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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Case report

Clinical Case of acute equine piroplasmosis in a Malaysian mare

Al-Obaidi QT[1*], Al-Sultan II[1], Arshad MM[1], Mohd Azam KGK[1] and Mimi AM[1]

[1]Faculty of Veterinary Medicine, University Malaysia Kelantan, Pengkalan Chepa, 16100, Kota Bharu, Kelantan, Malaysia

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Abstract

This is the first clinical report on acute piroplasmosis in a mare in Malaysia. A 20 years old local thoroughbred mare showed clinical signs of acute equine piroplasmosis, anaemia, increase in erythrocytes sedimentation rate (ESR) and reticulocytosis, neutrophilia and lymphocytosis, increase in aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALKP), blood urea nitrogen (BUN) and total bilirubin and decreased total protein (albumins and globulins), calcium, phosphorous, glucose and creatinine. Microscopic examination of blood smear was positive for *Theileria equi* and *Babesia caballi*. Competitive ELISA test was confirmative to antibodies of *T. equi* and *B. caballi*. Multiplex PCR test was positive for the causative agents of equine piroplasmosis. Microscopic localization of micro and macroschizontes in the cytoplasm of lymphocytes and macrophages in various organs provided a distinct hisopathological evidence of acute form of the disease.

Keywords: Equine piroplasmosis; clinico-pathology; *Theileria equi*; *Babesia caballi* Malaysia

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Introduction

Equine Piroplasmosis (EP) is commonly known as biliary fever, it is an acute, sub-acute or chronic tick-borne disease of *Equidae* (de Waal, 1992). Two different blood protozoan parasites from genus *Babesia* and *Thieleria* cause the disease (*Babesia caballi* and *Theileria equi*, previously known as *Babesia equi*). The aforementioned parasites are transmitted by ticks of the Ixodidae family (Mehlhorn and Schein, 1998; Jongejan and Uilenberg, 2004). In general, *T. equi* infections are clinically severe and more common than those caused by *B. caballi* (de Waal, 2000; Donnellan and Marais, 2009). In Malaysia, there is no registered report in OIE document (OIE, 2014). The present report puts on record for the first time a clinical case of acute piroplasmosis in a mare at Kelantan, Malaysia.

Materials and Methods

The local thoroughbred mare aged 20 years with 350 Kg body weight infested with ticks was suspected to be suffering from piroplasmosis. Another mare in the same stable was found piroplasmid-free based on negative results of microscope examination, cELISA and PCR techniques. Haematological and biochemical parameters along with DNA extraction from the blood of both mores were compared. After seeking permission of the owner following investigations were carried out.

***Corresponding author:** Qaes Talb Al-Obaidi, Parasitology Department, Faculty of Veterinary Medicine, University Malaysia Kelantan; E-mail: d12e003f@siswa.umk.edu.my; qaes_talb@yahoo.com; Mobile No: 00601123137293; Fax- 09771732

Clinical sings

Routine clinical investigation and faecal samples were screened for internal parasitic infestation using standard techniques (Zajac and Conboy, 2012).

Blood smears preparation

Thick and thin blood smears were made from ear vain puncture. Smears were dried, fixed with absolute methanol (3-5 min) and stained with Giemsa 5% solution (Azur-eosin-methylene blue solution, Merck Sdn. Bhd., Germany) and examined under microscope. Parasitemia was calculated by the equation of Fritsche and Smith (2001):

$$parasitemia\ \% = \frac{number\ of\ infected\ RBC}{number\ of\ calculated\ RBC} \times 100$$

Blood sample collection

Venous blood was collected from jugular vein using 18G needle into two vacationer tubes (5 ml each) one with anticoagulant (ethylene diamine tetraacetic acid) and another without any anti-coagulant. Blood parameters performed were total red blood cells (TRBCs), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), thrombocytes count, total white blood count (TWBC) and differential leucocyte count (DLC) using haematology analyser (Mythic18VET, France). Erythrocyte sedimentation rate (ESR) estimation was done using Westergren pipette. Reticulocytes count was performed by mixing 2 drops of blood with 2 drops of methylene blue (0.5%) left for 20 minute and examined under microscope. The percentage of reticulocytes was calculated as mentioned by Jain (2000):

$$Reticulocyte\ \% = \frac{number\ of\ reticulocyte}{number\ of\ calculated\ retticulocytes} \times 100$$

Biochemical analysis and competitive ELISA

The tube without anticoagulant was used for serum separation and stored at -20ºC until analysis (Kouam et al., 2010). Aspartate amino trasferase (AST), alanine amino trasferease (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total bilirubin, total protein, albumins, globulins, calcium, phosphorous, glucose and creatine were measured in serum using special Cassettes for each in a chemistry analyzer (Vet Test, Arachem, USA). Serum was also used for detection of antibodies against *T. equi* and *B. caballi* using micro plate reader (Bio Tek, ELx808/ USA) and commercial c-ELISA kits (VMRD, Inc, Pullman and WA99163 USA).

DNA extraction and PCR assay

Blood with EDTA was used for extraction of DNA by using QIAamp DNA Mini Kit (QIAgen GmbH, Hilden, Germany). A single multiplex PCR reaction targeting 18S rRNA employed a mixture of primers composed of a single forward primer (TBM; 5'-CTTC AGCAC CTTGA GAGA AA TC-3'), and two reverse primers (Equi-R; 5'-TGCCTTAAACTTCCTTGCGAT-3' and BC-R; 5'-GATT C GTC GG TTTT GCC TTGG -3') with expected 360 bp and 650 bp long amplicons for *T. equi* and *B. caballi* respectively (Qablan et al., 2013). PCR reactions were conducted in a total volume of 25 µl, composed of 12.5 µl commercial Master Mix (1st BASE Pte Ltd, Singapore), 20 µM of each primer and ~20 ng of genomic DNA. The mixture was pre-denaturation for 5min at 95ºC, denaturation for 45 sec at 94ºC, annealing for 45 sec at 60ºC, extension for 30 sec at 72ºC, final extension for 10 min at 72ºC and each step was repeated 36 cycles in the thermocycler. The amplified DNA samples were electrophoresed on 1.5% agarose gel stained with Midori green to visualize the amplified DNA fragment in the Gel DocTM EZ imager (BIO RAD, USA). The amplified DNA was visualized with Gel Doc^{TM} EZ imager (BIO RAD, USA).

Post mortem lesions

The mare was euthanized using pentobarbital sodium (Dolethal) at the rate of 1 ml/kg body weight intravenous (Vetouinol, UK) and necropsy was conducted. Gross morphology was recorded and tissue samples were collected from brain, heart, lung, liver, spleen, lymph nodes, kidneys and intestines, fixed in 10% formalin for 48 hours, processed for haematoxylin and Eosin and Giemsa staining (Kiernan, 2009).

Results

Clinically infected mare showed signs of loss of appetite, emaciation, fever, congestion of mucous membranes with petechial haemorrhages on 3rd eyes lid and conjunctiva, difficulty in movement with muscular rigidity, incoordination, ataxia, oedema on the fetlock joint of the hind limbs, haemoglobinurea with passing of brown-coffee like urine and ticks infested different parts of the body. Furthermore, increase in the body temperature (40.1ºC), respiratory rate (46.6/min), heart rate (75.1/min) and capillary refilling time (4/sec) was observed. The gastrointestinal parasites were absent and the laboratory results for the haematological parameters included decreased in TECs, Hb, and PCV, thrombocytes, MCV and MCHC reflecting to microcytic hypochromic type of anaemia. An increase in ESR, percentage of reticulocyte and TWBCs were observed. Biochemical analysis revealed increase in AST, ALT, ALKP, BUN and total bilirubin and decrease in the total protein, albumins and globulins, calcium, phosphorous, glucose and creatinine. The findings were suggestive of acute equine piroplasmosis, which was

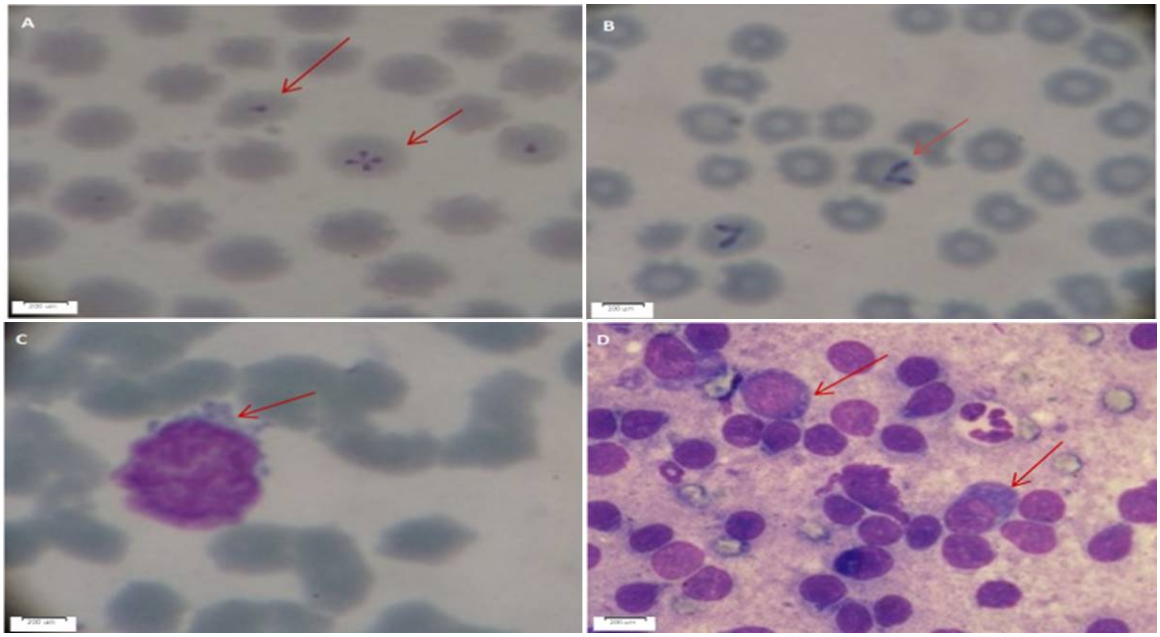

Fig. 1: (A, B) Blood smears showing merazoites stage of *B. caballi* and *T. equi* inside the RBCs. **(C)** Blood smear showing microschizontes and macroschizontes of *T. equi* (Koch's Blue Bodies) inside the lymphocytes. **(D)** Lymphocyte patches showing microschizontes and macroschizontes (Koch's Blue Bodies) inside the lymphocytes. All werestained with Giemsa's solution 5%.

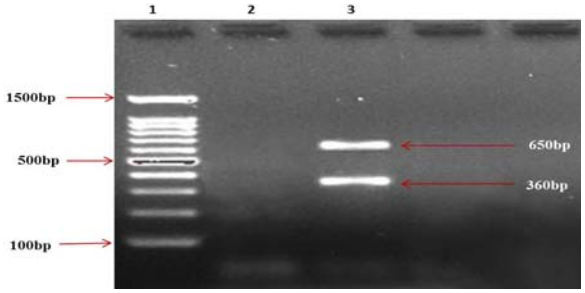

Fig. 2: Gel electrophoresis image showing: lane (1) ExactMark 100-1500bp DNA ladder. Lane **(2)** DNA extracted from piroplasmid-free horse used as negative control. Lane **(3)** DNA extracted from mare infected with *T. equi* and *B. caballi* in the band size was 360 bp and 650 bp respectively.

confirmed by microscopic examination and cELISA test and multiplex PCR. Microscopic examination of blood smears indicated infection of *T. equi* (Maltese cross shape) and *B. caballi* (double pear shape) inside the RBCs. The microschizontes and macroschizontes of *T. equi* (Koch's Blue Bodies) were seen clearly inside the cytoplasm of lymphocytes (Fig. 1). Parasitemia of both parasites was 18.2%. Results for both cELISA test and multiplex PCR technique were positive for *T. equi* and *B. caballi* (Fig. 2).

Necropsy disclosed general body weakness and yellowish discolouration of subcutaneous tissue. The cardiac lobe of the left lung was congested with presence of multiple solid nodules raised 1-3cm flash like in consistency and embedded into the pulmonary tissue. These nodules were disseminating through all lobes. Emphysematous pulmonary lesion and atelactic areas were evident mostly at the caudal lobe. The liver, spleen and kidney were enlarged. The bladder was distended, full of dark colour urine (coffee like). Most lymph nodes particularly mesenteric and those on the course of other internal organs were haemorrhagic and hypertrophied.

Histological examination revealed lesions in brain, heart, lung, liver, spleen, kidney, mesenteric lymph node and intestine. In the brain neuronal degeneration specifically in the Purkinje cells and geniestocytic astrocytes were in abundant amount of glial cells with presence of granular pink stained material in between neuronal cells, certain outlines of necrotic neuronal tissue were identified and macro and microschizontes (Koch's blue bodies) were seen in the cytoplasm of lymphocytes and macrophage. Myocardial muscles showed oedema, and hypertrophy of muscle fibres. The coronary blood vessels were congested. Pulmonary tissue showed oedema and emphysema. Hypertrophy of hepatic cells and congestion of lobular vein with activation of Kupffer cells between the hepatic cords and the sinusoids were evident. The only distinct lesion in the spleen was the abundant hemosiderin and activation of red and white bulbs. Kidney showed tubular degeneration and necrosis of epithelial lining, intravascular thrombosis and haemorrhage of renal

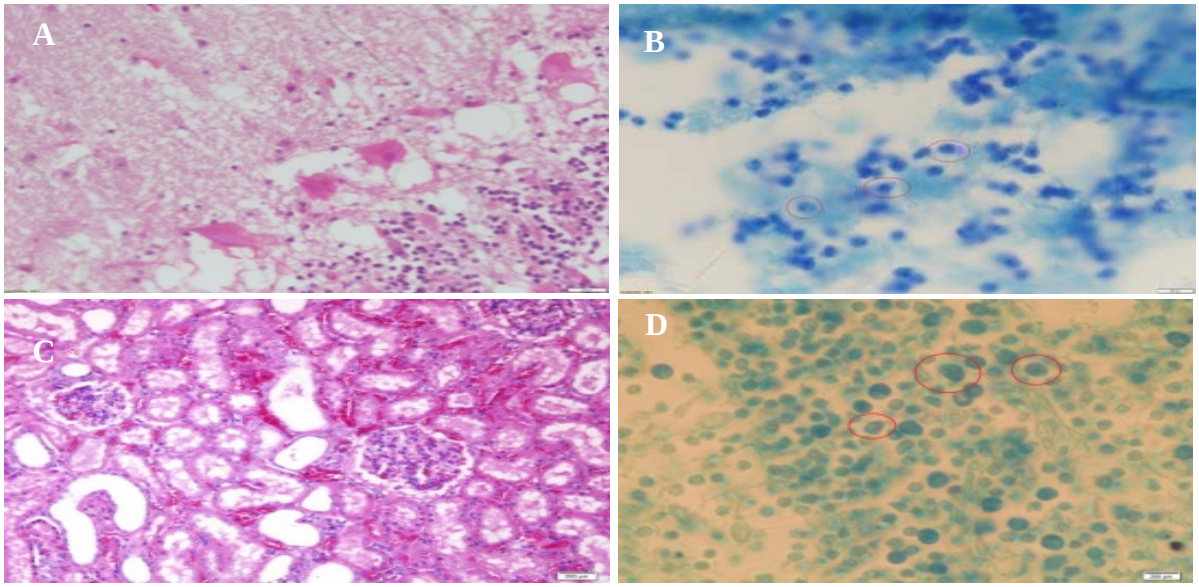

Fig. 3: (A) Neuronal degeneration, abundant amount of glial cells with granular pink stained necrotic material in-between neuronal cells. (B) Koch's blue bodies in lymphocytes and macrophages of brain tissue stained with Giemsa. (C) Renal tubular degeneration and necrosis of epithelial lining, intravascular thrombosis and haemorrhage of renal parenchyma. Glomeruli undergo oedema and thicking of Bowman's capsule. (D) Lymphocytes with Koch's blue bodies.

parenchyma. Glomeruli showed oedema and thickening of Bowman's capsule. The lymphoid glands appeared inflammatory with Koch's blue bodies infiltrating the cytoplasm of lymphocytes (Fig. 3).

Discussion

In Malaysia no acute case of EP has been reported and most of the research was conducted on clinically healthy equids (Chandarwathani et al., 1998; Zawida et al., 2010). In the current study, the naturally infected mare showed clinical sings of acute EP similar to other cases reported by Uilenberg (2006), Radostitis et al. (2007) and Ibrahim et al. (2011).

The oedema around the fetlock joint may be due to hypoprotenemia (Alsaad et al., 2010). Hemoglobinurea occurs in mare due to intravascular hemolysis of RBCs in infected animals and release of hemoglobin (Hemoglobinemia) is responsible for brownish or dark coffee like urine (Alsaad and Al-Mola, 2006).

Alsaad et al. (2010) stated that infected horses sufferd from lack of calcium has an impact on the function of muscle contraction. The detection of ticks on different body parts of mare refer to the fact that ticks play an important role for transmission of EP as reported by Kouam et al. (2010).

Reticulocytes detected in the blood circulation of the mare were similar to the result seen by Al-saad and Al-Mola (2006). The increased leukocytes which may probably due to neutrophilia and lymphocytosis was also recorded by Ibrahim et al. (2011). The liver enzymes activities in mare were observed in the form of AST, ALT, ALKP and total bilirubin may be resulting from the indirect hepatocellular damage and/or the excessive destruction of RBCs (Hailat et al., 1997; Zobba et al., 2008).

Results of microscopic examination of blood smears were positive for *T. equi* (Maltese cross shape) and *B. caballi* (double pear shape), with parasitemia, these results were in agreement with Alsaad et al. (2010) and Malekifard et al. (2014). Result of cELISA test was positive for antibodies of *T. equi* and *B. caballi.* Moreover, multiplex PCR was also positive for *T. equi* and *B. caballi.* Morphological diagnosis at necropsy was similarly reported by de Waal (1992) and OIE (2009). Results of histopathology were indicative of piroplasmosis in acute clinical infection and in agreement with Rothschild and Knowles (2007), Al-saad and Al-Mola (2006) and Sengupta et al. (1999).

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