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Paeniclostridium (*Clostridium*) *sordellii*–associated enterocolitis in 7 horses

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Abstract. Enteric disease in horses may be caused by a variety of microorganisms, including several clostridial species. *Paeniclostridium sordellii* (previously *Clostridium sordellii*) has been frequently associated with gas gangrene in humans and several animal species, including horses. However, its role in enteric diseases of animals has not been fully determined. We describe herein 7 cases of enteric disease in horses associated with *P. sordellii* infection. Grossly, the small and/or large intestines were necrotic, hemorrhagic, and edematous. Microscopically, there was severe mucosal necrosis and hemorrhage of the small and/or large intestine of all horses. *P. sordellii* was isolated and/or demonstrated by immunohistochemistry and/or PCR in the intestine of all horses. All other known causes of enteric disease in horses were ruled out in these 7 cases. *P. sordellii* should be considered among the differential diagnoses in cases of enteric disease in horses.

Key words: *Clostridium sordellii*; colitis; enteritis; enterocolitis; horses; *Paeniclostridium sordellii.*

Introduction

Enterocolitis is an important cause of morbidity and mortality in foals and adult horses.[19,33] Current understanding of this syndrome is limited by the complex and dynamic nature of the gastrointestinal flora, including the fact that several of the pathogens that cause enterocolitis may be found, at variable prevalence, in the gastrointestinal tract of normal horses.[33]

There is a large variety of infectious and non-infectious causes of enterocolitis in horses. These include, among others, *Salmonella* spp., *Neorickettsia risticii, Lawsonia intracellularis, Clostridium perfringens, Clostridioides difficile* (formerly *Clostridium difficile*), cantharidin toxicity, larval cyathostomiasis, rotavirus, and coronavirus.[1,10-13,28,32,33,35,38]

For many years, the percentage of horses with enteric disease for which an etiology was determined was relatively low, but increased knowledge and improved laboratory techniques available for routine use in diagnostic laboratories have led to increased numbers of cases with a confirmed etiology. Nevertheless, there is still a significant percentage of severe inflammatory conditions of the intestinal tract in which an etiology is never determined; this is frustrating for pathologists, clinicians, and owners.

Paeniclostridium sordellii (previously *Clostridium sordellii*) is a gram-positive, sporulating anaerobic rod that has been associated mainly with fatal toxic shock–like syndrome in humans,[3,20,30,39] gas gangrene in ruminants, pigs, and horses,[6,26] abomasitis in lambs,[24,27] necrotic enteritis in chickens,[25,34] ulcerative enteritis in quail,[8] omphalitis in foals,[21] and other histotoxic and enteric infections in wildlife, including bears and pelicans.[2,18] *P. sordellii* is a common inhabitant of soil, and it can be also found, albeit rarely, in the intestinal content of clinically healthy animals.[29] It has been suggested that this microorganism may be associated with enteric disease of several animal species, but this has been poorly documented and remains controversial. One case of *P. sordellii*–associated enteritis in horses has been published in the literature.[16] However, the pathology and pathogenesis of this condition have not been described thoroughly. We present herein a series of cases of *P. sordellii*–associated enteric disease in horses with a thorough description of the lesions and diagnostic work-up performed.

Materials and methods

Case selection and clinical history

The autopsy records of the San Bernardino Branch of the California Animal Health and Food Safety (CAHFS) Laboratory

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Table 1. Age, sex, breed, type of death, and clinical history of 7 horses with *Paeniclostridium sordellii*–associated enteric disease submitted for autopsy.

Case	Age (y)	Sex	Breed	Type of death	History
1	27	F	Arabian	Euthanasia	Colic; several days duration
2	2	M	Quarter Horse	Euthanasia	Acute colic
3	4	F	Andalusian	Euthanasia	Colic; 4-d duration
4	5	CM	Thoroughbred	Euthanasia	Acute colic; < 24 h duration
5	15	CM	Fjord	Spontaneous	Acute colic; < 24 h duration
6	7	CM	Mustang	Spontaneous	Acute colic; < 24 h duration
7	2	F	Peruvian Paso	Euthanasia	Lethargy, ventral neck edema; 3-d duration

CM = castrated male; F = female; M = male.

System were searched for horses submitted for autopsy between January 1, 1990, and December 2015 that had a diagnosis of inflammatory intestinal disease. The total number of horses that fit these criteria during that period was 710. From this set of submissions, 7 horses were selected based on 1) *P. sordellii* being isolated and/or detected by PCR in the intestine and 2) negative results for other agents known to produce intestinal disease in horses. These 7 cases were submitted between 2008 and 2015 from different locations in southern California (Table 1).

Gross and microscopic pathology

The autopsy report from each horse was reviewed. The interval between death and autopsy was 2–24 h, and all carcasses were in a mild-to-moderate state of postmortem decomposition. Samples of heart, lungs, adrenal glands, liver, kidneys, spleen, stomach, parasympathetic ganglia, oral mucosa, trachea, mesenteric lymph nodes, skeletal muscle, brain, thyroid gland, pituitary gland, pancreas, diaphragm, small and large intestine, and testis or uterus and ovary had been collected in all cases. The samples had been fixed by immersion in 10% neutral-buffered formalin, for 24–72 h, and processed routinely to produce 4 µm-thick sections stained with hematoxylin and eosin (H&E). The brain was fixed whole for 24–48 h, after which it was sliced at ~0.5-cm intervals and fixed in fresh formalin for another 7–10 d. Blocks from cortex, corpus striatum, thalamus, midbrain at the level of superior colliculi, cerebellum, cerebellar peduncles, pons, and medulla at the level of the obex, were obtained. Sections of small intestine and colon were also stained with Hucker-and-Conn Gram and phosphotungstic acid–hematoxylin (PTAH) stains.

Immunohistochemistry for *C. perfringens* and *P. sordellii*

Four µm-thick paraffin sections of jejunum, ileum, and/or colon from the 7 horses were processed by indirect immunoperoxidase techniques for *C. perfringens* and *P. sordellii* as described previously[10,21] (EnVision kit; Dako, Carpinteria, CA) according to the manufacturer's instructions. Primary antibodies were polyclonal rabbit anti–*C. perfringens* (Gen-Way Biotech, San Diego, CA) and goat anti–*P. sordellii* (VMRD, Pullman, WA), respectively. Small intestine from a goat inoculated experimentally with *C. perfringens* type C and muscle of a horse from which *P. sordellii* had been isolated were used as positive controls for each microorganism, respectively. Small intestine from a goat and a horse that were culture-negative for *C. perfringens* and *P. sordellii*, respectively, were used as negative controls. Additional negative controls consisted of test tissue sections of all of the horses, incubated with normal rabbit or goat serum instead of the specific antibodies.

Bacterial cultures

Small and large intestinal contents or swabs from all horses were inoculated onto pre-reduced, anaerobically sterilized (PRAS) *Brucella* blood agar (Anaerobe Systems, Morgan Hill, CA), PRAS phenylethyl alcohol sheep blood agar (Anaerobe Systems), egg yolk agar (Anaerobe Systems), and cycloserine–cefoxitin–fructose agar (CCFA; Veterinary Media Services, UCDavis, Davis, CA), and incubated anaerobically at 37°C for 48 h. All isolates were identified by conventional biochemical techniques. Samples of small and/or large intestinal contents from each animal, and individual or pooled samples of liver, spleen, or kidney, were inoculated onto Columbia 5% sheep agar (Hardy Diagnostics, Santa Maria, CA) and MacConkey (MAC) agar plates (Hardy Diagnostics) and incubated aerobically at 37°C for 48 h. Isolates were subjected to the RapID ANA II system (Remel, San Diego, CA); colonies suspected to be *P. sordellii* were tested using a fluorescent antibody for this microorganism using a conjugate (VMRD), per CAHFS SOPs.

In addition, small and large intestinal contents from 26 Thoroughbred racehorses that had been euthanized as a result of catastrophic leg injuries, but were otherwise in good health, were collected within 24 h of death and used as controls to monitor the prevalence of *P. sordellii* in horses. These horses were part of the California Horse Race Board postmortem program and had been submitted routinely to the

Table 2. Primers used for detection of *Paeniclostridium sordellii* in formalin-fixed, paraffin-embedded sections of intestinal tissue.

Primer name	Sequence (5'–3')	Target gene	Product size (bp)
sdlF	CCATAAGTGGTGGTGCTTCG	*sdl*	138
sdlR	TGATTGCAGCGTATAAGCAAAT		
CsLetF	AGAATGTGAGATAAATGTTGCTTCA	*tcsL*	228
CsLetR	ATCCTAAATCCATTTTCAGTCTTGG		
CsHemF	ATTGTGGCACGAGCTTCTGG	*tcsH*	153
CsHemR	TCCAGCTATAGAATTAGGTGGCA		

laboratory for musculoskeletal examination. These samples were subjected to anaerobic culture as described above.

Molecular tests

PCR to characterize P. sordellii isolates. Only one *P. sordellii* isolate (case 5) was available from our retrospective study. Crude DNA preparation of this isolate was subjected to PCR analysis for the *P. sordellii* sordellilysin gene (*sdl*), a cholesterol-dependent cytolysin, and for the genes encoding *P. sordellii* lethal toxin (*tcsL*) and *P. sordellii* hemorrhagic toxin (*tcsH*), as described previously.[7,37] DNA extracted from *P. sordellii* JGS6382 strain (*sdl+, tcsL+, tcsH+*) was used as the positive control.

PCR to confirm presence of P. sordellii and its major toxins in intestinal tissue. Formalin-fixed, paraffin-embedded (FFPE) sections (~25 µm thick) of small and/or large intestine of 6 horses (cases 2–7) were deparaffinized, and DNA was extracted (QIAamp DNA FFPE tissue kit; Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was used as a template for PCR detection of the *P. sordellii sdl, tcsL*, and *tcsH* genes. Primers were designed to amplify and screen short fragments of these 3 genes (Table 2). PCR was performed in a total volume of 25 µL containing 0.5 µL of each primer (0.5 µM), 5 µL of extracted DNA, 7 µL of nuclease-free water, and 12 µL of PCR master mix 2× (Promega, Madison, WI), which contains Taq DNA polymerase (pH 8.5, 50 U/mL), dNTPs (400 µM), and $MgCl_2$ (3 mM). Thermocycler profiles were as follows: 95°C for 10 min, 35 cycles of 94°C for 40 s, 52°C for 35 s, and 72°C for 40 s, and a final extension step at 72°C for 5 min. Samples were held at 4°C. DNA extracted from *P. sordellii* strain JGS6382 was used as a positive control for all reactions. Reactions in which DNA was replaced by FFPE intestine scrolls of a normal horse were used as negative controls. PCR amplicons were visualized in ethidium bromide–stained 1% agarose gels (Agarose SFP; Amresco, Solon, OH).

Salmonella PCR. PCR to detect a fragment of the *Salmonella*-specific *invA* gene was performed on small and large intestinal content samples from all horses, as described previously.[4] Briefly, enriched overnight cultures were centrifuged and processed for real-time PCR using the sediment as a template.

Clostridium perfringens toxins ELISA

Samples of small and/or large intestinal contents from all horses were tested for toxins alpha (CPA), beta (CPB), and epsilon (ETX) of *C. perfringens* via a commercial capture ELISA kit (BIO-X, Brussels, Belgium), following the manufacturer's instructions. Briefly, the test used 96-well plates sensitized by specific monoclonal antibodies for CPA, CPB, or ETX. Samples were added to wells, and plates were incubated followed by washing and incubation for 60 min with peroxidase-labeled anti-CPA, -CPB, or -ETX polyclonal antibodies. A mixture of chromogen-substrate (hydrogen peroxide and tetramethylbenzidine) was used for the colorimetric reaction. Optical densities were read using an ELISA reader with a 450-nm filter. Purified CPA, CPB, or ETX were used in positive control wells; toxins were replaced by buffer in negative control wells. Results were calculated according to the manufacturer's instructions.

Clostridioides difficile toxins ELISA

Samples of small and/or large intestinal contents from all horses were tested for toxins A and B of *C. difficile* using a commercial ELISA kit (TECHLAB, Blacksburg, VA) according to the manufacturer's instructions. A mixture of purified toxin A and B was used in the positive control wells; toxins were replaced by buffer in negative control wells. Optical densities were read using an ELISA reader with a 450-nm filter. Results were calculated according to the manufacturer's instructions.

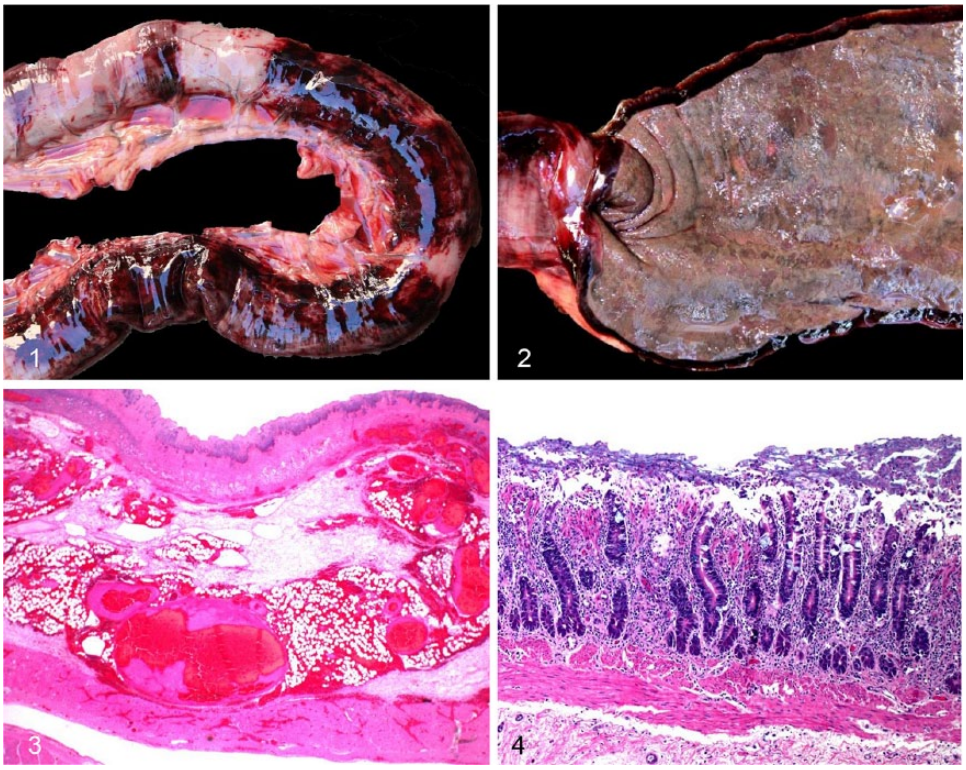
Other ancillary tests

Cantharidin testing was performed on the content from the small intestine of one horse (case 6), by gas chromatography–mass spectrometry following CAHFS SOPs.

Results

Gross pathology

Three horses (cases 4, 5, and 7) had lesions in both the small and large intestine (jejunum/ileum and colon/cecum); 2 horses (cases 1 and 2) had lesions only in the large intestine, and the 2 other horses (cases 3 and 6) had lesions only in the



Figures 1–4. Intestine of horses with *Paeniclostridium sordellii*–associated enteric disease. **Figure 1.** Small intestine of horse 6. Severe, multifocal-to-coalescing serosal hemorrhage. **Figure 2.** Small intestine of horse 6. Severe, diffuse mucosal necrosis with pseudomembrane and transmural hemorrhage. **Figure 3.** Colon of horse 2. Diffuse mucosal necrosis and transmural hemorrhage. H&E. **Figure 4.** Colon of horse 3. Diffuse mucosal necrosis with luminal pseudomembrane. H&E.

small intestine. Gross lesions in the small and large intestine were similar and characterized by segmental mucosal or transmural hemorrhages (Fig. 1), with mucosal erosions and ulcers, and diffuse pseudomembrane formation (Fig. 2). The intestinal wall was thickened by edema and hemorrhage, and the intestinal content was dark red and thin to pasty. The serosa of the affected intestinal segments was diffusely and severely congested and hemorrhagic (Fig. 1). In addition, 2 of the horses (cases 1 and 2) had a few, ~1-cm diameter, shallow ulcers with elevated borders on the pars esophagea of the stomach.

Significant lesions outside the gastrointestinal tract included segmental cranioventral-to-caudodorsal consolidation of both lungs in case 4, cortical hemorrhages in the kidneys of cases 1 and 4 and adrenal gland in case 7, and subendocardial ecchymoses in both ventricles of the heart of cases 4 and 6.

Microscopic pathology

Microscopic lesions in small intestine and colon were observed in all the horses that had gross lesions in those organs. These consisted of mucosal hemorrhage and/or superficial-to-deep lytic necrosis (Fig. 3), with loss of superficial epithelium and lamina propria, and formation of a pseudomembrane composed of fibrin, sloughed and necrotic epithelium, red blood cells, leukocytes, and myriad rods (Fig. 4). Severe villus blunting was observed in the small intestine; epithelial and lamina proprial necrosis in this part of the intestine was most prominent at villus tips. There was thrombosis of mucosal and submucosal vessels, and marked submucosal edema and hemorrhage accompanied by extensive pleocellular infiltrates. In case 3, fibrin and inflammatory exudate could be seen breaking into the lumen from the necrotic lamina propria ("volcano lesions"). Hemorrhages were often transmural. Free in the intestinal lumen, throughout the necrotic mucosa, and often deep within the lamina propria and submucosa, were small-to-moderate numbers of mixed bacteria with a prevalence of gram-positive rods.

Microscopic lesions outside the gastrointestinal tract included moderate-to-marked, multifocal-to-diffuse pulmonary congestion, edema, and hemorrhage. Case 4 had severe, fibrinopurulent, and hemorrhagic bronchopneumonia. The heart, liver, and kidney of all horses had diffuse congestion and multifocal hemorrhages. The liver of case 1 had chronic-active, periportal, pleocellular cholangiohepatitis. Periportal and biliary hyperplasia was observed in cases 1 and 3. The stomach of cases 2 and 4 had chronic ulceration of the pars esophagea with exuberant granulation tissue ulcer beds.

Table 3. PCR results for *Paeniclostridium sordellii* and its major toxins using formalin-fixed, paraffin-embedded tissues from 6 horses.

	Gene		
Case	*sdl*	*tcsL*	*tcsH*
2	−	−	−
3	+	+	−
4	+	+	−
5	+	−	−
6	+	−	−
7	+	−	−

Immunohistochemistry

The great majority of the rods seen in H&E- and Gram-stained intestinal sections stained positively with *P. sordellii* IHC. Only a few isolated rods were positive with *C. perfringens* IHC in these sections.

Bacterial cultures

P. sordellii was isolated from the small and/or large intestine of all of the horses in moderate-to-large numbers. *Streptococcus equi* ssp. *zooepidemicus* and *Escherichia coli* were isolated from liver, lung, and colon in case 2. Mixed aerobic flora was isolated from the liver, colon, small intestine, and/or lungs of all horses. *C. difficile* was not isolated from the small or large intestine of any horse, and *Salmonella* spp. was not isolated from the small or large intestine or liver of any horse. Neither *P. sordellii* nor *C. difficile* were detected in the small or large intestinal content from the 26 control racehorses that were euthanized as a result of catastrophic leg injuries.

Molecular tests

The single *P. sordellii* isolate evaluated was positive for an internal fragment of *sdl*, but negative for the genes that encode TcsL and TcsH. *P. sordellii sdl* was detected in 5 of the 6 samples of FFPE tissues evaluated for this test (Table 3). The *tcsL* gene was detected in the extracted DNA from the intestine of 2 horses; all samples were negative for *tcsH* (Table 3). *Salmonella* spp. PCR was negative in all samples.

Clostridial toxin ELISAs

Toxins of *C. perfringens* or *C. difficile* were not detected in the intestinal contents of any horse.

Other ancillary tests

Cantharidin was not detected in the liver of the horse tested for this substance.

Discussion

Among the most common infectious causes of enteritis, colitis, and/or enterocolitis in horses are *C. difficile, C. perfringens* type C, *Salmonella* spp., and *Rhodococcus equi*.[32] We ruled out these etiologies based on bacterial culture, toxin ELISAs, and/or PCR. Other infectious causes of colitis including *Lawsonia intracellularis, Strongylus* spp., *Cryptosporidium parvum, Rickettsia risticii*, and coronavirus[32] were ruled out by histology and/or other ancillary tests. Cantharidin and NSAIDs were ruled out based on specific tests and clinical history, respectively.[32] The death-to-autopsy interval in all cases was 2–24 h, and the tissues were moderately to well preserved in all horses. This suggests that the microbiologic results were not significantly affected by this interval.

A presumptive *P. sordellii* etiology was established in the 7 cases of our study based on ruling out the most common etiologies for inflammatory enteric disease in horses mentioned above, coupled with detection of *P. sordellii* by culture, IHC, and/or PCR in the intestine of all affected horses. The reason for the negative PCR result in case 2 was not determined, but it might be associated with low DNA recovery from the intestinal tissue, a frequent complication when working with FFPE tissues in which significant DNA damage usually occurs.

P. sordellii has been found, albeit rarely and in small numbers, in the intestine of normal horses,[29] and this is the reason that the mere isolation of this microorganism is not considered enough to establish a diagnosis of *P. sordellii*–enteric disease. We did not find this microorganism in the intestine of any of the 26 healthy horses cultured as controls, suggesting that the carrier rate for this microorganism is indeed very low or nil. In addition, *P. sordellii* was isolated in moderate-to-large numbers from the intestine of our horses, and it was demonstrated by IHC to be associated with the enteric lesions of all horses. Although it would have been ideal to have matched controls of the same breed, age, sex, and exact geographic location as the *C. sordellii*–infected individuals, such controls were not available for our study. However, the control horses were of the same age class and originated from the same general geographic region (southern California) of the affected animals, and are therefore considered acceptable, albeit not ideal, controls.

P. sordellii is responsible for histotoxic disease in humans and other animals. This microorganism has been associated with septic shock in women after medical abortion.[39] In animals, *P. sordellii* has been associated with gas gangrene in chickens, ruminants, and horses.[6,15,21,26] Although it has been suggested frequently that this microorganism is responsible for enteric disease and sudden death in several animal species,[5,9,17] evidence to support this claim is scant. Koch postulates have not been fulfilled, and definitive evidence of the role of *P. sordellii* in enteric disease of animals is therefore lacking. However, our results strongly suggest that this microorganism is involved in enteric disease of horses. Predisposing factors may

include antimicrobial therapy, and other factors not identified to date. Although this is speculative, similar risk factors have been identified for other enteric clostridial infections (e.g., *C. difficile*).[32]

P. sordellii produces several toxins, although it is usually assumed that 2 of them, namely lethal toxin (TcsL) and hemorrhagic toxin (TcsH), are mainly responsible for the virulence of this microorganism.[14,22,23,36] The toxins of *P. sordellii* are often compared to toxins A and B (TcdA and TcdB) of *C. difficile*, and it has been reported that a specific antiserum against the lethal toxin of *P. sordellii* successfully neutralized the cytotoxic and lethal activities of *C. difficile* TcdB.[22,23,29,31] In our study, only 2 samples of DNA extracted from FFPE intestinal tissue tested positive for a fragment of the *tcsL* gene, but this gene was not identified in the 1 isolated strain available for testing. Identification of toxin genes in tissue sections by PCR may help to detect the presence of virulence factors that may be lost upon subculture.[7] The great majority of *P. sordellii* isolates are non-toxic after isolation from infected patients or even in vivo; this is probably because toxin genes are on mobile elements that are easily lost. This could have been the cases in some of our horses. *P. sordellii sdl* was detected in 5 of 6 samples of FFPE tissues evaluated for this test. This is a pore-forming toxin that causes cell necrosis, although little is known about its effect on living animals.[22] It is possible that sordellilysin was at least partly responsible for the lesions observed in our cases. We also cannot rule out that other, yet unknown, virulence factors were involved in the pathogenesis of the *P. sordellii*–associated enteric disease of these animals. Conventional and molecular Koch postulates should be fulfilled to determine the role of *P. sordellii* and its toxins in enteric disease of horses.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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