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Myonecrosis caused by double infection of S. equi subspecies zooepidermicus and Clostridium novyi type A in a horse

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Summary: This case report describes the lesions and clinical findings in a horse sent to the Clinical Veterinary Hospital of the University of Extremadura for an infected wound, on the lateral aspect of the left hind limb. The infection was caused by Streptococcus equi subsp. zooepidemicus and Clostridium novyi type A, a bacterial co-infection that could eventually lead to synergistic infection and fatal myonecrosis. It is possible that prior infection with aerobic or facultative bacteria, such as Streptococcus equi subsp. zooepidemicus, as part of the bacterial flora of horses, may promote, by creating a low potential redox environment, the germination of Clostridium novyi type A spores. To the best of the authors' knowledge, this is the second report of Clostridium novyi type A as a causative agent of myonecrosis in horses. The anaerobic bacteria involved in clinical cases may be underreported due to the difficulty and inefficiency of culturing these microorganisms on some occasions.

Keywords: myonecrosis, bacterial coinfection, Clostridium novyi type A, limb wound

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Introduction

The equine wounds, particularly those located in the limbs, present a high risk of infection due to the surrounding contaminated environment. In addition *Babić* et al. (2017) have suggested that wounds on the distal limbs of horses could have difficult healing because of poor circulation, joint movement and minimal soft tissue protection between skin and bone.

Myonecrosis or gas gangrene is a highly lethal infection of the muscles, typically caused by Clostridium sp. and it is characterized by profound toxaemia, extensive oedema, massive death of tissue, and a variable degree of gas production.

Clostridium novyi type A had previously been described as the causal agent of an acute myonecrosis in a horse (*Farias* et al. 2014), which most likely started after an intramuscular injection. Deep puncture wounds can provide an ideal environment for the growth of bacteria, particularly anaerobic microorganisms. In the clinical case described below, no intramuscular injection could be associated with the origin of the infection, but co-infection of aerobic and anaerobic bacteria was most likely the cause to the settling of the fatal myonecrosis.

Case Presentation

A 1.5-year-old Lusitano purebred horse was referred to the Veterinary Clinical Hospital of the University of Extremadura for evaluation of a wound on the lateral aspect of the left hind limb. According to the owner, the horse was standing in the field and he noticed the presence of the wound the day before, as the horse obviously had a lameness. There was no history of recent vaccination or deworming.

The owner called a veterinarian who examined the animal and treated him with 100,000 IU of tetanus antitoxin injected intravenously. He also reported the presence of a viscous fluid in the subcutaneous tissue which made him suspect a possible affectation of some adjacent synovial structures. Based on this finding, the veterinarian decided to send the horse to the Hospital without administering any further medication.

During the physical exam, the horse appeared lethargic but had normal rectal temperature, the physical condition was good, with a body weight of 350 kg and there was no evidence of dehydration. The mucous membranes were pink and presented a capillary refilling time under 2 seconds, heart rate was 68 beats/min, respiratory rate was 12 breaths/min. and intestinal motility was correct.

Hematological parameters showed no significant changes, with the sole exception of an increase in the total number of white blood cells (WBCs) count ($12 \times 10^3 \mu l$; range 4.9–$10.3 \times 10^3 \mu l$). The biochemical analyses also showed normal values; however, marked elevations in aspartate transaminase were noted 550 U/L (range 195–402 U/L).

The affected limb had severe diffuse swelling extending from the hoof to the stifle joint and signs of pain and discomfort were evident during palpation. The horse had a grade of 3/5 lameness on the affected limb.

A deeper examination of the affected limb revealed that the wound at the tip of the calcaneus bone had a purulent material of high density with a foul odour and presented several pathways. Radiographs taken in the tarsal area excluded the presence of bone lesions while an ultrasound scan did not show any effusion.

The presence of excessive synovial fluid inside of the tibiotarsal joint with hyperechoic images compatible with the presence of fibrin clots, led to the suspicion of joint contamination. A pale yellow viscous fluid was obtained by a puncture, and its laboratory analysis revealed 11,000 white blood cells (WBC/L) and 3.6 mg/dL of total protein concentration (positive samples present > 30,000 cells/L and/or a concentration of total protein exceeding 4.5 mg/dL), so the infection of the joint was excluded. The calcaneal bursa seemed to be affected, but tissue necrosis and the presence of dense purulent secretions all over the surrounding area made its evaluation difficult.

After the initial examination the horse was hospitalized for treatment. Treatment consisted of sodium penicillin (22,000 UI/kg, IV, q 6 h), gentamicin (6.6 mg/kg, IV, q 14 h) and phenilbutazone (2.2 mg/kg, IV, q 12 h). During the hospitalization the horse developed fever (up to 39.9 °C) that responded well to the administration of non-steroidal anti-inflammatory drugs. During this period, the horse was obviously lethargic and had a decreased appetite, although gastrointestinal motility was correct.

During the hospitalization, sterile protective bandages were applied all over the limb (from the hoof to the tibia) every 24 hours. At the second bandage change, skin necrosis on the lateral aspect of the tarsus was noticed, about 10 cm proximal to the point of the calcaneus. New sterile bandages were applied in the same region with moderate tension applied as treatment for the swelling. Under sedation and local anaesthesia, the necrotic tissue was surgical sharply debrided with a scalpel blade and a curette. The affectation of the subcutaneous tissue was evident as well as the communication with the wound on the tuber calcanei. A new bandage was applied, the treatment was continued with the same drugs and metronidazole (20 mg/kg, PO, q 12 h) was also added to the treatment due the suspicion of an anaerobic/aerobic infection.

When the bandage was removed for the next review of the wound at 60 hours of hospitalization, the presence of subcutaneous fluid and crepitus was noticed on palpation, indicating the presence of gas in subcutaneous tissues. The presence of crepitation and subcutaneous fluid was evident in all the extremity mainly in the lateral aspect of the stifle. An ultrasound performed showed a large amount of subcutaneous fluid distributed throughout the limb, with echoic points consistent with gas accumulation. An incision in several points of the leg with a blade allowed to drain a foul-smelling liquid stained with blood (serosanguinous fluid). Malodorous exudate suggests anaerobic infection (*Aleman* et al. 2003).

Serosanguinous fluid was aseptically obtained with needle and syringe and cultured onto sheep blood agar plates. These were incubated both aerobically and anaerobically at 37 °C. and the colony growth was evaluated after 24–48 h post-inoculation. Pure pinpoint, beta-hemolytic colonies grew on sheep blood agar after 24 h of incubation under aerobic conditions. Anaerobic cultures failed to grow on blood agar after 48 h of incubation. Using the API 20 Strep system (bioMérieux, France) the aerobic isolate was identified as S. equi subsp. zooepidemicus. Definitive identification was made by sequencing of the PCR product obtained by amplification with universal bacterial 16S rRNAs primers (*Garcia* et al. 2012). A BLAST homology search (www.ncbi.nlm.nih.gov/BLAST) revealed that the nucleotide sequence of the amplified product matched (99 % identity) the 16S rRNA gene (partial sequence) of Streptococcus equi subsp. zooepidemicus strain ICMP 20920 (GenBank accession no. MG786415.1).

The poor prognosis of the horse after three days of hospitalization, contributed most to the owner's decision to euthanize the animal. Gross post-mortem examination showed extreme swelling and crepitus over the affected limb. Skin ulcers with marked serosanguinous fluid, degeneration and necrosis of skeletal muscles, formation of subcutaneous gas (subcutaneous emphysema) and pyogenic debris (figure 1). Post-mortem skeletal muscle samples were taken from the affected limb and placed into a fixative solution of 10 % neutral buffered formalin. Samples were dehydrated and embedded in paraffin, and tissue sections of 5-μm were stained by hematoxylin and eosin, and Gram's stain to carry out histopathology analysis.

Histopathological evaluation revealed ephitelial cells loss (epidermolysis) with infiltration of mononuclear cells in the musculature. Muscle fibres exhibited multifocal degeneration and presence of liquefying necrosis. According to these results, a necrotic myositis was described together with a pyogenic swelled without well-formed surrounding fibrous capsule (figure 2). Considering most of the observed signs, as the palpation of gas in the infected tissues; the histopathological presence of multifocal degeneration and necrotic myofibers without polymorphonuclear leukocytes and the presence of Gram-positive, rod-shaped microorganisms in muscle tissue (figure 3), strongly suggested the possibility of a mixed clostridial infection although no growth occurred under anaerobic conditions.

To confirm the suspicion of a clostridial infection, paraffin-embedded sections of the skeletal muscle tissues were used to detect the presence of pathogenic clostridia DNA by the multiplex polymerase chain reaction (PCR) previously described by (*Sasaki* et al. 2002). The sample was deparaffinized by two xylene and ethanol washing steps followed by DNA extraction using the QIAamp FFPE column (Qiagen, Spain).

A 343 bp amplification band was obtained and identified as Clostridium novyi type A on the basis of its specific length. The PCR product was directly sequenced in both directions with the primers FlaF and FlanaR. A BLAST search against the GenBank database showed (96 % identity) with the Clostridium novyi gene for flagellin protein fliA (A) (GenBank accession no. AB058936.1).

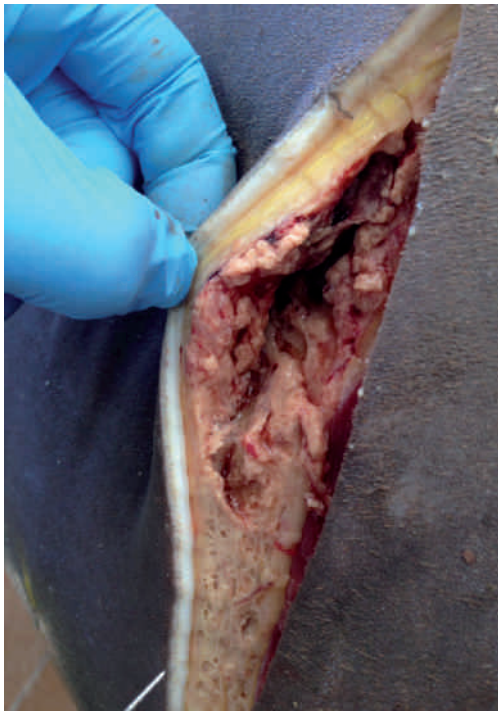

Fig. 1 Myonecrosis and gas formation. | *Myonekrose und Gasbildung.*

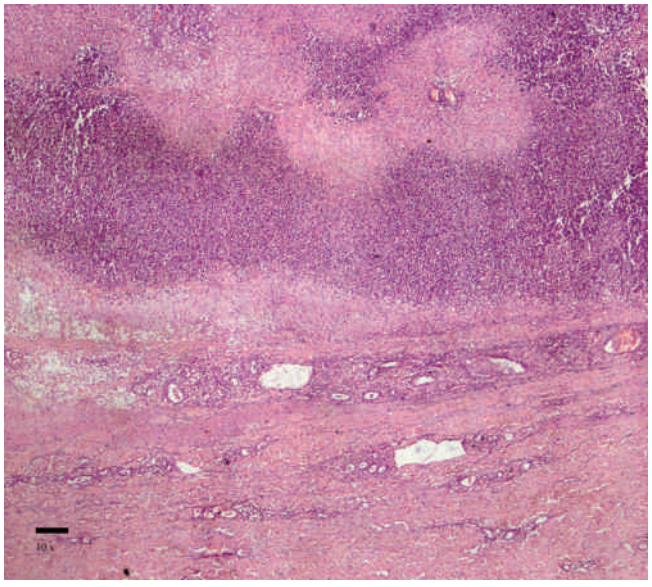

Fig. 2 Muscle necrosis and pyogenic inflammation without fibrous capsule (10 ×). | *Muskelnekrose und pyogene Entzündung ohne faserige Kapsel (10 ×).*

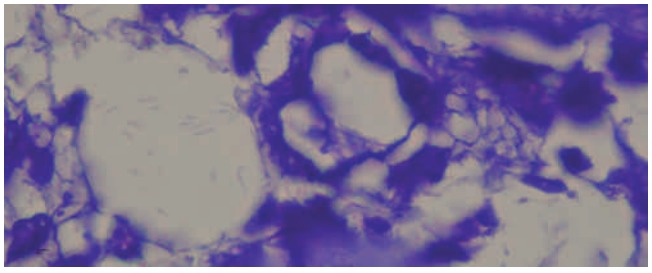

Fig. 3 Positive **rod**-shaped bacilli in Gram-stained smear of subcutaneous tissue (100 ×). | *Positive stäbchenförmige Bazillen im Gram-gefärbten Abstrich des subkutanen Gewebes (100 ×).*

Discussion

The only altered parameters that were detected in the analysis carried out to the animal were an increase in the total white cells count and also in the AST. These abnormal values are indicative of an active infectous condition and muscular damage, respectively. Increases in WBCs (leukocytosis) usually indicate infection or inflammation resulting from a bacterial infection (*Ricketts* 2004). Aspartate aminotransferase (AST) and creatine kinase (CK) are enzymes present in muscle tissue. When muscle tissue is damaged, these enzymes are released into the blood and can be detected in high concentrations (especially AST) in horses that suffer from myopathy. The CK and AST rise in concentration proportionally with the amount of damage. In the described case, the laboratory data, showed a normal CK level accompanied with an AST elevation, which indicates that muscular damage was already present. It is known that serum AST elevations are not specific for myonecrosis, its increments could also be due to hemolysis or internal organ damage (*Valberg* et al. 2009), specially in the liver, but in this case the absence of other biochemical and hematological abnormalities ruled out this possibility.

Streptococcus equi subsp. zooepidemicus is part of the commensal bacteria in horses, where it could act as an opportunistic pathogen that can cause disease in the upper respiratory tract, in the uterus, in the umbilicus, and in wounds. In fact, subspecies zooepidemicus is the microorganism most frequently recovered from wounds and abscesses from horses (*Lavoie* et al. 1991). This fact leads to the general recommendation of choosing antimicrobial therapy active against this microorganism in every traumatic open wound that is either infected or likely to become infected (*Clark* et al. 2008).

Clostridium novyi is commonly found in soil and feces of animals, existing in the form of exo-spores that can remain viable indefinitely. (*Navarro* and *Uzal* 2016). Anaerobes are also opportunistic pathogens that can cause infections when these bacteria find anaerobic conditions in tissue, usually as a result of the presence of necrotic tissue and the co-infection with aerobic or facultatively anaerobic bacteria. It has been previously described that severe S. equi subsp. zooepidermicus infections might decrease the blood flow and the perfusion of tissues in distal limb segments (*Fiorello* et al. 2008) promoting an anaerobic environment that allows the germination of C. novyi spores. This disruption of the blood supply results in protection of bacteria not only from the natural defense mechanisms of the horse, but also from antibiotics that need to be carried to the site of infection by the bloodstream (*King* 2006). C. novyi type A produces mainly the lethal, oedema-inducing alpha toxin (TcnA), and the non-lethal phospholipase, gamma toxin; this bacterium is associated with gas gangrene of human and animals.

In contrast to the first case of myonecrosis in horses caused by C. novyi type A, where it is assumed that the clostridial muscle infection resulted from the intramuscular injection of a vitamin complex in this case, previous infection with an opportunistic pathogen such as S. equi subsp. zooepidermicus is thought to have caused muscle damage or inflammation and, as a result, the blood and oxygen supply to certain parts of the muscle may have been low enough to allow spores

of Clostridium spp. to germinate. Several different species of Clostridium bacteria can cause myonecrosis (gas gangrene), as a result of the infection of muscle tissue. In Equine species, the following species have been reported: C. fallax, C. septicum, C. chauvoei, C. sporogenes, C. novyi Type A and, more commonly, C. perfringens (*Farias* et al. 2014).

Isolating and identifying C novyi is difficult due to its severe anaerobic nature. It is also laborious and demanding to culture, requiring the presence of thiols (*Moore* 1968). Clostridium novyi type A requires total handling in an anaerobic chamber. If anaerobic jars are used, these should be left unopened for at least 48h before the plates are examined (*Brazier* et al. 2002).

Probably, in our case, exposure to oxygen could explain the lack of growth of C. novyi type A on blood agar plates incubated for 48 hours in an anaerobic bag system (Bio-Bag system, EEUU). The strict anaerobic requirement as well as the difficult culture of the C. novyi type A may have resulted in the agent being overlooked or misidentified.

Conclusion

In the reported case, the absence of growth of C. novyi type A, the rapid advance of muscle lesions and the lack of an aggressive and rapid treatment based on the opening of the swollen tissues to get oxygen at the infected site, finished with the emergency euthanasia of the animal.

Clostridial myonecrosis is a quickly advancing condition associated with liquefying necrosis of muscle or other soft tissue, gas formation and clinical signs of toxaemia. Because of the life-threatening nature of this infection, rapid diagnosis and treatment should be considered by the clinician to increase the survival of the affected horses.

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Conflict of interest

The authors declare that there is no conflict of interest.

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