Immune-mediated hemolytic anemia associated with chronic fibrosing mediastinitis in an Arabo-Friesian horse

Immuungemedieerde hemolytische anemie geassocieerd met chronisch fibroserende mediastinitis bij een Arabo-Fries paard

[1]C. Ribonnet, [2]V. Saey, [2]R. Ducatelle, [2]K. Palmers

[1]Dierenkliniek De Morette, Edingsesteenweg 237, B-1730 Asse
[2]Vakgroep Pathologie, Faculteit Diergeneeskunde, Universiteit Gent, Salisburylaan 133, B-9820 Merelbeke

caroline.ribonnet@demorette.be

ABSTRACT

In this report, a ten-year-old gelding with immune-mediated hemolytic anemia associated with chronic fibrosing mediastinitis of unknown origin is described. The patient suffered from chronic weight loss and intermittent diarrhea for already several months. He was presented with severe anemia, anorexia and fever of a 24-hour onset. A direct Coombs test was highly positive for IgG auto-agglutination. No evidence of an underlying process was found on clinical examination. Post-mortem examination revealed green liquid material containing food particles in the cranial mediastinum and histology suggested chronic fibrosing mediastinitis. Even though perforation of the esophagus is a plausible cause, no signs of esophageal rupture were noted on macroscopic examination. This case shows that non-responsive, immune-mediated hemolytic anemia can be due to an undetected underlying disease, such as chronic mediastinitis.

SAMENVATTING

In deze casuïstiek wordt een tienjarige ruin beschreven met immuungemedieerde hemolytische anemie geassocieerd met chronische fibroserende mediastinitis van onbekende oorsprong. De patiënt leed aan chronisch gewichtsverlies en intermitterende diarree en werd aangeboden in de kliniek met uitgesproken anemie, anorexie en koorts die reeds 24 uur aanwezig was. Een directe Coombs' test was sterk positief voor IgG-autoagglutinatie. Bij het klinisch onderzoek werd geen onderliggend pathologisch proces van de immuungemedieerde hemolytische anemie gevonden. Post-mortemonderzoek toonde groene vloeistof met voedselpartikels in het craniale mediastinum aan. Histologisch onderzoek suggereerde chronische fibroserende mediastinitis. Hoewel slokdarmperforatie hiervoor een mogelijke oorzaak is, werden bij macroscopisch onderzoek geen tekenen van ruptuur waargenomen. In deze casereport wordt aangetoond dat bij niet-responsieve, immuungemedieerde hemolytische anemie, een niet-gedetecteerde onderliggende pathologie, zoals chronische mediastinitis, aanwezig kan zijn.

INTRODUCTION

Hemolysis refers to the intravascular or extravascular destruction of erythrocytes. Extravascular hemolysis, where red blood cells are phagocyted by macrophages in the spleen, liver and bone marrow, is always present with hemolytic anemia. Intravascular hemolysis refers to the lysis of red blood cells within the circulation, resulting in hemoglobinemia and hemoglobinuria (Valdez et al., 2015). Hemolysis can be immune-mediated (IMHA) or may occur secondary to infectious disease, oxidative injury, toxicities or conge-nital enzyme defects (Stockham et al., 1994; McCullough, 2003; Dunkel, 2018).

IMHA or antibody-mediated destruction of red blood cells can occur primary or secondary. Primary IMHA occurs when antibodies are directed against an intact erythrocyte surface. Examples of primary IMHA are neonatal isoerythrolysis, hemolysis after blood transfusion and hemolysis as part of autoimmune disorders like systemic lupus erythematosus (Geor et al., 1990; Dunkel, 2018). IMHA is classified

as idiopathic when it is of unknown origin (Dunkel, 2018).

Secondary IMHA occurs when antibodies react against an antigenically altered erythrocyte surface (Stockham and Scott, 2008; Dunkel, 2018). This has been reported after administration of certain drugs. Several cases associated to penicillin (Blue et al., 1987; Step et al., 1991; McConnico et al., 1992; Robbins et al., 1993) and potentiated sulfonamides have been documented in horses (Thomas and Livesey, 1998; Brumbaugh, 2001; Stockham and Scott, 2008; Cudmore et al., 2015; Dunkel, 2018). Certain infectious agents can induce secondary IMHA, e.g. *Rhodoccoccus equi* (Johns et al., 2011), *Clostridium perfringens* (Reef, 1983; Weiss and Moritz, 2003, Cottle and Hughes, 2010; Anderson et al., 2013), *Streptococcus equi* subspecies *equi* (Caniglia et al., 2014), and equine infectious anemia virus (Sellon et al., 1994). Also neoplastic processes can be involved in the pathogenesis including lymphosarcomas (Reef, 1984; Mair and Hillyer, 1991), lymphomas (Meyer et al., 2006; McGovern et al., 2011), leukemia (Cooper et al., 2018), malignant melanomas and splenic sarcomas (Mair et al., 1990; Underwood and Southwood, 2008; Stockham and Scott, 2008; Dunkel, 2018).

Immune-mediated hemolytic anemia can be diagnosed using different tests. In severe cases, erythrocyte auto-agglutination can be seen when looking at a slide with a drop of blood diluted with 0.9% NaCl to disperse normal physiological rouleaux or stacking of red blood cells (Hewetson, 2013; Dunkel, 2018). Another diagnostic test is the erythrocyte osmotic fragility test. It measures the degree of resistance of erythrocytes to osmotic swelling and lysis in saline solutions with increasing degrees of hypotonicity. In healthy animals, the percentage of hemolysis increases with hypotonicity following a sigmoid curve while in most horses with IMHA, the presence of subpopulations of erythrocytes with differing fragility ranges cause an altered multiphasic curve (Taylor and Cooke, 1990; Dunkel, 2018). The direct Coombs test or direct agglutination test (DAT) looks for antibodies attached to red blood cells circulating in the bloodstream and it is the most commonly used method for diagnosing IMHA (McCullough, 2003; Lording, 2008). In a small study on the detection of antibodies bound to erythrocytes in horses and dogs with IMHA, direct immunofluorescence flow cytometry showed higher sensitivity (100%) than the direct Coombs test (58%). Yet, immunofluorescence flow cytometry is not routinely available in every veterinary laboratory, and in Belgium, only available for research purposes (Wilkerson et al., 2000; Underwood and Southwood, 2008).

Treatment of IMHA is based on immunosuppressive drug therapy. Corticosteroids are still the first-line treatment for IMHA (McCullough, 2003; Dunkel, 2018). They reduce immunoglobulin production and their affinity for the red blood cell membrane, and inhibit the recognition system of macrophages for IgG, IgM and C3b (McCullough, 2003). Dexamethasone (0.1-0.2 mg/kg IV q24h) or prednisolone (1-2 mg/kg IV, IM or PO q24h) has been described for the treatment of IMHA in horses (McCullough, 2003; Zanella and Barcellini 2014; Dunkel, 2018; Swann et al., 2019). In refractory cases, other immunosuppressive drugs, such as azathioprine, cyclophosphamide or cyclosporine A, can be added to the treatment or replace the glucocorticosteroids (McCullough, 2003; Dunkel, 2018).

Mediastinitis is an uncommon and life-threatening pathology (Burnett, 1990; Pierce, 2000). The mediastinum divides the thoracic cavity in two lateral parts and contains the thoracic part of the esophagus and trachea, the pericardial sac, lymph nodes, blood vessels and nerves. It is divided into a cranial, middle and caudal part. Fenestrations are always present in the caudal mediastinum of a horse. These connect the left and right pleural cavities, which explains why pleural infections are often bilateral in the horse (Barone, 1966). In horses and humans, mediastinal infections are seen secondary to perforations of the thoracic esophagus or spreading of infection from the head and neck area along the fascial planes causing descending necrotizing mediastinitis (Pierce, 2000; Scaglione et al., 2007; Roman et al., 2015; Sumi, 2015; Choe, 2017; Sanchez, 2018). In human medicine, mediastinitis is often described after thoracic surgery. In horses and humans, primary respiratory tract infections rarely extend to the mediastinum (Pierce, 2000; Griffin, 2002). Primary mediastinitis is rare in humans (Pierce, 2000). Some cases of *Staphylococci*- and *Streptococci*-associated purulent mediastinitis and mediastinal abscesses have been reported in humans and horses primary of unknown origin or secondary to bacteriemia, pleuritis, strangles (Shishido et al., 1997; Griffin, 2002; Chang et al., 2009; Kouritas et al., 2012; Waller et al., 2014). Firshman (2003) reported a single case of fungal mediastinitis of unknown origin in a horse. In humans, the prognosis is poor and depends on a rapid diagnosis, start of antibiotic treatment and surgical intervention (Pierce, 2000). In this report, a case of IMHA associated with mediastinitis in an Arabo-Friesian gelding is described.

CASE HISTORY

A ten-year-old Arabo-Friesian (>50% Friesian blood) gelding was presented at the Equine Clinic de Morette with hyperthermia, anorexia, trembling and depression for already 24 hours. The horse had a history of intermittent diarrhea for already one year and weight loss over the last months. Because of his decreased appetite, the horse had been treated with omeprazole 2 mg/kg per os once a day for ten days.

Clinical examination

The horse was presented with fever (temperature 39.6°C) and a body condition of 2/9 (Henneke et al., 1983). Heart rate was 52 beats/min and respira-

tory rate was 16 breaths/min. Cardiac, thoracic and abdominal auscultations were within normal limits. Mucous membranes were pale and the capillary refilling time was less than two seconds. The jugular vein filling was normal.

Hematologic examination revealed severe macrocytic, hypochromic anemia and hemoglobinemia (hematocrite 13.8%, reference value 30-46%; Hemoglobin 4.7g/dL; reference value 11-17g/dL; MCV 67.7fL, reference value 37-50 fL; MCH 23.2 pg, reference value 14-20pg; MCHC 34.3g/dL, reference value 36-41g/dL), mild leukocytosis (11,630/µL, reference value 5000-10000/µL) with neutrophilia (10,653/µL, reference value 3000-7400/µL; bands: 0/µL), and lymphopenia (686/µL, reference value 1500-4000/µL). Thrombocytes were within normal limits (224,220/µL, reference value 100,000-300,000/µL). Blood biochemistry showed no abnormalities with a total protein of 63g/L (reference value 52-70g/L), albumin 30.3g/L (reference value 25-39g/L) and an albumin/total protein ratio of 0.48. Other inflammatory parameters, like globulins, fibrinogen and serum amyloid A, were normal as well. Renal blood parameters were increased (creatinine: 210.4 µmol/L, reference value: 76.9-155.5µmol/L; ureum: 11.66 mmol/L, reference value: 3.16-6.49mmol/L). Cardiac Troponin I was normal on admission (< 0.01µg/L). A direct Coombs' test was performed with monovalent Coombs' reagents (Piek et al., 2012). The test was highly positive for IgG auto-agglutination at 37°C with a titer of 1024 and 2048 for the fragment crystallisable (Fc) portion and the heavy and light (H+L) chain, respectively; but negative for IgM at 4°C. Urine was macroscopically normal. Dipstick analysis was positive for hemoglobin. Other parameters of (semi-)quantitative examination were normal and urine sediment examination was within reference range.

On rectal examination, no abnormalities were noted. A fecal sample was negative for worm egg count and culture of *Salmonella*, *Yersinia* and *Campylobacter*. A rectal biopsy did not reveal any significant pathology.

Abdominal ultrasound revealed a moderately distended stomach (five intercostal spaces, reference range 3-4 intercostal spaces), considering the anorectic state of the horse (Reef et al., 2004). No other abnormalities were seen during the abdominal ultrasound examination. Thoracic ultrasonography showed normal reverberation lines including in the cranial lung overlying the mediastinum in the third and fourth intercostal spaces. There was no pathology detected on radiography of the chest neither.

Abdominocentesis showed pure transudate with normal lactate and cytology.

The day after admission, endoscopy of the upper airways and guttural pouches showed no pathology and an intact trachea, but slightly increased mucopurulent tracheal secretions were present (grade 1/5) (Gerber et al., 2004). Bacteriological examination of the tracheal aspirate showed a complex mixed flora of bacteria with numerous colonies of *Kluyvera intermedia*. No significant abnormalities were found on cytological examination of the tracheal aspirate.

Endoscopy of the esophagus was normal and no gastric ulceration was noted; however, delayed gastric emptying was suspected since impacted food material was present after 24 hours and even after 48 hours of starvation. An oral glucose tolerance test showed no signs of malabsorption.

A treatment with sodiumbenzylpenicillin (22.000 UI/kg IV QID), intravenous fluid therapy (Ringer Lactate 90mL/kg/day) and dobutamine continuous rate infusion (CRI rate 2µg/kg/min) were initiated. Renal parameters improved only slightly after 72 hours of treatment; creatinine decreased from 210.4 µmol/l to 197 µmol/l. After three days of treatment, dexamethasone (0.05mg/kg IM SID) was added to the regime, fluid therapy was decreased to 50mL/kg/day and dobutamine CRI was ceased. Thereafter, creatinine improved further and returned within normal limits ten days after admission.

As the anemia didn't improve after seven days of corticosteroid treatment (hematocrite 15 %), a bone marrow aspiration was performed. The smear revealed erythroid hyperplasia: moderate cellularity, an increased amount of erythroblasts (44%, reference value 2-20%), 5% of proerythroblasts (reference value <5%) and no pyknotic cells and thus normal red blood cell lines with normal maturation status and a myeloid/erythroid ratio of 0.9 (reference value M:E 0.5-1.5). These findings are suggestive of a regenerative anemia; whereas the peripheral blood smear revealed clear agglutination indicative of immune-mediated hemolytic anemia.

After ten days of corticosteroid therapy, the hematocrite remained unchanged. The dexamethasone treatment was therefore increased to 0.2mg/kg IM SID. The next day, the horse developed sinus tachycardia (HR 70–80 bpm) unrelated to clinical symptom, such as pain, dehydration, hypovolemia or worsened anemia. Cardiac troponin I was moderately elevated (0.731µg/l, reference range <0.0086µg/l). Echocardiography revealed mild pericardial effusion and marked thickening of the interventricular septum (IVSd: 4.3cm, reference value 2.8 ± 0.2cm; BW-corrected IVSd: 2.7cm; IVSs: 6.7cm, reference value 4.6 ± 0.5cm, BW-corrected IVSs: 4.3cm) and left ventricular free wall (LVFWd: 4.5cm, reference value 2.5 ± 0.3cm, BW-corrected LVFWd: 2.2cm; LVFWs: 5.95cm, reference value 3.8 ± 0.3cm, BW corrected LVFWs: 3.7cm). Dimensions of the left ventricular lumen were diminished in systole (LVIDd 10.5cm, reference value 11.2 ± 0.8cm, BW-corrected LVIDd: 10.4cm; LVIDs 4.4cm, reference value 7.3 ± 0.8cm, BW-corrected LVIDs: 6cm). Fractional shortening was increased (58%, reference value 34.8%) (Slater and Herrtage, 1995; Al-Haidar, 2017). The combination of these findings is suggestive for myocardial

disease.

Azathioprine (3mg/kg PO SID) was added to the treatment protocol after twelve days of corticosteroid therapy due to non-responsive anemia.

Because of the lack of response to medical treatment and the persistent tachycardia, the horse was euthanized after fifteen days of hospitalization.

Post-mortem findings

Necropsy

A complete necropsy was performed. The mediastinum showed green discoloration and emphysema. In the ventral part of the mediastinum, just cranial to the pericardium, there was a well-delineated swelling. The swelling contained one liter of green fluid with food particles (Figure 1). The lung parenchyma showed diffuse edema and congestion; lymph nodes were macroscopically normal. A mild increase in pericardial effusion was present but no other macroscopic abnormalities of the myocard were noted. Discrete swelling of the ventral part of the neck was noted due to subcutaneous edema. The complete esophageal mucosa and adventitia were intact. Focally, about 10 cm cranial to the cardia, the muscular layer of the esophagus showed moderate hypertrophy. There were no macroscopic lesions detected in the trachea. The small intestinal wall was moderately edematous and the content was fluid. Post-mortem evaluation of the kidneys was impossible due to severe autolysis.

Histology

Tissue samples of the mediastinal swelling and heart were fixed in phosphate-buffered formalin, embedded in paraffin wax and cut into four-micrometer-thick sections. Slides were stained with hematoxylin and eosin (HE) and Von Kossa (VK) stain. Samples of the mediastinal swelling consisted of fat and dense, fibrous tissue, in which mixed, basophilic bacterial colonies (cocci and bacilli) were present (Figure 2). The fibrous tissue multifocally showed a degenerated, hyalinized aspect with secondary dystrophic calcification indicating a chronic process (Yi, 2017) (Figure 3). Multiple plant fibers were noticed inside this fibrous tissue and some of these fibers also showed a mineralized aspect.

There were mild mixed inflammatory infiltrates multifocally in the myocardial interstitium and a focal, discrete interstitial zone of fibrosis and inflammatory infiltrates.

DISCUSSION

To the authors' knowledge, this is the first case report in which immune-mediated hemolytic anemia associated with chronic fibrosing mediastinitis of unknown origin is described in a horse.

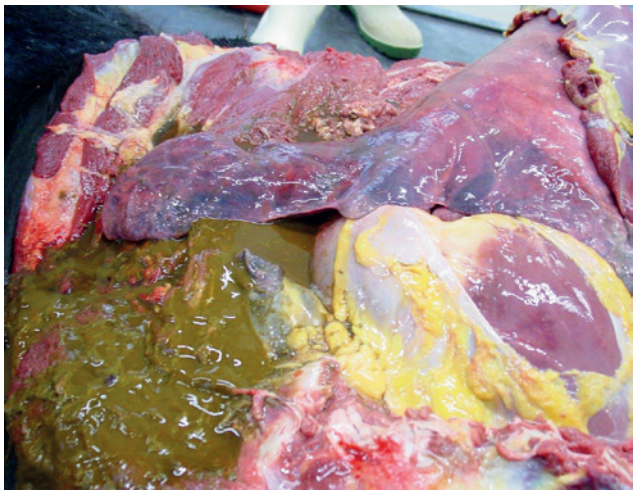

Figure 1. Mediastinal swelling, containing a green sticky fluid with food particles at necropsy.

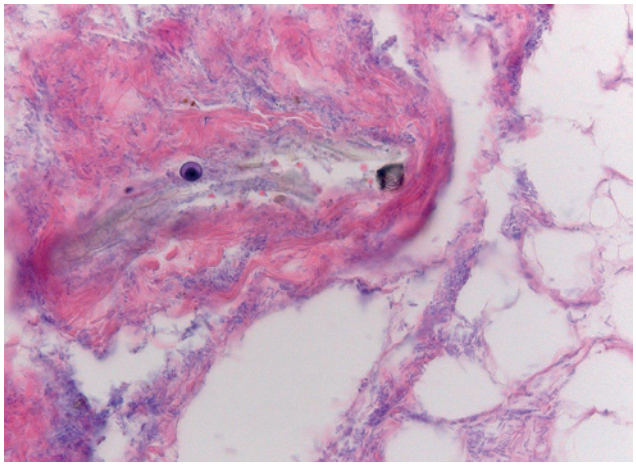

Figure 2. Hematoxylin and eosin stain x 200 of the cranial mediastinal swelling. Dense, fibrous tissue admixed with foreign bodies of plant origin and mixed, bacterial colonies.

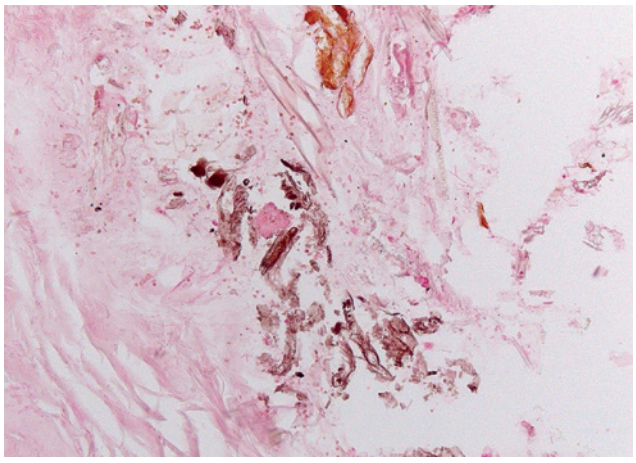

Figure 3. Von Kossa stain x 200 of the cranial media-stinal swelling. Areas of dystrophic mineralization (staining brown-black) surrounding foreign bodies of plant origin.

In the absence of a pre-mortem underlying cause of the IMHA, the horse was treated as suffering from idiopathic IMHA. Therefore, treatment was focused on decreasing the immune-mediated destruction of erythrocytes by increasing dosages of immunosuppressive medication. The elevation of serum creatinine could be related to immune-complex glomerulonephritis. This could explain the decrease in serum creatinine values with increasing dosages of corticosteroids, despite cessation of fluid therapy. On the other hand, no important protein loss was present in the urine as described in cases of glomerulonephritis (Schott et al., 2018). Anorexia and protein catabolism due to muscle wasting are a potential explanation for the azotemia as well, since the horse had an improved appetite and gained weight during his stay (Stockham and Schott, 2008; Hall, 2016). With exception of the mediastinitis, no other possible etiologies for IMHA could be identified in this case. The horse had no history of antibiotic administration prior to admission. He had only been administered omeprazole prior to hospitalization and to the authors' knowledge, omeprazole has not been reported as a cause of IMHA in horses. However, it has been described as a cause of IMHA in humans (Marks et al., 1991; Butt et al., 2007). Despite the discontinuation of omeprazole upon arrival at the hospital, the signs of IMHA did not improve in this horse.

The most likely anatomical cause of food contamination in the mediastinum is rupture of the esophagus, even though no macroscopic signs of rupture were found along the esophageal mucosa or adventitia in this case. Considering the chronicity of the clinical signs, a healed perforation of the esophagus was a plausible underlying cause of the mediastinitis. Food contamination of the thoracic cavity due to rupture of the esopaghus is an uncommon event in horses. By the time the unspecific clinical signs become more obvious, severe complications like pleuritis, endotoxemia and sepsis, are usually present resulting in a very poor prognosis (Dechant, 1998; Gonzalez et al., 2008; Cathcart et al., 2013). In most equine cases, thoracic esophageal rupture leads to accumulation of fluid in the pleural cavity. In those cases, ante-mortem diagnosis can be made with help of medical imaging and thoracocentesis, where septic exudate with alimentary particles can be aspirated (Dechant, 1998; Gonzalez et al., 2008; Cathcart et al., 2013; Hepworth-Warren et al., 2015). There were no findings at pre-mortem examination indicating esophageal rupture or septic contamination of the thorax in the present case. Food contamination stayed constrained to the mediastinum preventing detection by thoracic ultrasonography and radiography. The compartmentalization of the pathological process could also be responsible for the presence of the mild leucocytosis and normal acute phase proteins on the blood analysis.

In human medicine, mediastinitis is usually diagnosed by CT-scan of the chest (Pierce, 2000; Kocher et al., 2012; Choe et al., 2017). Radiography and ultrasonography of the chest have poor sensitivity for the diagnosis of disease limited to the mediastinum in horses; and more advanced techniques, as CT-scan or MRI, are not routinely available for the diagnosis of thorax pathology. In addition, the cranial mediastinum is not clearly visualized in the horse using thoracoscopy (Vachon and Fischer 1998; Griffin, 2002).

During hospitalization, gastroscopy was performed on two occasions and no lesions in the esophagus were detected. Moreover, the horse had a normal appetite throughout the hospitalization. Nevertheless, earlier in the disease process, contrast radiography of the esophagus could have been used to detect a possible perforation (Butler et al., 2017). It is interesting to note that the current case presented moderate idiopathic hypertrophy of the muscularis of the distal esophagus (IMHO). IMHO is characterized by a thickening of the distal portion of the esophagus involving the circular layer of the tunica muscularis without signs of fibrosis or inflammation. The etiology, pathophysiology and clinical significance of IMHO in horses are still questionable (Benders et al., 2004). Cathcart et al. (2013) described the first case of IMHO as a cause of spontaneous thoracic esophageal rupture. The defect in the esophagus was located within the hypertrophied part creating a small insidious channel between the submucosal and muscular layers. The authors suggested that this could have resulted in pulsatile release of small amounts of organic material and prevented the tear to extend further, causing a slow progression of disease for an unknown period of time (Cathcart et al., 2013). In the present case, the part of the esophagus in the cranial mediastinum did not show muscular hypertrophy, and therefore, this finding on post-mortem examination was probably not relevant as a cause of the mediastinitis.

As the horse in this report was an Arabo-Friesian horse, it is also interesting to look into breed-related disorders. Friesian horses are predisposed to esophageal disorders like megaesophagus and IMHO (Bezdekova and Janalik, 2016). In fact, in a study by Komine et al. (2014), esophageal disorders were identified in approximately 35% of the examined Friesian horses, where IMHO was the most prevalent finding, while the prevalence in other breeds was less than 3%. In the study, it has been suggested that IMHO in Friesian horses may be a different condition than in non-Friesian horses and may be of clinical significance in this breed (Komine et al., 2014). An underlying genetic neuromuscular disorder has been suggested (Van der Kolk et al., 2011), while other authors suspect a connective tissue disorder responsible for the high prevalence of esophageal and gastric disorders in Friesian horses (Komine et al., 2014; Ploeg et al., 2015a; Ploeg et al., 2015b; Saey, 2016; Winfield and Dechant, 2015). In recent research, where the connective tissue of the aorta and flexor tendon were analyzed in this breed, differences have been shown in

connective tissue metabolism, which makes it more prone to rupture (Ploeg et al., 2017). In a recent study by Saey et al. (2018), a higher rate of collagen degradation in Friesian horses has been suggested.

Anderson et al. (2013) reported a case of clostridial myonecrosis in a gelding with secondary complications, such as IMHA and transient hypertrophic cardiomyopathy. The fluid in the mediastinum was not cultured in the present case, but an infection with *Clostridium* bacteria is a plausible explanation for the mediastinitis, non-responsive IMHA and sudden onset of cardiomyopathy. The pericardial effusion and interstitial myocarditis in the present case could also have been due to the close communication between media-stinum and heart. In human medicine, mediastinitis with subsequent pericardial involvement has been described (Scaglione, 2007; Roman et al., 2015; Ulusan and Koc, 2016). In a study by Roman et al. (2015), out of fifteen patients with descending necrotizing mediastinitis, four showed pericarditis and two myocarditis.

The culture of tracheal aspirate revealed the presence of *Kluyvera intermedia*. This enterobacteria is rarely isolated in horses (Durham, 2018) and sporadically in humans (Carter and Evans, 2005; Thele et al., 2017). According to the human literature, this bacteria is considered part of the normal flora of the digestive tract in humans and ubiquitously present in water and soil. In this case, the isolation of *Kluyvera intermedia* was considered contamination as it was isolated in a complex mixed flora of bacteria and no signs of clinical infection were present on cytology of the tracheal aspirate.

CONCLUSION

In this case report, the difficulty of differentiating non-responsive idiopathic IMHA from IMHA where the underlying cause remains undetected, is illustrated. With the currently available imaging modalities, it may be difficult to diagnose mediastinitis, which might be involved in the pathogenesis of IMHA in the horse.

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