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Studies on haemato biochemical evaluation, diagnosis of ehrlichiosis and babesiosis in dogs: Its successful therapeutic management

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Abstract
Ten dogs were presented with the symptoms of inappetance, dullness and anaemia. Upon clinical examination, they were suspected and screened for haemoparasitic diseases. Haematology, biochemistry, blood smear examination and PCR were done. Marked neutrophilia, monocytosis and thrombocytopaenia were noticed. Two dogs were found positive for *B. gibsoni* and two for *E. canis.* The treatment of those positive cases for 28 days and post treatment parameters were evaluated and discussed.

Keywords: dog, *Ehrlichia canis*, *Babesia gibsoni*, haematology, Berenil, triple therapy, doxycycline

1. Introduction
Vector borne infections are caused by a variety of etiological agents including bacteria, protozoa and rickettsial organisms. Haemoparasites transmitted by ticks are one of the most common vector-borne infections in dogs. (Chomel, 2011) [7]. Canine babesiosis and ehrlichiosis are global diseases with varying frequency and clinical symptoms according to geographic location. *Ehrlichia canis* is the etiological agent of the Canine Monocytic Ehrlichiosis (CME), affects animals all over the world and is known to appear in the cytoplasm of mononuclear cells as morulae, which are clusters of organisms (Harrus and Waner, 2011) [11]. The evidence of morulae of *E. canis* in stained blood smears is a useful diagnostic technique in the acute disease (Mylonakis *et al.* 2003) [17]. However, it lacks sensitivity for subclinical and chronic disease, in addition to being time consuming (Woody and Hoskins 1991) [21]. Instead, polymerase chain reaction (PCR) with parasite-specific primers provide a more sensitive and specific diagnostic tool in the laboratory diagnosis of canine ehrlichiosis (Carlos *et al.* 2007) [6].
B. gibsoni is a canine blood protozoan, a pear-shaped intra erythrocytic piroplasm occurs in pairs (Homer *et al.* 2000) [12] and causes canine babesiosis with clinical manifestations of fever, pale mucous membranes, anorexia, anaemia, icterus, lymphadenopathy, and spleenomegaly (Boozer and Macintire 2003) [5]. The classical presentation is a febrile illness with detectable anaemia; nevertheless, clinical symptoms are extremely varied. Babesiosis can range in severity from a subclinical illness to organ failure and death. Abd Rani *et al.* (2011) [1] reported that PCR is more sensitive than microscopic inspection of the blood film in finding haemoparasites.
Dogs infected with *Babesia* or *E. canis* generally have nonspecific clinicopathologic abnormalities. Although bleeding is not considered as a usual indication of canine babesiosis (Matthewman *et al.* 1993) [16], thrombocytopenia and hemolytic anemia are more evident in babesiosis than in dogs with ehrlichiosis (Manzillo *et al.* 2006) [15]. Both severity of the disease and degree of hypoxia are linked to biochemical abnormalities in *Babesia sps.* and *E. canis* infections (Harrus *et al.* 1997) [10]. Treatment for Babesiosis or spontaneous recovery from an acute episode frequently fails to eliminate the organism from the host, resulting in a carrier stage (Birkenheuer, 1999) [4]. Ehrlichiosis and babesiosis in dogs have a variety of treatment options. Hence, the objective of the study is to evaluate haematology, biochemistry, PCR sensitivity and success rate of treatment in clearing the infection.

2. Materials and Methods
Ten dogs from same kennel were presented to Madras Veterinary College Teaching Hospital, Madras Veterinary College, Chennai with the clinical signs of inappetance, dullness and

anaemia were included for the study. The dogs presented were 9 females and 1 male with age groups varying from 12 months to 132 months of different breeds (5 Labradors, 3 German Shepherds and 2 Golden Retrievers). Clinical parameters were in normal range. Peripheral blood smears were collected from the tip of ear for staining technique. Whole blood (3 ml) was collected from the cephalic vein of the dogs, transferred to EDTA coated tubes for haematology and DNA extraction, clot activator tubes for serum biochemistry. Blood samples for DNA extraction were stored at -80 ℃ (Voltas- TATA, India) until further processing.

2.1 Staining method
The blood smears were prepared on glass slides and stained with Leishman- Giemsa cocktail stain as per the procedure described earlier by Senthil *et al.* (2015) [18]. Stained smears were examined under light microscopy (Olympus, Japan) for the presence of the organisms.

2.2 Haematology and Biochemistry
The whole blood was analysed for haemoglobin, RBC, WBC, platelet count and differential leucocyte count by auto haematology analyser (Mindray BC-2800 Vet, China). Serum biochemical parameters like BUN, creatinine, total protein, SGPT and bilirubin were analysed by A15 auto analyzer (Biosystems, Spain).

2.3 DNA extraction from blood
Genomic DNA was extracted from EDTA coated blood samples using DNeasy blood and tissue kit (Qiagen, Netherland) as per the manufacturer's instructions. The quality of DNA in the final elutes was estimated using Nanospectrophotometer (Nano drop™ one, Thermoscientific, USA). Extracted DNA samples were preserved at -80 ℃ until further molecular analysis.

2.4 Conventional PCR for the detection of the organisms
The extracted DNA samples from blood samples were screened for the presence of *B. gibsoni* and *E. canis* with species specific primers (Table-1). Amplified PCR products were visualized by gel-electrophoresis in 1.5% agarose gel and the same was documented in gel doc mega UV transilluminator (Bio- Rad, USA) with 100 bp reference molecular weight marker.

Table 1: List of Primers and its cyclic conditions

Parasite	Primer	Product size	Cyclic conditions	Reference
B. gibsoni	Gib599:5'-CTCGGCTACTTGC CTTGTC-3' Gib1270:5'-CCGAAACTGAAATAACGGC-3'	662 bp	Initial denaturation at 95 °C for 5 min 40 cycles of Denaturation at 95 °C for 30 sec Annealing at 56 °C for 30 sec Extension at 72 °C for 1.45 min Final extension at 72 °C for 5 min	Inokuma *et al.* 2004 [13]
E. canis	ECAN5:5'-CAATTATTTATAGCCTCTGGCTCTGGCTATAGGA-3' HE3:5'- TATAGGTACCGTCA TTATCTTCCCTAT-3'	959 bp	Initial denaturation at 94 °C for 5 min, 35 cycles of Denaturation at 94 °C for 1 min, Annealing at 58 °C for 1 min, and Final extension at 72 °C for 7 min.	Silva *et al.* 2012 [20]

3. Results and Discussion
Out of ten blood smears examined, one was found positive for *B. gibsoni* (Figure-1) and one for *E. canis* (Figure-2) by Leishman- Giemsa cocktail stain under oil immersion (100X). Variations in haematology and biochemical parameters of 10 dogs are depicted in Table-2. The mean neutrophil and monocyte values were above the reference range, indicating neutrophilic leucocytosis and monocytosis. Similar findings were noticed by Das and Konar (2013) [8] and Aguiar *et al.* (2020) [2] respectively. Platelet count, on the other hand, was below the range suggesting severe thrombocytopaenia. This was in concurrence with the findings of Bilwal *et al.* (2017). This could be due to platelet sequestration in the spleen or immune-mediated platelet destruction resulting in the development of disseminated intravascular coagulation (Boozer and Macintire, 2003) [5]. All the serum biochemical values were well within reference range. This was in accordance with Shah *et al.*, (2011) [19] who reported normal serum biochemistry in babesiosis. Out of ten, two samples were positive for *B. gibsoni* (Figure-3) and two for *E.canis* by PCR (Figure-4) and PCR was found more sensitive in detecting the infection than routine blood smear examination. The samples that were positive with blood smear also tested positive with PCR. There was no evidence of co-infection with two organisms. The dogs found positive for *B. gibsoni* were treated with two doses of Inj. Berenil (Diminazene aceturate) @ 5mg/Kg B.Wt deep intramuscularly 7 days apart along with triple therapy which includes PO BID of each Doxycycline @ 5mg/Kg, Metronidazole @ 15mg/Kg, Enrofloxacin @ 25mg/Kg for 28 days as per Lin and Huang (2010) [14]. The dogs with *E. canis* were treated with oral Doxycycline @ 5mg/Kg B.Wt for 28 days as per Fourie *et al.* (2015) [9] who stated that using doxycycline orally for 28 days cleared the infection. All the dogs were given iron, B-complex supplements and anti-tick precautions. The dogs tested negative for both the organisms in blood smears and PCR after 28 days of treatment, and their blood parameters returned back to normal limits.

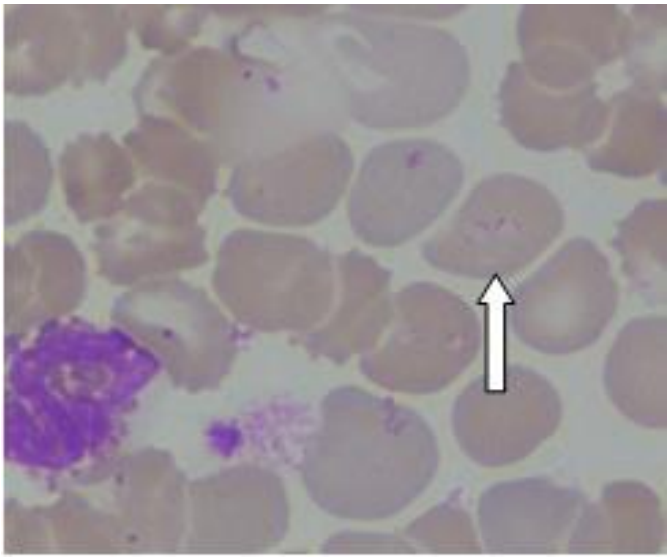

Fig 1: *B. gibsoni* inside RBC's of dog blood smear (100X)

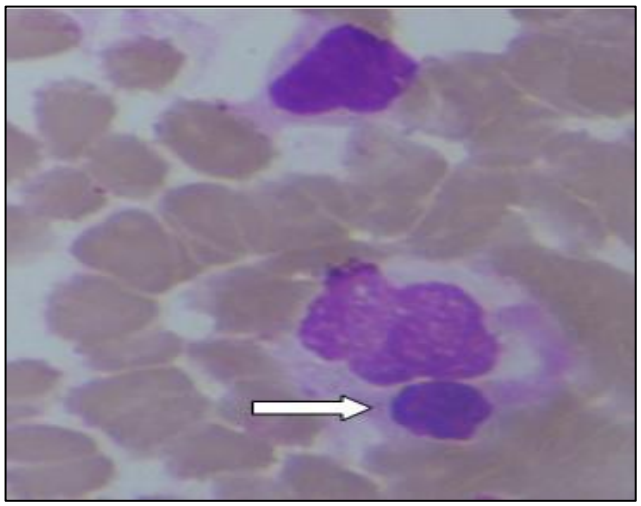

Fig 2: Morula of *E. canis* in Monocyte of dog blood smear (100X)

Table 2: Haemato-biochemical values of infected dogs

Parameter	Reference	Mean±SE
HB (mg/L)	12-18	14.2±0.7
WBC (cells/cmm)	6000-17000	14360±2093
RBC (m/ cmm)	5.5-8.5	6.247±0.289
N (%)	60-70	76.2±1.31
L (%)	20-30	16.3±1.4
M (%)	0-5	6.7±0.63
E (%)	0-1	0.4±0.2
Platelets (cells/ cmm)	200000-500000	147200±34446
Blood glucose (mg/dL)	76-120	120.9±15.7
BUN (mg/dL)	8-28	16.945±2.3
Creatinine (mg/dL)	0.5-1.5	1.134±0.08
SGPT (IU/L)	10-109	42±5.27
Bilirubin (mg/dL)	0.15-0.5	0.426±0.18
Total Protein (g/dL)	5.4-7.4	7.34±0.27

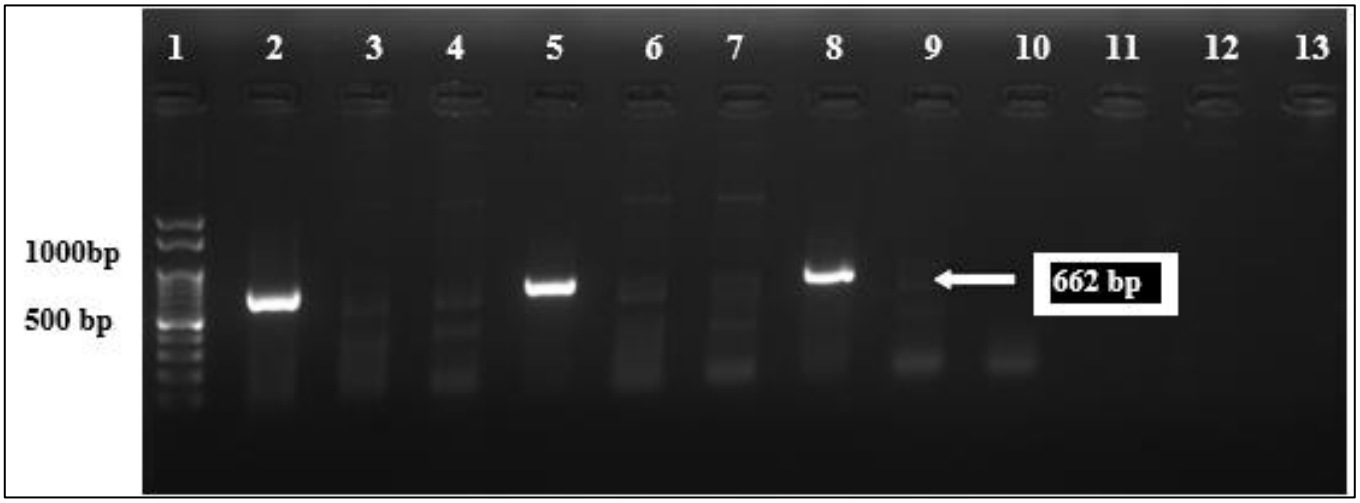

Fig 3: Showing 662 bp PCR product of 18S rRNA gene of *B. gibsoni* in 1.5 percent agarose gel Lane-1 Molecular weight marker, Lane-2 Positive Control, Lane-13 No template Control, Lane- 5 and 8- Positive samples

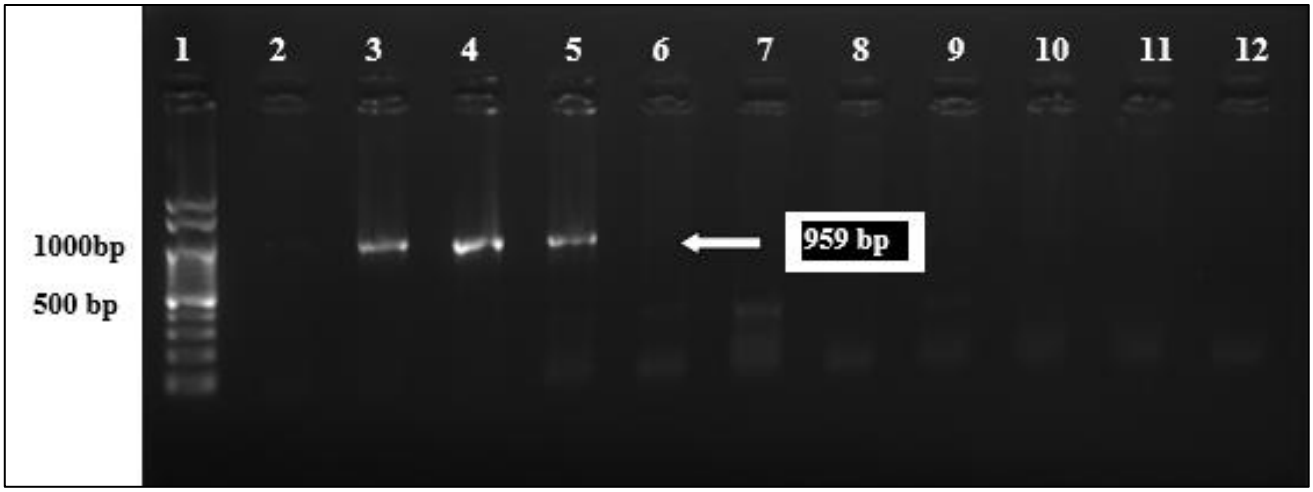

Fig 4: Showing 959 bp PCR product of virB9 gene of *E. canis* in 1.5 percent agarose gel Lane-1 Molecular weight marker, Lane-2 No template Control, Lane-3 Positive Control, Lane-4 and 5- Positive samples

4. Conclusion

According to the present study, canine babesiosis and ehrlichiosis can manifest as a mild subclinical form characterised by anaemia, thrombocytopaenia, and monocytosis. Since not all cases of subclinical infection can be detected solely by morphological identification in blood smears, a polymerase chain reaction assay should be considered to identify subclinical carriers. To clear the infection by negative PCR, a therapeutic regimen of intramuscular diminazene diaceturate, doxycycline-enrofloxacin-metronidazole combination in management of canine babesiosis caused by *B. gibsoni*, and doxycycline orally for *E. canis* can be recommended.

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