Co-Infection with *Theileria equi* and *Babesia caballi* in a Yearling Filly

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ABSTRACT

Equine piroplasmosis (EP) is a widely spread tick-borne disease of horses, caused by the hemoparasites *Theileria equi* and *Babesia caballi*. Although most horses in endemic areas are subclinical carriers of parasites, acute or peracute disease may occur, especially when naïve horses are infected. This case report presents a characteristic case of peracute and fatal EP in a yearling filly, that was transferred from stable to pasture with first exposure to ticks. The filly was diagnosed with high parasitemia of both *T. equi* and *B. caballi,* with co-infection of both parasites within the same erythrocyte. Post mortem findings revealed multi-organ damage (spleen, kidney, lungs and muscles) and systemic bleeding. Quantitative PCR revealed high parasitemia of both parasites, consistent with acute and clinical infection in the sick filly. An epidemiological investigation including molecular analyses of blood samples was conducted on the farm, in which of thirteen horses, two were carriers of *T. equi* and one was a carrier of *B. caballi* with similar genotypes to the filly. The described case emphasizes the importance of implementation of preventive measures when transferring naïve horses to tick infected pasture in endemic areas. In addition, identifying the previously undocumented presence of co-infection within the same erythrocytes raises questions regarding the pathogenesis and cell invasion mechanisms of these parasites, with possible future therapeutic implications.

Keywords: Equine Piroplasmosis; *Theileria equi*; *Babesia caballi*; Co-Infection, Horse.

INTRODUCTION

Equine piroplasmosis (EP) is a tick-borne disease of equids (horses, donkeys, mules and zebras), caused by the hemoprotozoan parasites *Theileria equi* and *Babesia caballi*. Clinical manifestation may range from subclinical carriage to peracute, life-threatening disease (1-3). Clinical signs are mostly non-specific and mainly attributed to hemolytic anemia caused by parasite replication in erythrocytes. The severity of clinical signs usually correlates with the parasite load and the magnitude of the anemia (2-4).

Equine piroplasmosis is endemic in most parts of the world and is one of the most widely distributed equine infectious disease (2, 3, 5). In endemic areas, most horses are subclinical carriers of parasites, while acute or peracute diseases are more often reported when naïve animals are infected for the first time (2, 3). Since carrier animals can serve as a source of infection to ticks (6), there is a risk of spread to non-endemic areas when horses are transported, leading to importation restrictions in several non-endemic countries (6, 7).

Both parasites are usually endemic in similar areas, where vector ticks are present (8). In endemic areas, a

large proportion of the horses are infected with one or both parasites and usually remain carriers for prolonged periods of time (2, 3, 5). If untreated, carriage of *T. equi* is considered life-long, while natural clearance is possible for *B. caballi* (2, 3). EP is endemic in Israel, with reported molecular prevalence of 65% and 9.3% for *T. equi* and *B. caballi,* respectively (9, 10).

In recent years, molecular characterization of both parasites identified several genotypes of each parasite, as well as novel, closely related species (11-17). In Israel, three *T. equi* genotypes (A, C and D) based on its *18S rRNA* gene sequence (4, 18) and two *B. caballi* genotypes (A1 and A2) based on its rhoptry associated protein-1 (*rap-1*) gene sequence (4, 9) has been identified. Co-infection with both *T. equi* and *B. caballi* have been reported in numerous studies (4, 19-25), however, the significance of simultaneous infection has never been investigated. This case describes a case of peracute EP with co-infection of both parasites.

CASE REPORT

A yearling Quarter horse filly was referred to the Koret School of Veterinary Medicine – Veterinary Teaching Hospital (KSVM-VTH) with severe lethargy and pedaling.

The filly, which was raised in a stalled facility, had been fully vaccinated and had no prior medical problems, according to the owners. A few weeks before presentation to the KSVM-VTH, the filly was transferred to a new farm in northern Israel, where it was kept in a stall overnight and turned out in pasture during the day.

On the day before her admission, the filly collapsed, while in pasture, and was found recumbent and weak. On physical examination by the referring veterinary clinician, severe dehydration, pale mucus membranes (MM), hypothermia (T=34.7°C, reference range (RR): 36.5-38.5°C), respiratory effort and unresponsiveness were noted. Complete blood count (CBC) revealed leukocytosis (white blood cells (WBC)=21.5x10^3/µl, RR: 4.3-14.8x10^3/µl), anemia (hematocrit=12.7%, RR: 31.1-50.5%; red blood cells=3.41x10^6/µl, RR: 7.2-12x10^6/µl); and thrombocytopenia (platelets (PLT)=40 x10^3/µl, RR: 69.9-250.8x10^3/µl). The filly was treated with 20 liters of lactated ringer solution (LRS) with 5% dextrose, sodium bicarbonate (25ml), vitamin B complex (Duphalyte, Zoetis, 1 L, IV), flunixin meglumine (Norbrook, 1mg/kg, IV), Dimethyl sulfoxide (DMSO, Vetmarket, 20 mg diluted in 1 L saline solution, IV) and antibiotics (Marbocyl, Vetoquinol, 4 ml, IV). After treatment the filly improved slightly, its body temperature normalized (37°C); it was able to stand and was referred to the KSVM-VTH for further treatment on the following morning.

Upon arrival at the hospital the filly was recumbent, weak, paddling and with respiratory effort. Physical examination revealed mild dehydration, pale-yellowish mucous membranes, tachycardia (heart rate (HR)=100 bpm, respiratory rate (RR): 24-40 per minute) and hypothermia (T=35.7°C). Neurological evaluation did not reveal abnormalities, and the filly was alert and responsive. Complete blood count revealed leukocytosis (WBC=28.8 x10^3/µl), anemia (packed cell volume (PCV)=13%, RBC=3.1 x10^6/µl) and thrombocytopenia (PLT=52 x10^3/µl). The presence of piroplasm parasites and toxic changes in neutrophils were apparent in the blood smear. Blood chemistry evaluation revealed elevated gamma-glutamyl-transferase (GGT) activity (13439 IU/L, RR:8-22 IU/L), aspartate-aminotransferase (AST) activity (2694 IU/L, RR:168-494 IU/L) and total bilirubin (4.2 mg/dL, RR:0.5-2.3 mg/dL).

The clinical and clinical pathology evaluation led to the assessment that the filly was suffering from peracute equine piroplasmosis. Treatment with intravenous infusion of LRS with 5% dextrose was initiated to stabilize her condition following by packed cell transfusion (800ml) and supportive care. The filly was treated with imidocarb dipropionate (Imizol, MSD, 4mg/kg, IM) and flunixin meglumine (Norbrook, 0.5 mg/kg, IV), but died within few hours.

Ante-mortem blood smear findings

Giemsa-stained blood smear was taken ante-mortem. Marked neutrophilia was noted, with severe toxic changes in neutrophils including cytoplasmic basophilia, cytoplasmic vacuolation, Döhle bodies and toxic granulation. Piroplasm parasites were detected in erythrocytes, which were identified by their morphology as both *T. equi* and *B. caballi*. Parasitemia of *T. equi* was assessed as 7% (35/500 RBCs), and of *B. caballi* as 3.4% (17/500 RBCs). One to five merozoites were identified within erythrocytes, including the characteristic "Maltese

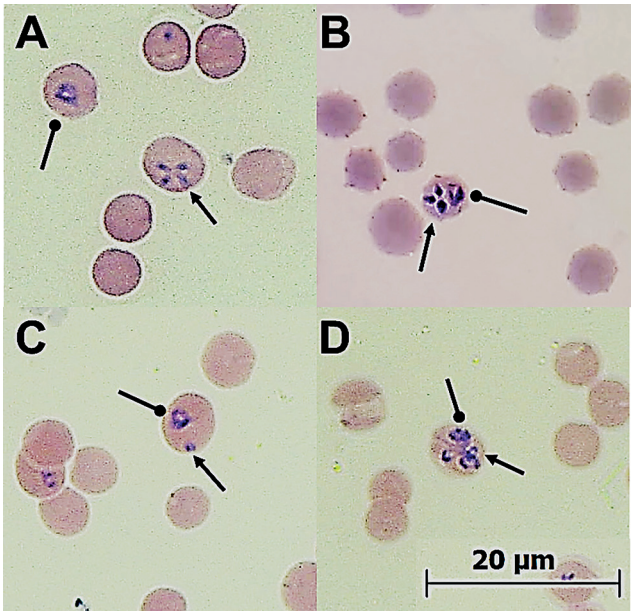

Figure 1: Piroplasm infection observed microscopically in Giemsa-stained blood smear (X1000). Some *T. equi* parasites are highlighted with arrows, and *B. caballi* parasites are highlighted with circular pointers. **A.** Characteristic forms of *T. equi* tetrads (the Maltese cross), and *B. caballi* merozoites. **B-D.** Co-infection with both parasites within erythrocytes.

cross" form of *T. equi* and including cells containing both *T. equi* and *B. caballi* parasites (Figure 1).

Post-mortem findings

Tissue samples from the filly were harvested during the post mortem examination and stored in 10% formaldehyde until processing.

Gross pathology findings included diffuse jaundice and edema of the sub-dermis, serosal tissues and internal organs. Petechial hemorrhages were present in skeletal muscles (mainly in the left hind limb). Petechial hemorrhages were also noted in the intestinal serosa and in the epicardium. The spleen was enlarged and congested. No other pathological changes were observed in other organs.

Histological findings included signs of chronic-active and interstitial pleuropneumonia characterized by thickened inter-lobular and pleural serosa, and inflammatory infiltration composed mainly of mononuclear cells and fibrin. Different stages of degeneration and necrosis at skeletal muscle level were observed, consistent with chronic myositis (Figure 2). The spleen was congested, and severe, extensive, acute tubular

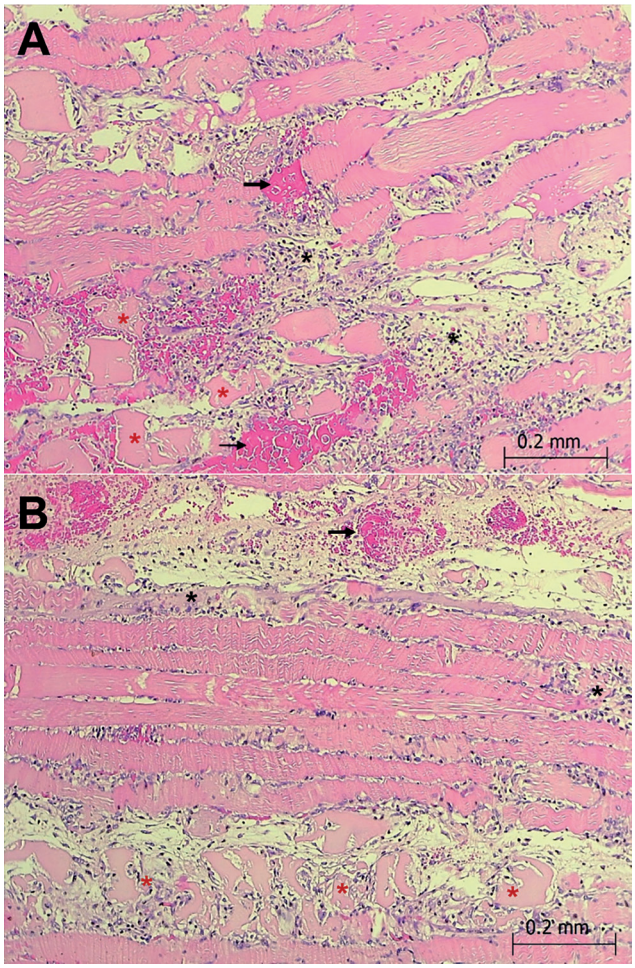

Figure 2: Formalin-fixed skeletal muscle (Equine), H&E. Histopathology: Myocyte degeneration, necrosis, and fragmentation (red asterisks), inflammatory reaction composed of mononuclear infiltration (black asterisks), Hemorrhages and fibrin (fasciae) separating myocytes bundles (arrows).

necrosis without inflammatory reaction was noted in the kidneys.

MOLECULAR CHARACTERIZATION OF EP INFECTION

DNA was extracted from 100µl of whole blood using a commercial kit (DNeasy blood and tissue kit, QIAGEN, Germany), according to the manufacturer's instructions. Detection of infection with EP parasites was performed using diagnostic PCR directed to identify a 400bp fragment of *T. equi 18S rRNA* gene (26), and *B. caballi rap-1* gene (13), as previously described (4, 9, 27). Positive PCR products were cleaned using Exonuclease I and

Shrimp alkaline phosphatase (New England Biolabs Inc., Massachusetts, US) and sent for sequencing (Macrogen Europe, Amsterdam, The Netherlands). Sequences were identified using BLAST http://www.ncbi.nlm.nih.gov/BLAST, (last accessed May 2019), analysis. Genotype classification of each parasite and gene was based on the identity of each sequence to previously classified sequences. Quantification of parasitemia was assessed via qPCR using TaqMan minor groove binder (MGB™) probes targeting *T. equi ema-1* gene (12) and *B. caballi 18S rRNA* gene (28), as previously described (4).

The filly was found to be infected with both *T. equi* and *B. caballi*. Quantification of parasite load by qPCR revealed *T. equi* parasitemia of 84,271 *ema-1* gene copies, equivalent to 5.36% parasitized erythrocytes (PE), and *B. caballi* parasitemia of 23,126 *18S rRNA* gene copies, equivalent to 0.32% PE. The parasites were classified as *T. equi 18S rRNA* genotype A and *B. caballi rap-1* genotype A1.

Sample collection of horses from the farm for epidemiological investigation

Blood samples were collected from all available horses at the farm of origin, five months after the hospitalization of the filly. Blood samples were taken upon owner's consent, and under the approval of the Internal Research Committee of the Koret School of Veterinary Medicine–Veterinary Teaching Hospital (KSVM-VTH/02_2018). Blood was collected from the jugular vein of each horse into sterile vacuum tubes containing Ethylenediamine tetraacetic acid (EDTA) and stored at -20ºC until processing. Detection of infection with EP parasites was performed using diagnostic PCR and sequencing, as described above.

Thirteen horses at the filly's farm, all kept in the same or adjacent pasture of the filly, were tested for the presence of piroplasms. The horses consisted of eight mares and five geldings, 10 Quarter horses and three mixed breeds, with their ages ranging between three and 19 years (mean=11.5, SDV=4.8). Two of these horses tested positive for *T. equi,* which were classified as *T. equi 18S rRNA* genotypes A (similar to the filly) and D. One horse tested positive for *B. caballi,* which classified as *B. caballi rap-1* genotype A1 (similar to the filly). All sequences were 99-100% identical to previously published sequences of the corresponding gene available in the GenBank, and were submitted to the GenBank (MN611352, MN624971, MT683513, MT683514, MT678837, MT678838).

DISCUSSION

This case report presents a characteristic example of peracute EP attributed to infection with both *T. equi* and *B. caballi* parasites. Novel documentation of co-infection of both parasites within a single erythrocyte was found.

Peracute EP is the most severe and life-threatening clinical presentation of EP. This presentation is usually the result of high parasite load and has been described mainly in cases of neonatal EP or in cases of infection of naïve horses (1-3). This case had a history of possible novel exposure, since the filly was transferred to a new farm with pasture, after being kept all her life in a stall. Laboratory findings where characteristic for EP infection and included anemia, thrombocytopenia and hyperbilirubinemia (29, 30). Death in cases of peracute EP is usually the consequence of acute anemia and organ damage (2). The filly in this case suffered from renal, lung, cardiac and muscular damage, with evidence of systemic bleeding. It has been described that anemia may lead to cardiac and renal complications (31), and elevated renal and cardiac biomarkers have been previously described as associated with the level of EP parasitemia (32). In addition, systemic inflammatory responses may result in disseminated intravascular coagulation (DIC), which may manifest in systemic bleeding and organ damage, while pigment nephropathy resulting from intravascular hemolysis, may further contribute to renal dysfunction (1, 3). Inflammatory myopathy has also been described in cases of chronic EP (33), which may be consistent with the histological finding found in this peracute case.

Evaluating parasitemia levels of infected horses is important to determine the magnitude of infection and accordingly, to decide upon the course of treatment (dose and number of treatment cycles). In endemic areas, the objective of treatment is not necessarily to achieve clearance of parasites, but to have clinical improvement in animal health, without breaking the long lasting immune response sustained by prolonged infection. In addition, in endemic areas, where parasite prevalence is high, it is sometimes difficult to determine whether EP is the cause of clinical signs or an incidental finding. Since clinically affected horses have been found to shown significantly higher parasitemia, it has been recently suggested

that molecular quantification of parasitemia may assist in identifying EP as the cause of disease (4). In the current case, assessment of parasitemia was compared between manual cell count in the blood smear and by qPCR. The results showed good agreement between both methods in the evaluation of *T. equi* infection (7 and 5.4% PE) and lower agreement for *B. caballi* infection (3.4 and 0.32% PE). The difference between methods may be attributed to underestimation of the calibration curve of the qPCR, or to overestimation of the cell-count due to non-uniform distribution of parasitized erythrocytes in the blood smear (since parasitized cells are heavier and tend to concentrate in the thinner part of the slide, where cell-counting is carried out for better assessment). Despite the differences in evaluation, parasitemia was high for both parasites by both methods, and was higher than the cutoff value (0.066% PE for *T. equi* and 0.004% PE for *B. caballi*) set to discriminate between clinical and subclinical cases of both parasites (4).

Co-infection with both *T. equi* and *B. caballi* has been reported in the past, as well as co-infection with several *T. equi* genotypes and *T. haneyi* (4, 14, 15, 19-25, 30, 34). In many of these reports, including a report from Israel, the vast majority of *B. caballi*-infected horses were co-infected with *T. equi* (4, 19, 23-25). Although the risk factors for exposure to either parasite are similar (mainly exposure to vector ticks) (5, 35), the rate of co-infection is high, suggesting some interaction between these parasites. The novel identification of both parasites within single erythrocytes in this case raises questions regarding the pathogenesis and cell-invasion pathways that may predispose parasitized erythrocytes to invasion of other, closely related, parasites. Although the exact mechanism of cell invasion has not yet been elucidated for EP, it is known that infection leads to alterations in erythrocytes membranes with an increase in erythrocyte rigidity, which eventually result in hemolysis (1, 3, 36-38). These alterations may possibly assist cell-invasion by other piroplasms and increase the likelihood of co-infection. This avenue calls for further research, which may perhaps lead to vaccine development based on these mechanisms.

Molecular characterization of both parasites indicated infection with *T. equi* 18S rRNA genotype A, and *B. caballi rap-1* genotype A1. Both genotypes were found to circulate in the farm based on the epidemiological investigation, in resident horses with no clinical signs. *Theileria equi* genotype A has been previously linked to clinical manifestation of EP in Israel (4) and in Italy (39). No specific *B. caballi* genotype has ever been linked to clinical disease. A study of *T. equi* subclinical carriers demonstrated that intermitted exposure to ticks (rearing in paddocks) was associated with elevated parasitemia and with *T. equi* genotype A (10). The farm in the current report has a population of resident horses that are either kept in stalls or paddocks and some were turned out in pasture during the day. In addition, the farm also boards horses and hosts equestrian events and riding courses with participation of non-resident horses. The horses sampled in the epidemiological investigation were resident horses, which were kept in paddocks or pasture. The EP-infected horses identified in this group were apparently healthy and probably chronically exposed to both ticks and piroplasm parasites. Naïve horses that are introduced to this environment are more likely to develop clinical disease, and the attending veterinarian reported treating several EP cases in this farm (data not presented), including the above mentioned. Although the exact mechanisms for the development of resistance against clinical disease are not well elucidated for EP, in cattle exposure to *B. bovis* early in life is known to be crucial for the development of premunition (40).

The fact that the filly was introduced to this endemic farm when it was a yearling might have contributed to the development of clinical fulminant disease, as resident horses that have been exposed to ticks from a younger age did not show clinical signs. Although Israel is endemic for EP, it is important to consider that there are "endemic" and "non-endemic" equine populations within an endemic region, thus, it is necessary to use proper prevention and control measures in the introduction of naïve horses to endemic farms. The best management practice should be to investigate the status of a farm before introduction of a new horse, taking in consideration the EP history and origin of the horses. Furthermore, if possible, naïve horses introduced to an infected farm should be treated with acaricides and routinely clinically examined to detect infection at an early stage, which may be resolved with minimal treatment, thus permitting a more favorable prognosis.

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