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Concomitant *Babesia gibsoni* and *Ehrlichia canis* infection in a dog

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ABSTRACT

Experimental transmission of *Babesia gibsoni* in a dog, in a state of subclinical infection by *Ehrlichia canis*, resulted in a severe concomitant infection of *E. canis* and *B. gibsoni* characterized by neurological signs, including nystagmus and seizures. Mononuclear cells harbouring the characteristic morulae of *E. canis* were numerous in lung impression smears. *B. gibsoni* were observed in the peripheral blood in erythrocytes and within macrophages in spleen, liver and kidney impression smears. Erythrocytes were found trapped within capillaries of the brain. *B. gibsoni* were seen in various stages of division and on average 16 merozoites were seen in a single infected erythrocyte.

Key words: ehrlichiosis, babesiosis, dog

Introduction

Haemoprotozoan diseases frequently complicate natural outbreaks of canine ehrlichiosis. Interactions among these various parasitic agents unquestionably affect the organisms individually and alter their effects on the host. The influence of other infections, especially haematozoan agents in the course of ehrlichiosis, needs to be studied. Both *E. canis* and canine babesiae are common in South India, either individually or as concurrent infections. Although concurrent infections of *E. canis* and *B. canis* have been referred to in the literature (SHIRLAW, 1938; BOOL and SUTMOLLER, 1957; EWING and BUCKNER, 1965; MATHEWMAN et al., 1993), there are few reports on pathogenesis caused by rickettsiae in concert with *Babesia gibsoni*. The influence of *B. gibsoni* in the course of canine ehrlichiosis needs to be known, and this paper reports on the experimental transmission of *B. gibsoni*

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in a dog that was in the subclinical phase of ehrlichiosis. It also focuses attention on the resultant syndrome caused by the mixed infection of *E. canis* and *B. gibsoni*.

Materials and methods

An isolate from a dog with naturally occurring canine ehrlichiosis diagnosed at the Madras Veterinary College Clinics was used. The dog had a history of tick infestation and had pyrexia and enlargement of popliteal lymph nodes. Microscopic examination of peripheral blood smears revealed the presence of Ehrlichial inclusions in mononuclear cells.

Babesia gibsoni was isolated from a dog that was naturally infected with *B. gibsoni*, at the Madras Veterinary College Clinics. The dog was an intact, one-year-old male Labrador retriever with a history of exposure to ticks. The dog was pyrexic and had blanched conjunctival mucous membranes. Microscopic examination of peripheral blood smears revealed the presence of *B. gibsoni* piroplasms in RBC.

The experimental dog was housed in a kennel with tick-proof conditions and was vaccinated against canine distemper, infectious canine hepatitis and leptospirosis. The dog was also treated with anthelmintic before its use in the experiment. Freedom from *E. canis* was confirmed on the basis of negative results with ELISA and Dot-ELISA and freedom from haemoprotozoan infections was ascertained by thorough screening of peripheral blood smears for 10 days before being used in the experiment.

Experiment I: Experimental dog aged about 6 months, seronegative for *E. canis* by ELISA and Dot-ELISA, was inoculated with five ml of heparinized blood collected from a dog naturally infected with *E. canis*.

Experiment II: The same dog was inoculated with 5 ml of *B. gibsoni*-infected blood after 90 days post-infection with canine ehrlichiosis.

Sera were analyzed for *E. canis* antibodies by ELISA and Dot-ELISA using DH 82 cells infected with *E. canis* (Oklahoma strain). The sera were examined before and after experimental infection with *E. canis*. Antigen and known positive serum was provided by the Department of Infectious Diseases and Physiology, College of Veterinary Medicine, Stillwater, USA. The two tests were used to examine sera from suspected dogs. ELISA was carried out as per standard methods (GRAY et al., 1980). Antigen diluted 1:500 in 0.05 M carbonate/bicarbonate buffer (pH 9.6) was dispensed in volumes of 0.1 ml into wells of polystyrene microplates (Nunc, USA). After washing with PBS-T 20 three times, plate was blocked for one hour with bovine serum albumin (Merck). Test sera were diluted 1:100 in PBS and incubated with antigen for 1 h at room temperature. Bound antibody was detected using 0.1 ml of anti-dog rabbit IgG conjugated with horse radish peroxidase (Sigma, USA)

and 0.1 ml of ABTS (Sigma, USA) in PBS. The absorbances were measured at 405 nm using a multiscan Elisa Reader (Flow Lab, U.K.)

Dot-enzyme linked immunosorbent assay (Dot-ELISA) was performed following the method of PAPPAS et al. (1984), with minor modifications. Nitrocellulose (Sigma, USA) discs were placed in each well of microtitre plate and 1.0 ul of *E. canis* antigen purified from infected DH 82 cells were coated using Hamilton syringe. After 1 h the unbound sites were coated with 0.1 ml of 0.2% PBS-T and incubated for 15 min at room temperature. Test sera diluted 1:50 in PBS-T (0.05%) were incubated with antigen for 2 hrs at room temperature. Bound antibody was detected with horseradish peroxidase labelled rabbit anti-dog IgG (Sigma, USA) diluted 1:400 in 0.5% PBS-T and 3,3' Diaminobenzidine tetrahydrochloride (Sigma, USA). Positive cases were indicated by the appearance of brown dots on the discs.

Sera showing double the optical density of known negative sera were taken as positive for the presence of *E. canis* antibodies.

Clinical evaluation. The infected dog was monitored for clinical signs and haematological abnormalities. Rectal temperature was recorded daily and Giemsa stained peripheral blood smears were examined every other day. Haematological evaluations were carried out at weekly intervals in Experiment I but haemogram was estimated only on the day of death in Experiment II. On necropsy, Giemsa stained impression smears (touch preparations) of various organs were examined for the presence of parasites.

Results

Experiment I. The inoculated dog with whole blood drawn from a dog naturally infected with *E. canis* developed ehrlichiosis. Febrile response occurred 13 days after blood inoculation and the response was slight and transient. Fever was accompanied by anorexia, serous oculonasal discharge and moderate lymphadenopathy. Ehrlichial inclusions were seen in mononuclear cells within 4 days after the onset of fever and were first sighted 17 days post-inoculation (DPI). Morulae were seen in the peripheral blood smears up to 25^{th} DPI and thereafter the dog became clinically normal, although a slight loss in body weight was apparent. Sequential haematological changes over a six-week period revealed progressive anaemia, leukopenia and increased erythrocyte sedimentation rate (ESR). In Giemsa stained smears, the morulae were 2-3 μm in diameter, dark purple in colour and clearly different from the colour taken up by the nucleus of the host cells.

The sera sample collected from the dog before the experiment was negative in both ELISA and Dot-ELISA. In ELISA, the OD value was 0.032 as against the value of 0.029 for known negative sera. Positive reactions were detected by both the tests in sera collected after 90 days. The OD value was observed to be 0.101. Similarly, in Dot-ELISA, distinct

brown dots were seen in the sera sample collected after 90 days post-infection. The sera sample from the experimental dog was one of the 56 samples examined using ELISA and Dot-ELISA. Positive reactions were seen in 23 of 56 (41.1%) and 21 of 56 sera (37.5%) in ELISA and Dot-ELISA, respectively.

Experiment II. Piroplasms of *B. gibsoni* were noticed after 13 DPI. The piroplasms were annular or signet ring-shaped in appearance and measured 1.2 to 2 μm in diameter. The maximum parasitaemia observed was only 2-3% and the dog died 19 days post-inoculation of *B. gibsoni* infected blood with signs of chorea and nystagmus. Anaemia was evident on the day of death. Blood was observed in the faeces also on the day of death. Haematologic examination revealed lowered RBC counts (4×10^6 cells/ml) and decreased PCV (24%). Total leucocyte counts were within the laboratory reference range (7500/ml).

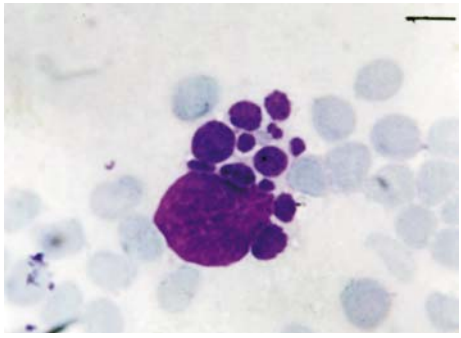

Fig. 1. Morulae of *Ehrlichia canis* in a lymphocyte, lung impression smear Giemsa, ×1000

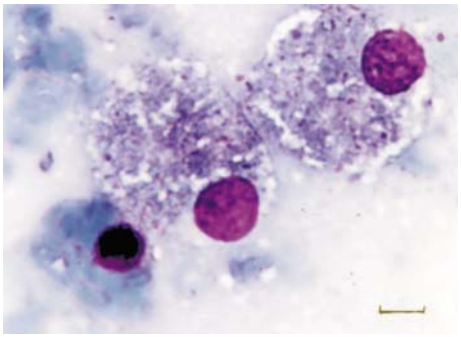

Fig. 2. Macrophages showing masses of *Babesia canis* in liver, Giemsa, ×1000

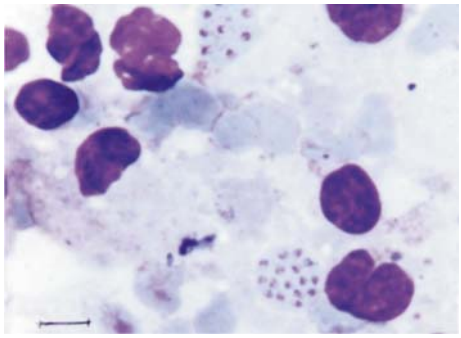

Fig. 3. *Babesia gibsoni* in multiples of 8 and 16 in erythrocytesspleen impression smear, Giemsa, ×1000

On necropsy, the mucous membranes appeared pale in general and splenomegaly and hepatomegaly were pronounced. Petechial haemorrhages were observed in kidneys. Lungs were emphysematous, the most striking feature being the presence of haemorrhagic streaks in duodenal region of the small intestine, although hookworms were not present. Microscopic examination of lung impression smears showed the presence of intracytoplasmic inclusions of *Ehrlichia canis* and *B. gibsoni* parasitized macrophages and erythrocytes, respectively. The inclusions were in mulberry configuration, which is characteristic of canine ehrlichiosis. Inclusions were readily found in lung impression smears. The morulae were readily found in mononuclear cells and among mononuclear cells; lymphocytes were commonly affected, followed by monocytes. The morulae were round to ovoid, red or dark purple in colour and measured 4-8 μ in diameter (Fig.1). In erythrocytes, both small and big babesias were present. In liver impression smear, merozoites of *B. gibsoni* were seen in macrophages; some were typically annular but in many the cytoplasm of the host cell was pale bluish in colour and contained numerous small, dense reddish masses of chromatin representing the nucleus (Fig. 2).

In spleen impression smear, merozoites of *B. gibsoni* were seen in erythrocytes in various stages of division. An average 16 merozoites were seen in a single erythrocyte, although there were as few as 8 and as many as 32 in a single erythrocyte (Fig. 3). In some erythrocytes, the merozoites appeared as distinct chromatin dots, whereas in some the chromatin mass had a little cytoplasm surrounding it, giving an annular shape which is characteristic of *B. gibsoni* piroplasms. In erythrocytes, which had few merozoites, the size of the chromatin dot was larger than in erythrocytes that had 16 or 32 merozoites. In kidney impression smear, the parasitaemia was very intense and many of the erythrocytes were parasitized with *B. gibsoni* merozoites. The merozoites were pleomorphic, being oval or spherical and some very elongated. Extracellular merozoites were also noticed.

Discussion

The experimental dog developed uncomplicated ehrlichiosis and recovered from the acute phase of the disease after 25 DPI and was clinically normal after 30 days. EWING and BUCKNER (1965) have shown that *E. canis* can persist for extended periods in convalescent animals which are clinically normal. KUEHN and GAUNT (1985) reported that dogs which recover from the acute phase of the disease become non-clinical carriers of the infection. Upon subsequent inoculation of *B. gibsoni*-infected blood, the dog died on the 19th day post-inoculation with signs of central nervous system derangement that was suggestive of canine distemper.

Neurological signs, including nystagmus and seizures, have been reported in dogs with canine babesiosis, which is reported to be due to sludging of parasitized erythrocytes in small blood vessels of the brain, resulting in anoxia, which often causes damage to the

organs (WRIGHT et al., 1979; HILDEBRANDT, 1981). According to EWING (1969), signs of CNS derangement are not uncommon in canine ehrlichiosis which occurs as a result of inflammation and bleeding in the meninges. A meningoencephalitis that could be confused with canine distemper was observed in puppies (GREENE and HARVEY, 1982), which were attributed to mononuclear cell infiltration in the meninges. In the present study, the presence of trapped erythrocytes inside blood vessels of the brain and the presence of numerous *E. canis* parasitized mononuclear cells in lungs is an indication that the dog had developed a severe concomitant infection of *E. canis* and *B. gibsoni*.

Intestinal haemorrhage was pronounced in the experimental dog. Severe intestinal haemorrhage is reported to occur in canine ehrlichiosis as evidenced by large quantities of blood in faeces (HUXSOLL et al., 1970) Massive internal haemorrhage has been observed in chronic ehrlichiosis that may occur as episataxis, haematuria, haematemesis or intestinal haemorrhage (PRICE et al. 1987) and gastrointestinal bleeding is reported to occur when the platelet count falls below 20,000 ml (HUXSOLL et al., 1970). In the present study, platelet counts were not examined and therefore platelet counts during intestinal haemorrhages could not be ascertained.

Mononuclear cells harbouring the characteristic morulae of *E. canis* were numerous in lung impression smears. The percentage of infected leukocytes is reported to be greater in lungs than in blood smears (EWING, 1969). In lung impression smears, the morulae of *E. canis* were predominantly seen in lymphocytes, and to a lesser extent in monocytes. EWING and BUCKNER (1965) reported that lymphocytes were commonly affected with the strain they isolated, but also recorded morulae in monocytes and neutrophils. In the present case, a maximum of twelve morulae were observed in a single host cell. Host cells containing multiple morulae were reported to occur in old world isolates (HAIG, 1955). EWING (1969) observed a maximum of 5 morulae in a single host cell with the strain he isolated.

In spleen impression smears, dividing forms of *B. gibsoni* were seen. The presence of 8-32 *B. gibsoni* parasitized erythrocytes in spleen represents a series of binary fission rather than multiple infection of the host cell. The presence of numerous *B. gibsoni* parasitized host cells in liver and lungs may probably be due to erythrophagocytosis. The presence of pleomorphic forms in the kidney, with some being very large and ovoid and some being elongated, was suggestive of *B. canis*, but SOULSBY (1982) has reported that although the trophozoites of *B. gibsoni* are characteristically annular or oval, large ovoid to circular forms about half the diameter of the host cell or elongate forms that stretch across the cell may be seen rarely in *B. gibsoni* infections.

In the present study, concurrent infections of *E. canis* and *B. gibsoni* have resulted in a syndrome, wherein each organism has potentiated the development of the other. Infections of various kinds may intensify the effects of *E. canis* (EWING, 1969). DONATIEN and LESTOQUARD (1937) has recognized concomitant attacks of *Rickettsia canis* and

Piroplasma canis in dogs, and during one such experimental concomitant transmission of *P. canis* and *R. canis* in an animal which was immune to *P. canis*, rickettsiosis attack was found to be extremely severe, although *P. canis* was found to resolve after appropriate therapy. On the contrary, an attack of rickettsiosis in a dog in a state of chronic infection by *P. canis*, has resulted in recrudescence of the latent infection of *P. canis* resulting in a severe and mortal attack of piroplasmosis in a dog. SHIRLAW (1938) also described a syndrome, caused by a mixed infection of Babesia, and Ehrlichia wherein clusters of Ehrlichia morulae were observed in the cytoplasm of polyblasts and reticular cells. Infections of various kinds may intensify the effects of *E. canis* (EWING, 1969) and in the present study concurrent infections of *E. canis* and *B. gibsoni* have resulted in a syndrome, wherein each organism has potentiated the development of the other, resulting in a fulminating attack of canine ehrlichiosis and canine babesiosis.

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HARIKRISHNAN, T. J., N. PAZHANIVEL, J. CHELLAPPA: Istodobna infekcija psa vrstama *Babesia gibsoni* i *Ehrlichia canis*. Vet. arhiv 75, 513-520, 2005.

SAŽETAK

Pokusna infekcija vrstom *Babesia gibsoni* u psa, supklinički zaraženog vrstom *Ehrlichia canis* dovela je do pojave teške kliničke slike koja se očitovala neurološkim znakovima uključujuči nistagmus i koreju. Mononuklearne stanice kao nalazište karakterističnih morula *E. canis* bile su brojne u razmascima plućnog tkiva. *B. gibsoni* je zapažena u eritrocitima periferne cirkulacije i unutar makrofaga u razmascima slezene, jetre i bubrega. Pronađeni su razoreni eritrociti unutar mozgovnih kapilara. *B. gibsoni* je uočena u različitim stadijima dijeljenja i prosječno je 16 merozoita pronađeno po jednom inficiranom eritrocitu.

Ključne riječi: erlihioza, babezioza, pas