Mycobacterium avium subsp. *hominissuis* infection in two sibling Fjord horses diagnosed using quantitative real time PCR: a case report

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ABSTRACT: This report describes new possibilities for intravital and *post mortem* diagnosis of avian mycobacteriosis in horses using the quantitative real time PCR (qPCR) method. Using this method, *Mycobacterium avium* subsp. *hominissuis* was diagnosed in two sibling Fjord horses. In the first horse, *M. a. hominissuis* was detected by qPCR in numbers of 2.89×10^5 and 1.47×10^4 cells per 1 g of intestinal content and mesenteric lymph nodes, respectively; in the second horse, faeces and mesenteric lymph node samples showed numbers of 6.31×10^5 and 3.36×10^6 cells per 1 g of tissue, respectively. Another aim of this study was to comprehensively describe clinical and pathological findings in both animals.

Keywords: *Mycobacterium avium* complex; mycobacteriosis; qPCR; IS*1245*; zoonosis

Horses seem to have a native resistance to mycobacterial infections (Buergelt et al., 1988). Despite this, pathomorphology, clinical signs and epidemiology have been described for many cases of infection in horses caused by different mycobacteria species (Buergelt et al., 1988; Lofsted and Jakowski, 1989; Flores et al., 1991; Gunnes et al., 1995; Leifsson et al., 1997). As reviewed by Pavlik et al. (2004), after the successful control of bovine tuberculosis in cattle and other animal species in Central European countries, *Mycobacterium avium* complex (*MAC*) members have been the most commonly found causal agents of mycobacterial infection in horses. As it stands, mycobacterial infections caused by *MAC* are occasionally diagnosed in horses in Europe (Aupperle et al., 2008; Pavlik et al., 2008; Ryhner et al., 2009; Kriz et al., 2010a). However, infections of horses caused by *M. bovis* are still reported from countries in which bovine tuberculosis in cattle has not yet been eradicated (Keck et al., 2010).

Taxonomically, the *MAC* consists of the following members: *M. a. avium,* which causes avian tuberculosis, especially in birds and other animals, and *M. a. hominissuis* and *M. intracellulare* which cause avian mycobacteriosis in pigs and humans in particular (Thorel et al., 1990; Pavlik et al., 2000; Mijs et al., 2002; Shitaye et al., 2006; Pate et al., 2009; Kaevska et al., 2010, 2011; Kriz et al., 2010b; Skoric et al., 2010).). Recently, new species, i.e., *M. colombiense* and *M. chimaera* belonging to the *MAC* were diagnosed in humans (Tortoli et al., 2004; Murcia et al., 2006; Vuorenmaa et al., 2009).

Clinical symptoms in horses infected with *MAC* usually involve chronic, progressive weight loss, poor appetite, diarrhoea and intermittent colic, recurrent episodes of pyrexia and oedema (Knottenbelt and Pascoe, 1994). Horses with *MAC* infection usually develop the intestinal form of the disease, suggesting oral uptake of bacilli as the portal of entry. The pathological lesions in intestines

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caused by *MAC* infection are of chronic proliferative character and are rarely found to caseate and calcify unlike those seen in bovine tuberculosis (Buergelt et al., 1988).

The intravital and post mortal diagnosis of infections caused by *MAC* members in horses is limited due to their rarity, non-specific clinical signs and time-consuming culture examination of faeces, biopsies, blood or other biological material, as reviewed previously (Pavlik et al., 2004). A new diagnostic method, i.e., quantitative real time PCR (qPCR), was developed for precise detection and quantification of *MAC* species in different tissues of pigs and poultry or in soil, dust or sediments (Slana et al., 2010).

We have recently described *M. a. hominissuis* infection in two sibling Fjord horses (Kriz et al., 2010a). The first aim of this study was to check new methods for intravital diagnostics of *M. a. hominissuis* using qPCR in bioptic material and faecal samples from these two sibling Fjord horses. The second aim was the comparison of the relevance of clinical and pathological findings with previously published observations (Buergelt et al., 1988; Lofsted and Jakowski, 1989; Flores et al., 1991; Gunnes et al., 1995; Leifsson et al., 1997).

Case description

Case 1

History and clinical findings. On 9th February 2009, a two-year-old Fjord colt (245 kg) with good deworming and vaccination history was admitted to the clinic. The horse showed apathy, diarrhoea, anorexia and wasting during the course of three weeks. Intermittent diarrhoea and poor appetite were observed during the clinical examination. The haematological profile showed thrombocytosis, whereas other parameters were within normal limits. Serum biochemistry revealed hypoalbuminaemia, increased activity of alkaline phosphatase and lactate dehydrogenase. Other parameters were within normal limits. These multiple diagnostic procedures were inconclusive. Cyathostomosis was suspected and appropriate treatment was administered.

The clinical state improved only slightly and temporarily. The results of repeated clinical examination were vague and similar to those presented before. Ultrasonographic examination showed a nodular mass and a thickened small intestinal wall, which was the reason for the diagnostic celiotomy. Firm multiple nodular formations were found on the distal part of the jejunum, large colon and caecum, which widened into the mesenterium and mesenteric lymph nodes along the blood vessels. A dubious to poor prognosis was made in this case and the horse was euthanized at the request of the owner. On the same day, a *post mortem* examination was performed.

Necropsy findings. The horse was cachectic and a small volume of serous liquid (approx. 0.5 l) was present in the abdominal cavity. Mesenteric, caecal and colonic lymph nodes were markedly enlarged, on the cutting surface displayed obvious yellow and white caseous nodules of different sizes from 0.1 to 0.5 cm in diameter. The oedema of the mesenteric root and dilation of the mesenteric lymphatic ves-

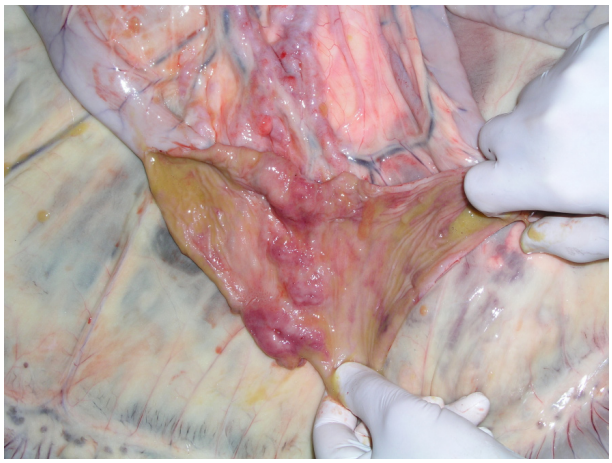

Figure 1. Horse 1, jejunum: granulomas in the jejunal wall and thickening of lymphatic vessels

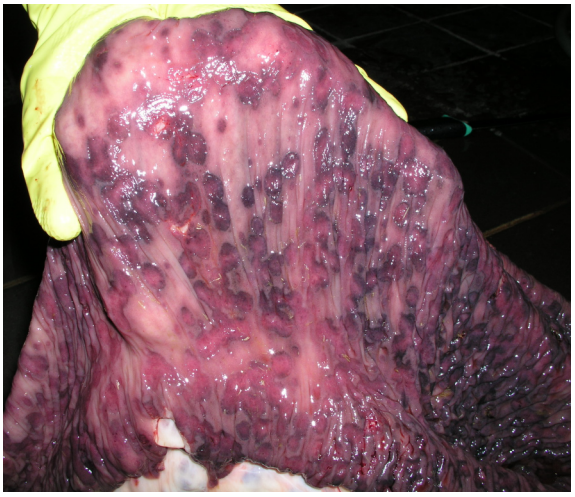

Figure 2. Horse 1, colon ascendens: circular lesions in mucosa

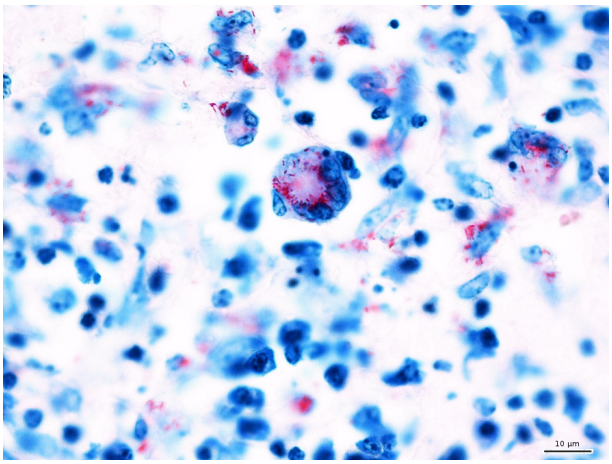

Figure 3. Horse 1, mesenteric lymph node: giant multinucleated cell of Langhans´ type with phagocytised mycobacteria, ZN, 1000× magnification

sels with numerous small haemorrhages on the mesenterium was a further noteworthy observation. A few firm, not well demarcated nodules (between 3 and 4 cm in diameter) protruding into the intestinal lumen, were found within the walls of the distal jejunum (Figure 1). On the mucous membrane of the ventral colon and caecum, multiple small petechial haemorrhages and hollowed circular lesions (0.5 to 1.0 cm in diameter) were present (Figure 2). The *Colon ventrale dextrum* was more affected macroscopically than the *colon ventrale sinistrum*. Erosions on the cutaneous part of the stomach mucosa around the *margo plicatus* were also found. The other organs in the abdomen and thoracic cavity were without macroscopic lesions.

In the mesenterium and mesenteric lymph nodes, nodular to diffuse granulomatous and sporadically pyogranulomatous inflammation was detected using light microscopy after the staining of paraffin-embedded tissue samples with haematoxylin and eosin (HE). The presence of a large amount of multinucleated giant cells of Langhans'-type, lymphocytes and epithelioid macrophages was detected in these lesions. Granulomas were necrotized centrally without signs of mineralization; pyogranulomatous formations were also seen. Fibroplasia of different intensity was found on the periphery of granulomas as well. Sporadic granulomatous and pyogranulomatous inflammatory lesions were also present in the jejunum and ventral colon. There were no signs of caseation or calcification in the intestine. Inflammatory lesions of the ventral colon were localized mainly within the submucosa and less often within the mucosa, which showed erosions.

Additional staining was also performed. PAS staining was negative. Gram staining detected mildly Gram positive coccoid bacteria. Staining with Ziehl-Neelsen (ZN) method detected a large amount of acid-fast small-sized bacilli in clumps localized mainly intracellularly (Figure 3).

Paraffin sections and impression smears of the mesenteric lymph nodes and jejunum were examined using qPCR (Slana et al., 2010), which revealed 2.89×10^5 and 1.47×10^4 *M. a. hominissuis* cells per 1 g of intestinal content and mesenteric lymph nodes, respectively.

Case 2

History and clinical findings. On 7th June, 2009, a one-year-old Fjord filly (210 kg), the sister of the colt described above, was admitted to the clinic. Before admission, the filly exhibited lethargy, intermittent diarrhoea and bilateral nasal discharge. The body condition was poor with increased heart rate and intermittent diarrhoea being recorded. Haematological examination revealed a decreased PCV, thrombocytosis and bands in peripheral blood, whereas serum biochemistry showed decreased albumin, increased alkaline phosphatase and lactate dehydrogenase activity. Slightly increased amounts of anechogenic peritoneal fluid and thickening of small parts of the left colon with decreased peristalsis were identified by abdominal ultrasonography. A diagnostic celiotomy was completed on 18th June, 2009. Thickened intestinal walls of both caudal jejunum and ileum and slightly enlarged mesenteric lymph nodes were found. Multiple biopsies were taken from altered tissues for histopathological and microbiological examinations.

The histopathological examination showed mild infiltration of the intestinal wall by lymphocytes and eosinophilic granulocytes and dilation of lymphatic vessels in the stroma of the intestinal villi. Red blood cells, phagocytosis of erythrocytes in subcapsular sinuses, mild hyperplasia of lymphatic tissue and, occasionally, clumps of epithelioid cells were observed. Staining with ZN and impregnation using Warthin-Starry method was negative.

Tissue biopsies and faecal samples were examined using PCR for *Lawsonia intracellularis* using a manual technique, including specific primers (KRD molecular technologies, Czech Republic), with

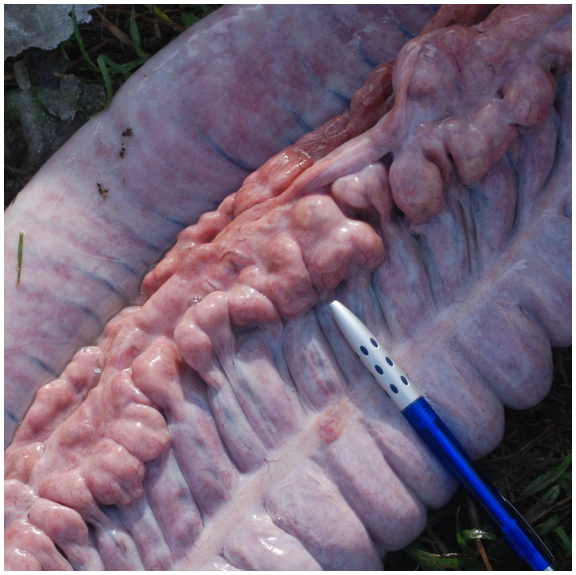

Figure 4. Horse 2, colonic lymph nodes: markedly enlarged lymph nodes of the ventral colon

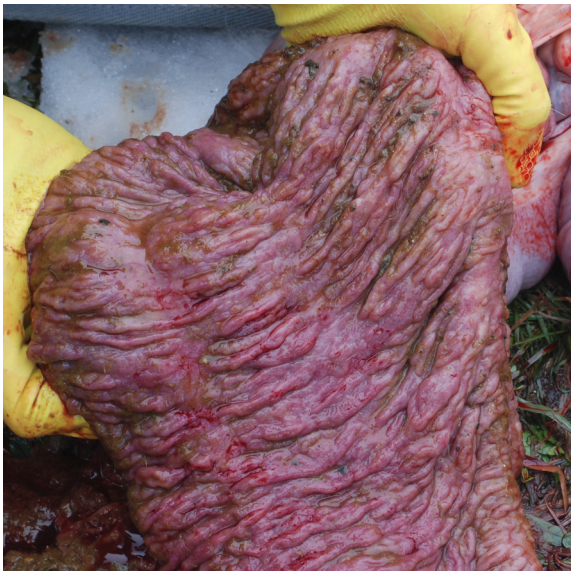

Figure 5. Horse 2, ventral colon: corrugated mucosa

negative results. The qPCR examinations according to Slana et al. (2010) confirmed the presence of *M. a. hominissuis* in faeces and the mesenteric lymph node in the numbers of 6.31×10^5 and 3.36×10^6 cells per 1 g of tissue, respectively. However, qPCR examination of a small intestinal biopsy and additional samples (guttural pouch flush and tracheal wash) were both negative for *M. a. hominissuis*. Microscopic examination of mesenteric lymph nodes showed the sporadic presence of characteristically shaped acid-fast bacteria. The filly was discharged with a diagnosis of mycobacterial bowel infection. The ensuing treatment included clarithromycin (7.5 mg/kg bw PO every 12 h) and rifampin (10 mg/kg bw PO every 12 h).

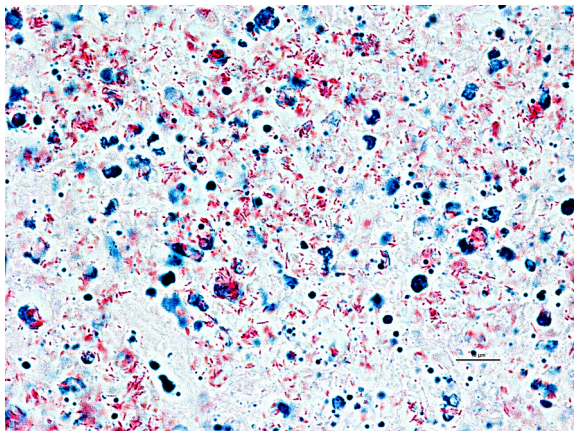

Figure 6. Horse 2, mesenteric lymph node: a massive presence of rod-shaped mycobacteria localized extracellularly and within the cytoplasm of macrophages, ZN, 1000× magnification

The clinical status of the filly was stable under this type of treatment for the next three months. Subsequently, the filly's condition deteriorated and the animal was euthanized on 22nd October, 2009. A *post mortem* examination was performed immediately.

Necropsy findings.The filly was cachectic, with oedema of the pelvic limbs. Mucous membranes and conjunctiva were pink in colour and a small volume of watery peritoneal fluid (approx. 0.5 l) was found in the abdominal cavity. Mesenteric, caecal and colonic lymph nodes were severely enlarged (Figure 4). Nodules of different sizes with caseous centres were visible on the cutting surface, in some cases the serous and serohaemorrhagic fluid escaped during cutting. Lymphatic vessels and the small intestine were without lesions when observed macroscopically. The mucosa of the ascending colon was slightly thickened and corrugated (Figure 5). The stomach, spleen, liver, kidney and lungs did not display any changes when observed macroscopically; the right ventricle was dilated.

Microscopically, after HE staining, multiple granulomas formed by lymphocytes and epithelioid macrophages were visible in the mucosa of the ascending colon, without the presence of multinucleated giant cells and central necrosis. The ZN stained tissue sections contained a small volume of tiny red rods localised intracellularly.

No gross lesions were found in the small intestine. Granulomatous inflammatory lesions were seen in the mesenteric lymph nodes. Granulomas had necrotic centres without signs of mineralization; a high amount of lymphocytes and epithelioid macrophages were present around them, as well as fibroplasia; multinucleated giant cells occurred sporadically.

After ZN staining, numerous acid-fast rods were observed in the cytoplasm of the epithelioid macrophages as well as extracellularly (Figure 6). Numerous small granulomas without necrosis were found in the liver; the spleen showed reactive hyperplasia of lymphatic tissue and hyperaemia of the sinuses. The kidneys were without gross lesions. The lungs showed signs of focal bronchointerstitial pneumonia with the presence of a mixed peribronchial and interstitial inflammatory infiltrate. Specimens of tissues (mesenteric and colonic lymph nodes, colon ascendens, liver, spleen, kidney and diaphragm) were examined using cultivation and the qPCR method; *M. a. hominissuis* was detected in all samples.

DISCUSSION AND CONCLUSIONS

M. a. hominissuis is a potential pathogen commonly occurring in the environment, above all in soil and water (Kazda et al., 2009; Kaevska and Hruska, 2010a,b; Krizova et al., 2010). It can infect humans, and is frequently isolated from children with peripheral lymphadenopathy (van Coppenraet et al., 2008). *M. a. hominissuis* infections have been documented in pigs and cattle and more rarely in dogs, birds, deer and other animals (Dvorska et al., 2004; Shitaye et al., 2006, 2009; Haist et al., 2008; Moravkova et al., 2008).

Until the middle of 2010, the most commonly identified member of the *MAC* in horses was *M. a. avium* (Aupperle et al., 2008; Pavlik et al., 2008; Ryhner et al., 2009), whereas *M. a. hominissuis* was the most common isolate from tuberculous lesions in pigs (Pavlik et al., 2005; Shitaye et al., 2006). Recently, *M. a. hominissuis* infection in this two sibling Fjord horses was described in the Czech Republic (Kriz et al., 2010a).

Mycobacteriosis is an uncommon disease in horses, possibly due to a natural form of immunity. The most common sites of infection in cases reported to date were the alimentary tract and the mesenteric lymph nodes (Flores et al., 1991; Figure 1). A generalized form of mycobacteriosis (Potter, 1948; Mair et al., 1986) and bone cyst-like lesions caused by *MAC* members have also been described (Hewes et al., 2005). Intestinal infection may cause localized ulcers or thickened mucosal lesions resembling lesions of paratuberculosis in cattle (Buergelt et al., 1988; Brown et al., 2007).

Clinically, horses tend to exhibit chronic weight loss, diarrhoea, intermittent colic, recurrent episodes of pyrexia and hypoproteinemia (Knottenbelt and Pascoe, 1994). Similar signs, excluding fever and colic, were present in both sibling Fjord horses described in this study. A probatory laparotomy was performed for macroscopic viewing and tissue biopsy. During surgery, abdominal lymphoma, a chronic form of *Lawsonia intracellularis* infection and proliferative and inflammatory bowel diseases were included in the main differential diagnoses.

As a result of these diagnoses, the owner opted to euthanize the colt. Further examination confirmed mycobacterial infection in the first case and this led to similar diagnostic procedures being applied to mycobacterial detection in the second animal resulting in subsequent treatment. The treatment of mycobacteriosis in horses is usually not performed due to the frequent resistance of mycobacteria to the medication and the rapid deterioration of health status of the treated animal after withdrawal of the treatment (Pavlik et al., 2004).

In previously described case studies of *M. a. hominissuis* infection most horses showed alteration of the gastrointestinal tract with dissemination of the infection to other organs (Buergelt et al., 1988; Lofsted and Jakowski, 1989; Gunnes et al., 1995). The spleen, liver and lungs are considered to be the organs most frequently involved in the dissemination of tuberculous lesions (Flores et al., 1991), although Leifsson et al. (1997) described mycobacterial bilateral uveitis and disseminated granulomatous foci to be present also in the myocardium. Flores et al. (1991) and Hewes et al. (2005) reported avian tuberculosis dermatitis and septic arthritis, respectively.

The histopathological lesions observed in our sibling Fjord horses were similar to those previously described by other authors (Buergelt et al., 1988; Lofsted and Jakowski, 1989; Flores et al., 1991; Gunnes et al., 1995). Granulomatous inflammation of the mucosa and submucosa of the intestine with the presence of lymphocytes, epithelioid cells and multinucleated giant cells was observed by Flores et al. (1991). In the filly, multinucleated giant cells were only sporadically present in the lymph nodes. In both horses, there were no signs of caseation or

calcification in the intestine, while considerable caseation without evidence of calcification was found in the lymph nodes.

Equine *MAC* infections caused by *M. a. avium* of serotypes 1 and 2 and *M. a. hominissuis* of serotypes 4, 5 and 8 are not associated with overt congenital or acquired immunodeficiency syndromes. However, more subtle defects that predispose horses to *MAC* infections may go unrecognized. For example, human susceptibility to disseminated *MAC* infections has been associated with genetic defects in the IFN gamma and IL-12 immune responses (Oaks, 2007). However, it is likely that an altered immune system predisposed these horses to *M. a. hominissuis* infection because other horses (including foals) were not affected. As the horses were siblings, hereditary immunosuppression is likely.

In both our cases, the horses laboured with a gastrointestinal form of the disease, including severe lesions of the large intestine and mesenteric lymph nodes. In the colt, there was also involvement of the small intestine and mesenteric lymph nodes, whereas the filly exhibited gross lesions only in the large intestine and mesenteric lymph nodes. However, the infection was disseminated to the liver, kidneys, spleen and diaphragm, where *M. a. hominissuis* was confirmed using culture and/or qPCR methods.

A high number of microscopic granulomas were present in the liver. The lungs showed signs of focal bronchointerstitial pneumonia, but a mycobacterial etiology was not confirmed. Despite the necropsy findings in the filly, abdominal ultrasonography and laparotomy also showed a thickening of the small intestine wall. Biopsy samples taken from enlarged mesenteric lymph nodes during the laparotomy three months prior to euthanasia helped confirm *M. a. hominissuis* infection using qPCR, whereas the small intestine biopsy was negative. This suggests the usefulness of the qPCR method also for intravital diagnosis of *M. a. hominissuis* infection.

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