blood agar plates (containing 5% sheep blood) and on *C. difficile*-selective agar (no. 43213; bioMérieux, Marcy l'Etoile, France) and were incubated at 37°C under anaerobic conditions. In addition, tetrathionate broth enrichment with subsequent subculture on *Salmonella-Shigella* agar and brilliant green agar was performed to attempt isolation of *Salmonella* spp.

The following *C. perfringens* reference strains were used in this study as controls for the different toxin genes: strain NCTC 10239 for α-toxin gene *cpa* and the enterotoxin gene *cpe*, strain ATCC 3626 for *cpa* and β-toxin gene *cpb*, and strain NCTC 8346 for *cpa* and ε-toxin gene *etx*, as described previously (4, 8). As a control for the β2-toxin gene *cpb2* we used our β2-toxigenic internal reference strain *C. perfringens* E 482/97, isolated from a horse, and strain *C. perfringens* S 1040/95, isolated from a piglet that died of necrotizing enteritis. Both strains contain the α- and β2-toxin genes and are devoid of the genes for the other major *C. perfringens* toxins, the β- and ε-toxins and enterotoxin. As a control for *C. difficile* toxin A and toxin B, strain ATCC 43255 was used.

Preparation of bacterial specimens and PCR amplifications. Template DNA from bacterial cultures was obtained by a direct lysis method. About 10 colonies were taken from primary cultures and were suspended in 450 μl of lysis buffer (0.1 M Tris-HCl [pH 8.5], 0.05% Tween 20, 0.24 mg of proteinase K [Boehringer Mannheim, Mannheim, Germany] per ml). Two to three colonies were taken from pure cultures and suspended in 450 μl of lysis buffer. The samples were incubated at 60°C for 1 h and then heated at 97°C for 15 min in order to inactivate the proteinase K.

The specific oligonucleotide primers for PCR amplification of the toxin genes are listed in Table 1. PCR assays were performed with the DNA Thermal Cycler Gene Amp 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). The reactions were performed in 50-μl volumes containing 5.0 μl of lysate, 45.0 μl of *Taq* PCR mixture (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40 detergent, each deoxynucleoside triphosphate at a concentration of 170 μM, 0.25 μM each oligonucleotide primer, and 1.25 U of *Taq* DNA polymerase [Boehringer Mannheim]). The reactions were subjected to 35 cycles of amplification consisting of 30 s of denaturation at 94°C, 30 s for primer annealing at the respective temperature (Table 1), and 30 s of chain extension at 72°C. The ramping time used to raise the temperature from the annealing temperature to 72°C is given in Table 1. PCRs for the detection of the *C. perfringens* α-, β-, and ε-toxins and enterotoxin genes were described previously (4). For the detection of the β2-toxin gene *cpb2* by PCR, we have developed a pair of specific primers, P319BETA2 and P320BETA2 (Table 1), which match nucleotide positions 377 to 392 and 950 to 935, respectively, of the DNA sequence of *cbp2* in the GenBank/EMBL database accession no. L77965) (9). This primer pair amplified a specific 573-bp internal fragment of the *cpb2* gene from our β2-toxigenic internal reference strain *C. perfringens* E 482/97 and from strain S 1040/95. No fragment was amplified with primers P319BETA2 and P320BETA2 from the reference strains of *C. perfringens* type A, type B, type C, type D, or type E or from enterotoxigenic *C. perfringens* or from the type strains of the *Clostridium* species *C. difficile*, *C. chauvoei*, *C. septicum*, *C. sordelli*, *C. sporogenes*, *C. novii*, *C. haemolyticum*, *C. tetani*, and *C. histolyticum*, showing the specificity of this primer pair for the β2-toxin gene *cpb2* (8a). PCR identification of the toxin A and toxin B genes of *C. difficile* was done as described previously (18). PCR products were analyzed by applying 20 μl on a 0.7% agarose gel (agarose type II medium EEO; no. A-6877; Sigma, St. Louis, Mo.) for electrophoresis and were visualized with ethidium bromide and UV light.

RESULTS

PCR analysis of the reference strains for the different toxin types of *C. perfringens* showed the presence of the α-toxin gene *cpa* in all *C. perfringens* reference strains as well as in our β2-toxigenic internal reference strain *C. perfringens* E 482/97 and in strain S 1040/95. The ε-toxin gene *etx* was found in the reference strains of type B (ATCC 3626) and type D (NCTC 8346). The β-toxin gene *cpb* was found in the reference strain of type B (ATCC 3626). The enterotoxin gene *cpe* was found in enterotoxigenic type A strain NCTC 10239. The β2-toxin gene *cpb2* was detected by PCR in our β2-toxigenic internal reference strain *C. perfringens* E 482/97 and in strain S 1040/95. PCR analysis of *C. difficile* ATCC 43255 showed the presence of the two genes for toxins A and B. These results thus confirm the specificity of the analytical methods.

The results of bacteriological and PCR examinations are given in Tables 2 and 3. *C. perfringens* was isolated from 5 of 21 (24%) fecal samples from 21 horses with intestinal diseases. All these isolates proved to harbor the β2-toxin gene *cpb2* and the α-toxin gene *cpa* (Table 2). Feces from 4 of the 21 (19%) horses contained *C. difficile*, and 12 (57%) further horses were negative for both *C. perfringens* and *C. difficile*. All isolated *C.*

TABLE 2. Bacteriological results for equine fecal samples, samples of intestinal ingesta, or biopsy specimens of the intestinal wall (small intestine and large colon)

Sample(s) and Group	No. of animals			
	Animals infected with β2-toxigenic *C. perfringens*	Animals infected with *C. perfringens* type A	Animals infected with *C. difficile*	Animals without *C. perfringens* and *C. difficile* infection
Fecal samples				
I (n = 9)	2	0	3	4
II (n = 4)	2	0	1	1[a]
III (n = 8)	1	0	0	7
IV (n = 53)	0	1	0	52
Ingesta or biopsy samples				
I (n = 9[b])	6[c]	2	1[c]	1
II (n = 3)	3[c]	0	1[c]	0
III (n = 8)	5	1	2	0
IV (n = 5)	0	0	0	5

[a] One animal was infected with *Salmonella typhimurium*.
[b] Includes five biopsy samples.
[c] One animal was infected with both β2-toxigenic *C. perfringens* and *C. difficile*.

perfringens strains were devoid of the β- and ε-toxin and enterotoxin genes, as assessed by PCR. Examination of feces from the 53 control horses without intestinal disease showed that they contained no β2-toxigenic *C. perfringens* isolates and no *C. difficile* isolates. A *C. perfringens* type A strain without an enterotoxin gene was isolated from one of these control animals.

The results of bacteriological examinations of specimens of intestinal ingesta or biopsy specimens of the intestinal wall (small and large intestine) revealed that of 20 specimens from 20 different horses with intestinal disease, 14 specimens proved to be positive for β2-toxigenic *C. perfringens* (Table 2). One horse with typical typhlocolitis which was positive for β2-toxigenic *C. perfringens* also contained nontoxigenic *C. difficile*. Both β2-toxigenic *C. perfringens* and toxigenic *C. difficile* (containing the genes for toxins A and B) were isolated from one horse with atypical typhlocolitis, and one horse was negative for *C. perfringens* and *C. difficile*. *C. difficile* alone was isolated from two of eight horses with intestinal diseases other than typhlocolitis. All specimens of ingesta from the five horses from the control group (group IV) were bacteriologically negative for *C. perfringens* and *C. difficile*.

In total, β2-toxigenic *C. perfringens* was isolated from samples from 13 of 25 (52%) horses with typical or atypical typhlocolitis (Table 3). Samples from two horses from this group contained *C. perfringens* type A and six contained *C. difficile*; of the latter, two were found to contain *C. difficile* in combination with β2-toxigenic *C. perfringens*. In horses with other intestinal diseases, the incidence of β2-toxigenic *C. perfringens* was 6 of 16 (37%) animals. *C. perfringens* type A was isolated from the specimen from one animal in this group, and *C. difficile* was isolated from two samples. β2-Toxigenic *C. perfringens* was not isolated from any of the 58 horses in the control group, and *C. perfringens* type A was found only once. All *C. perfringens* strains analyzed were devoid of the β- and ε-toxin and enterotoxin genes.

Among the animals in group I (typical typhlocolitis), 8 of 18 horses died or were subjected to euthanasia due to severe shock or laminitis. Of these, 4 were positive for β2-toxigenic *C. perfringens* and had been medicated with an NSAID and antibiotics (gentamicin and penicillin). All horses in group II (atypical typhlocolitis) died or were euthanized (n = 7); five of them were positive for β2-toxigenic *C. perfringens*. Four of these five animals were treated with an NSAID and antibiotics.

Taken together, from a total of 25 horses with typical and atypical typhlocolitis, 14 were treated with antibiotics and 9 harbored β2-toxigenic *C. perfringens*. Of these animals all eight horses which had received antibiotic treatment and yielded β2-toxigenic *C. perfringens* died. Among the animals in group III (n = 16), 10 horses were euthanized, and four of these were positive for β2-toxigenic *C. perfringens* and received antibiotic and anti-inflammatory therapy (gentamicin, penicillin, and NSAID).

DISCUSSION

Bacteriological examination of feces, specimens of intestinal ingesta, or biopsy specimens of the intestinal wall from horses with intestinal disorders and subsequent toxin gene typing of the isolated *Clostridium* spp. by PCR showed a high incidence of a new, yet unassigned type of *C. perfringens* containing the α-toxin gene *cpa* and the β2-toxin gene *cpb2*. Since the α-toxin is produced by all *C. perfringens* isolates and does not seem to play a major role in enteric diseases (10), we attribute the pathogenicities of these *C. perfringens* isolates to the β2-toxin. This newly characterized β2-toxigenic *C. perfringens* is different from *C. perfringens* type C (which contains the β-toxin), which is involved in necrotizing enteritis of pigs and which was also recently reported in horses with enterocolitis (7). *C. perfringens* type C was not found in this study. We found β2-toxigenic *C. perfringens* mainly in horses with typical and atypical typhlocolitis, representing 52% of the isolates. To a lesser extent they were also isolated from horses with other intestinal disorders, in which they represented 37% of the isolates. No β2-toxigenic *C. perfringens* was found in healthy horses or in horses that were hospitalized for reasons other than intestinal problems. *C. perfringens* type A was found only incidentally. No enterotoxigenic *C. perfringens* was found in this study, confirming the observations of Beckmann et al. (2), who showed that enterotoxigenic *C. perfringens* does not play a role in intestinal disorders of horses. It is important that, apart from β2-toxigenic *C. perfringens*, only a few *C. perfringens* type A isolates, which are assumed to be of low significance for enteric disorders, were found. *C. difficile*, which was also reported to be associated with typhlocolitis (13), was found in some horses, often together with β2-toxigenic *C. perfringens*.

TABLE 3. Results of bacteriological examinations of all equine specimens

Group	No. of animals			
	Animals infected with β2-toxigenic *C. perfringens*[a]	Animals infected with *C. perfringens* type A	Animals infected with *C. difficile*	Animals without *C. perfringens* and *C. difficile* infection
I (n = 18)[a]	8[b]	2	4[b]	5
II (n = 7)[a]	5[b]	0	2[b]	1[c]
III (n = 16)[a]	6	1	2	7
IV (n = 58)	0	1	0	57

[a] Results for all groups with clinical disease are significantly different from those for the controls ($P < 0.001$ by χ^2 test with Yates correction).
[b] One animal infected with both β2-toxigenic *C. perfringens* and *C. difficile*.
[c] One animal with *Salmonella typhimurium* infection.

A particularly high incidence of β2-toxigenic *C. perfringens* was found in specimens of intestinal ingesta and biopsy specimens of the intestinal wall from horses with typical or atypical typhlocolitis (75%), and a lower incidence was found in horses with other intestinal disorders (62%). These data indicate a correlation between the presence of β2-toxigenic *C. perfringens* in specimens of intestinal ingesta and biopsy specimens of the intestinal wall from horses with typhlocolitis. The data also suggest that β2-toxigenic *C. perfringens* might be particularly fatal in combination with antibiotic treatment. Preliminary in vitro experiments indicate that gentamicin enhances expression of the β2-toxin. Hence, β2-toxigenic *C. perfringens* not only may play an important role in the pathogenesis of equine typhlocolitis and other intestinal disorders of equines but also might be involved in the fatal progression of typhlocolitis in horses.

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