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Gas gangrene in horses by *Clostridium sordellii*

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Running head: Gas gangrene in horses

Abstract. Gas gangrene occurs in several animal species, and it is caused by one or more clostridial species. In horses, the disease is most often caused by *Clostidium perfringens* type A. Although *Clostridium sordellii* has been associated with gas gangrene in ruminants and humans, cases of the disease associated with this microorganism have not been described in horses. We report 8 cases of gas gangrene by *C. sordellii* in horses. These cases were characterized by myonecrosis and cellulitis, associated with systemic changes suggestive of toxic shock. The diagnosis was confirmed by gross and microscopic changes combined with anaerobic culture, fluorescent antibody test, immunohistochemistry and/or PCR. The predisposing factor in these cases was an injection or a traumatic skin injury. *C. sordellii* should be considered as a possible etiologic agent in cases of gas gangrene in horses.

Key words. *Clostridium sordellii*, gas gangrene, horse, muscle, subcutaneous tissue

Introduction

Gas gangrene (formerly known as malignant edema) is a rapidly progressing infection of muscle and subcutaneous tissue produced by one or more clostridial species, characterized by severe myonecrosis and/or cellulitis in humans and several animal species.[28, 32] The pathogenesis of gas gangrene involves skin or mucosal wounds through which vegetative forms or spores of the clostridial species involved gain entry to the organism. At the port of entry, the organism multiplies rapidly and produces toxins that act locally and access the blood, producing toxic shock syndrome and multiorgan failure.[22, 29] Septicemia is also a common complication of the disease.[8, 28]

Gas gangrene in horses is most often caused by *Clostridium perfringens* type A,[23] although sporadic cases have been described in association with other clostridial species, including *Clostridium septicum, Clostridium chauvoei, Clostridium novyi, Clostridium ramosum, Clostridium sporogenes* and *Clostridium fallax.*[2, 5, 7, 14, 23, 24, 28, 37] The majority of cases of equine gas gangrene described in the literature have been produced by a single clostridial species, although mixed infections with two or more clostridial species have occasionally been reported.[15, 23, 27, 37]

Clostridium sordellii is one of the members of the gas gangrene complex, and it has been described as a cause of gas gangrene in humans,[3, 10, 16, 30] cattle[38] and sheep,[20, 35] and also in a series of cases of omphalitis in foals.[22] However, to the best of our knowledge, no cases of gas gangrene associated with *C. sordellii* have been described in horses.

C. sordellii is a gram-positive, anaerobic bacillus, which is a common habitant of soil[29] and rarely, the intestinal content of healthy animals. Most cases of clostridial gas gangrene, including those produced by *C. sordellii* occur via contamination of wounds, including those associated with parturition and injections. Trauma-associated tissue necrosis generates local

hypoxia, alkaline pH and protein breakdown products required for clostridial proliferation.[26] In humans, clostridial toxic shock is a rare syndrome occurring post-partum and post-abortion, characterized by tachycardia, hypotension and lack of fever.[39] The patients frequently progress to fatal toxic shock syndrome.[12]

All strains of *C. sordellii* encode sordellilysin (*sdl*), phospholipase C and neuroaminidase.[9] In addition, some *C. sordellii* isolates may produce lethal toxin (TcsL) and/or hemorrhagic toxin (TcsH), both of which are considered the main virulence factors for the toxic shock syndrome in humans.[29, 31] Although the role of these toxins in cases of animal gas gangrene has not been determined, it is likely that they play a role similar to the one they play in human disease. In this paper, we describe here 8 cases of gas gangrene in horses produced by *C. sordellii*.

Materials and methods

We searched the records of the California Animal Health and Food Safety Laboratory System (CAHFS) at UC Davis for cases of horses submitted for autopsy between 1998 and 2019 that had a diagnosis of gas gangrene that was attributed to *C. sordellii*. This included 8 cases in which i) the horses had severe necrotizing cellulitis and/or myositis, ii) *C. sordellii* had been isolated from the affected muscle and/or detected intralesionally by immunohistochemistry, fluorescent antibody test and/or polymerase chain reaction, and iii) the horses had died spontaneously or been euthanized because of severe clinical disease associated with this infection. Information on signalment and clinical history is summarized in Table 1. A full autopsy was performed in all cases. Three horses died (cases 2, 4 and 5) and 2 were euthanized (cases 3 and 8). Information on the manner of death was not available in 3 cases (cases 1, 6 and 7).

Samples of lung, liver, kidney, heart, skeletal muscle, stomach, small and large intestine, spleen, thymus, lymph node, uterus, ovary, adrenal gland, pituitary gland, thyroid gland, salivary gland, peripheral nerve, trachea, spinal cord, sciatic nerve, trigeminal ganglia, tongue, pancreas, urinary bladder, subcutaneous tissue and/or the whole brain were collected in most cases and fixed in 10% buffered formalin pH 7.2 for several days. The brains were then cut into ~ 0.5 cm thick slices, and fixed in fresh formalin for additional 7-10 days; after this, samples of parietal cortex, corpus striatum, thalamus, mid-brain at the level of anterior colliculi, pons, cerebellar peduncles, cerebellum and medulla at the level of the obex were collected. All tissues were routinely processed to obtain 4 µm thick, hematoxylin and eosin-stained sections. In all cases, selected sections of subcutaneous tissue and muscle were also stained with Gram.

Samples of muscle and subcutaneous tissue from grossly affected areas, and multiple organs including one or more of liver, spleen, lung, skin, peripheral lymph nodes, peritoneal fluid, aqueous humor, and small intestinal and cecal content from most horses were aseptically collected and inoculated onto 5% sheep blood agar, and incubated aerobically and/or anaerobically at 37ºC for 48 hours (Table 2). Sub-samples of most of these specimens were also inoculated into cooked meat medium and incubated anaerobically at 37ºC for 48 hours. All isolates were identified by conventional biochemical techniques.

Muscle smears of 4 cases (cases 1, 2, 7 and 8) were also subjected to direct fluorescent antibody test (FAT) for *C. sordellii, C. chauvoei. C. novyi and C. septicum* as previously described[22] (Table 2). Reference strains of the clostridial species mentioned above were used as control for each FAT preparation.

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded sections of skeletal muscle and subcutaneous tissue of 4 cases (cases 3, 4, 6 and 8) as previously described.[22] Briefly, a streptavidin–biotin kit was used according to the manufacturer's

instructions (LSAB-peroxidase K675; Dako, Carpinteria, CA). Primary rabbit polyclonal antibodies against *C. sordellii* (VMRD, Seattle, WA) were used. Positive controls consisted of muscle sections of a horse from which *C. sordellii* had been isolated. Negative controls consisted of sections incubated with normal rabbit serum instead of the primary antibody and of muscle sections of a healthy horse from which no anaerobes had been isolated.

PCR for 3 genes specific of *C. sordellii, i.e.* sordellilysin (*sdl*), lethal toxin of *C. sordellii* (*tcsL*) and hemorrhagic toxin of *C. sordellii* (*tcsH*) was performed on muscle of 3 of the horses (cases 4, 6 and 8). For this, 3, 5-µm-thick scrolls of formalin-fixed, paraffin embedded (FFPE) skeletal muscle were placed into 1.5-ml microcentrifuge tubes for dewaxing by adding 1 ml of xylene, followed by centrifugation for 2 minutes at 13,000 x*g*. The xylene was then removed and the pellet was washed with 1 ml of 100% ethanol and centrifuged for 2 minutes at 13,000 *g*. The ethanol was discarded and the samples were air-dried at room temperature for 45 minutes. Then, the dewaxed tissues were subjected to DNA extraction using a commercial kit (QIAamp DNA FFPE Tisse Kit, QIAGEN, Hilden, Germany) following the instructions of the manufacturer. The extracted DNA was used as template for conventional PCR detection of *sdl*, *tcsL* and *tcsH* genes using the following set of primers, respectively: 5'-CCATAAGTGGTGGTGCTTCG-3' (sdlF) and 5'-TGATTGCAGCGTATAAGCAAAT-3' (sdlF) (138bp); 5'-GACCCAACGAAGAGTGGAGC-3' (TcsLF) and 5'-TCAAGTGTACCAGCAGGAGC-3' (TcsLR) (146bp); 5'- GGGACACCTTCTGTAAGTGTAGG -3' (TcsHF) and 5'-AGGTTCAACTGTATGCCCAACT -3' (TcsHF) (133bp). PCR was performed in a total volume of 25 µl containing 5 µl of extracted DNA, 0.25 µl of each primer (10 µM), 7 µl of nuclease-free water and 12.5 µl of DreamTaq Green PCR Master Mix 2X Thermo Scientific (Waltham, MA) which contains DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dNTPs (0.4 mM each) and $MgCl_2$ (4 mM). The following thermocycler profiles were used: 95º C for 4 min, 35 cycles at

95º C for 30s, 54º C for 30s, and 72º C for 1 min followed by a final extension step at 72º C for 5 min and a final hold at 4º C. DNA extracted from the *C. sordellii* JGS6382 strain was used as positive control. This strain is positive for *sdl*, *tcsL* and *tcsH.* Scrolls from the *C. sordellii*-negative skeletal muscle used for IHC (see above) and reactions in which nuclease-free water was used instead of DNA were used as negative controls. PCR amplicons were visualized in ethidium bromide-stained 1.5% agarose gels (Agarose SFR™, Amresco®, Solon, Ohio).

Results

In 7 of the 8 cases (cases 1, 2, 3, 5, 6, 7 and 8) there was a history of skin injury which was thought to be the port of entry for *C. sordellii.* No information about a possible port of entry was available in one case (case 4).

Grossly, the lesions involved muscle and/or subcutaneous tissue underneath areas of skin injuries except for case 1 (case 4), in which no skin lesions were seen. In all cases, the affected subcutaneous tissue presented extensive, moderate to severe, foul smelling, yellow and gelatinous edema, and hemorrhage, which frequently extended into the underlying musculature, separating muscles bundles (Fig. 1). The muscle of these areas was multifocally dark red with irregular pale areas, and it was friable, soft and dry, often showing gas bubbles (Fig. 2). The lungs were diffusely congested and edematous, and presented multifocal petechiae throughout the parenchyma and on the pleura. The heart showed multifocal epicardial, myocardial and sub-endocardial petechiae and ecchymosis that were most marked in the left and right ventricle, but were also observed in both atria. In addition, ascites, hydrothorax and hydropericardium was observed in 4 cases (cases 1, 4, 6 and 8). Diffuse mucosal edema and multifocal sub-serosal petechiae were observed in the colon of 5 horses (cases 1, 2, 4, 5 and 8). A few multifocal shallow ulcers with elevated borders and schirrous ulcer beds were present in the esophagic

167 portion, close to the *margo plicatus* of the stomach in 2 cases (cases 3 and 6). Hemorrhagic,
168 focally extensive ulceration of glandular gastric mucosa was seen in 1 horse (case 8).
169 Microscopically, the lesions in skeletal muscle were similar in all animals. There was
170 multifocal to coalescing necrosis of muscular fibers, characterized by diffuse, dense, eosinophilic
171 and glassy appearance of the cytoplasm, with loss of cross-striations, fragmentation, vacuolation,
172 hypercontraction bands, mineralization, karyorrhexis and karyolysis (Fig. 3). Multifocally, within
173 the cytoplasm of the necrotic myofibers there was a moderate number of degenerate and viable
174 neutrophils, and fewer macrophages. The interstitium and fascia was expanded by moderate to
175 severe hemorrhage, edema, fibrin, neutrophils, and fewer lymphocytes, plasma cells and
176 macrophages. The interstitium also showed multifocal, large empty clear vacuoles with well-
177 defined borders in 7 cases (cases 1, 2, 3, 5, 6, 7 and 8) and large numbers of gram-positive rods,
178 singly or in clusters (Fig. 5). These bacteria were approximately 5–7 µm X 0.8–1 µm, with
179 parallel borders and round ends, and many of them had central or sub-terminal spores (Fig. 4).
180 Fibrinoid, suppurative-necrotizing vasculitis was observed in areas of muscular necrosis in 2
181 cases (cases 1 and 6). The subcutaneous tissue overlying the areas of myonecrosis in all cases
182 showed pronounced expansion with edema, hemorrhage, fibrin, neutrophils, lymphocytes, plasma
183 cells and macrophages. The deep dermis was distended by fibrin, edema and hemorrhage; blood
184 vessels showed multifocal and perivascular neutrophil infiltrates. In addition, 3 animals (cases 3,
185 5 and 7) had mild, multifocal myocardial necrosis, characterized by swollen myofibers with
186 hypercontraction bands, which were surrounded by a mild neutrophilic and lymphoplasmacytic
187 infiltrate. Multifocal, mild to severe interstitial hemorrhage was seen in endocardium,
188 myocardium and epicardium. The kidneys of 6 horses (cases 1, 2, 3, 4, 5 and 6) were congested,
189 and homogeneous eosinophilic protein casts were observed in the lumen of renal tubules. Acute
190 proximal tubular necrosis was observed in 2 cases (cases 1 and 3).

A summary of bacteriologic and molecular findings is shown in Table 2. Briefly, *C. sordellii* was isolated from muscle in all horses. In addition, *C. perfringens* type A, *Streptococcus* spp., *Enterococcus* spp., *Echerichia coli,* and mixed aerobic and anaerobic flora were also isolated from affected muscle in 5 cases (cases 3, 4, 5, 6 and 8). All 3 FFPE samples analyzed by PCR were positive for *C. sordellii sdl* and *tcsL* genes, but negative for the *tcsH* gene.

FAT for *C. sordellii* was positive in 3 (cases 2, 7 and 8) of the 4 cases tested for this technique. FAT for the other clostridial species tested was negative in the 4 cases. Sections of skeletal muscle from 3 cases (cases 4, 6 and 8) and subcutaneous tissue were positive for *C. sordellii* IHC (Fig. 6) and 1 case was negative. The positive-stained bacteria were in the same location and had similar morphology to those described for the sections stained with Gram. Positive control tissues was stained positively with this technique and no staining was observed in any of the negative controls.

Discussion

In this study, a diagnosis of gas gangrene by *C. sordellii* was established on the basis of clinical history, gross and microscopic findings, and the detection of the microorganism by bacterial culture, IHC, FAT and/or PCR. Although Gram stain and IHC were negative in 1 case, *C. sordellii* was isolated from that animal, which, coupled with the gross and microscopic lesions, confirmed the diagnosis. It is likely that the sections used for IHC and Gram stain in that case were prepared from an area with low or no bacterial load which resulted in a negative IHC and Gram stain. The isolation of *C. sordellii* in pure culture from muscle of 3 horses and the supportive gross and microscopic lesions suggest that this microorganism can act as a primary pathogen to produce gas gangrene in horses. *C. perfringens* type A was isolated in two cases. Although this organism was isolated in small numbers, it is possible that it acted synergistically

with *C. sordellii* to produce gas gangrene in these two horses. Other microorganisms that can produce similar lesions in horses, *e.g. C. septicum. C. novyi* and *C. chauvoei,* were ruled out by culture and/or FAT.

Clostridial gas gangrene has been reported in horses before.[4, 18, 23, 25 27, 33, 34] However, *C. sordellii* has not been reported associated with gas gangrene in horses until now. In a previous report of 37 cases of gas gangrene.[23] *C. perfringens* type A was isolated in purity in 25 cases, and in combination with other clostridia in 4 cases. Based on those results, the authors,[23] concluded that *C. perfringens* type A is the most common cause of gas gangrene in horses. In that study,[23] *C. sordellii* was not isolated from any case.

In the study by Peek et al (2003), the lesions consisted of severe necrotizing fasciitis and myositis in the region of the inciting wound, coupled with splenic, hepatic, renal and/or myocardial necrosis.[23] In our cases, similar local and systemic lesions were observed, the latter suggesting that toxic shock syndrome also occurred. These lesions are similar to those described in cases of gas gangrene in several animal species.[20, 28]

C. sordellii has been associated with multiple histotoxic infections in a variety of animals, including omphalitis in foals,[22] gas gangrene in ruminants,[20, 35] emphysematous abomasitis in lambs[36] and metritis in sheep[6]. This microorganism has also been blamed for sudden death syndrome in cattle [38] and lions[11]. Solid evidence for the role in the latter is, however, lacking.

In humans, *C. sordellii* has been associated with fulminant necrotizing omphalitis in babies[1, 17, 19] and endometritis and toxic shock syndrome in women[21]. The cause of death of humans with *C. sordellii* infection is thought to be septic shock, including DIC. The toxins generated by the microorganism at the site if infection are thought to spread systemically leading to septic shock.[13] The gross and microscopic findings described in the 8 horses of this study,

suggest that a similar mechanism of death occurred in these horses. In our study, the predisposing factor was an injection in the great majority of cases.

In this study, a skin injury, either iatrogenic (injection) or accidental was considered the port of entry of the infection. This is consistent with most cases of gas gangrene previously reported in horses and other animal species.[24, 25, 27, 33, 34]

In humans, it is believed that one or two of the two main virulence factors of *C. sordellii* (TcsL and TcsH), are responsible for the main lesions and clinical signs observed in cases of gas gangrene.[10] The TcsL triggers apoptosis on endothelial cells, leading to vascular compromise, edema and shock.[12] The gene encoding TcsL was identified in the three cases available for PCR in this study, suggesting that this toxin might have been the main virulence factor responsible for these infections.

In summary, the 8 animals included in this study presented gas gangrene characterized by severe myonecrosis and cellulitis associated with *C. sordellii* infection, which is thought to have led to toxemia and septic shock.

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Declaration of conflicting interests

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

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357

358 **Table 1.** Signalment, clinical history, main clinical signs and affected region of 8 horses with gas

359 gangrene caused by *Clostridium sordellii.*

Case	Age (years)	Sex	Breed	Clinical History	Main clinical signs	Anatomic region affected
1	NR	NR	NR	Vaccination (rhinopneumonitis, influenza, tetanus) 3 days before onset	Local edema and pain	Left side neck and chest
2	14	F	Quarter Horse	Vaccination (rabies) 3 days before onset	Anorexia and seizures	Left gluteal region
3	3	F	Arabian	Injection (selenium-tocopherol, DMSO) 2 days before onset	Local pain, colic and shock	Lumbar region and both thighs
4	5	F	Quarter Horse	NR	Sudden death	Both thighs
5	20	F	NR	Traumatic skin wound before onset (interval NR)	NR	Left thigh
6	2	M	Appaloosa	Traumatic skin wound 5 days before onset	NR	Left thigh
7	19	F	Quarter Horse	Chronic cellulitis of unknown origin and duration	Anorexia	Both thighs
8	7	F	Quarter Horse	Traumatic skin wound 7 days before onset	Local edema and pain	Right shoulder

360 NR= Not reported; F= Female; M= Male.

362 **Table 2.** Microbiological and molecular findings in skeletal muscle of 8 horses with gas

363 gangrene caused by *Clostridium sordellii.*

Case	*C. sordellii* isolation	Gram stain	FAT		*C. sordellii* PCR				Other bacteria isolated
			C. sordellii	*C. chauvoei; C. septicum; C. novyi*	*sdl*	*tcsL*	*tcsH*	*C. sordellii* IHC	
1	+	+	-	-	NP	NP	NP	NP	-
2	+	+	+	-	NP	NP	NP	NP	-
3	+	-	NP	NP	NP	NP	NP	-	*C. perfringens*
4	+	+	NP	NP	+	+	-	+	*C. perfringens; Enterococcus* spp.
5	+	+	NP	NP	NP	NP	NP	NP	Mixed flora; *Streptococcus* sp. gamma-hemolytic *
6	+	+	NP	NP	+	+	-	+	*E. coli*
7	+	+	+	-	NP	NP	NP	NP	-
8	+	NP	+	-	+	+	-	+	Mixed flora; *Enterococcus* spp.

364 FAT= fluorescent antibody test; IHQ = immunohistochemistry; (+) = Positive; (-) = Negative;

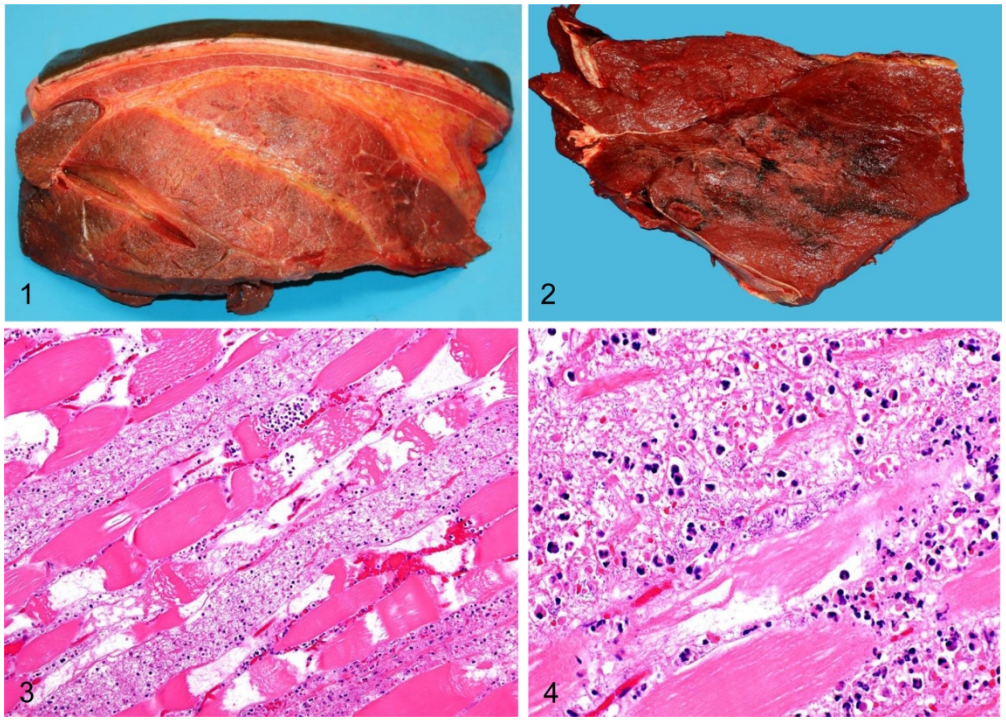
365 NP= Not performed; *bacteria isolated from a muscle different from which *C. sordellii* was

366 isolated.

Figure legends

Figure 1-6. Muscle from horses with gas gangrene produced by *Clostridium sordellii.*

Figure 1. Severe subcutaneous and interstitial edema. **Figure 2.** Focally extensive necrosis and hemorrhage. **Figure 3.** Coagulation necrosis, hemorrhage, edema and neutrophilic infiltration. H&E. **Figure 4.** Hypercontraction bands and neutrophilic infiltration within necrotic fibers, and large numbers of intralesional rods. H&E. **Figure 5.** Clusters of gram-positive rods. Gram. **Figure 6.** *Clostridium sordellii* stained by immunohistochemistry.



Figs. 1-4

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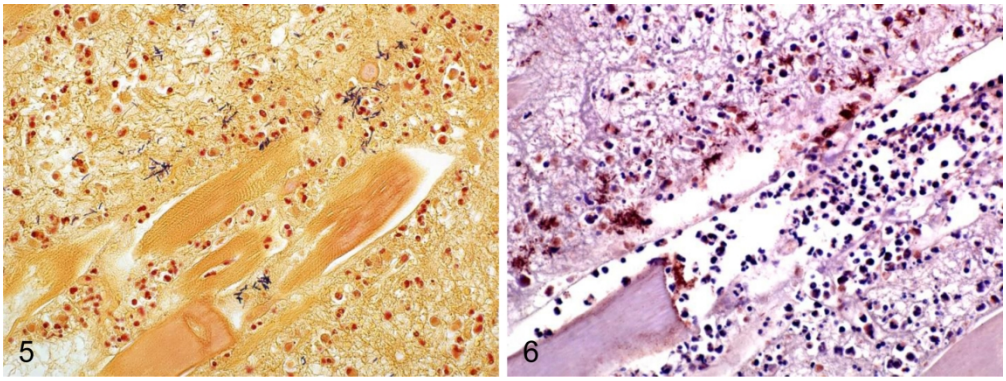

Figs. 5-6

166x62mm (300 x 300 DPI)