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Clinicopathological and molecular detection of cerebral form of ehrlichiosis in a german shepherd

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Abstract
A 3 year old intact female German shepherd dog with history of inappetance, pyrexia, paraplegia and uveitis was presented to the small animal outpatient unit of the Department of Clinics, Madras Veterinary College, Chennai. Haemato-biochemical values revealed relative thrombocytopenia with low hemoglobin and red blood cell count and moderate elevation in the Blood urea nitrogen. Radiographic imaging revealed mild splenomegaly. A standard cerebrospinal fluid examination was performed. Cerebrospinal fluid was slightly turbid with elevated glucose and protein levels. CSF Cytology revealed monocytic pleocytosis with several intracytoplasmic basophilic round to oval inclusion bodies in multifocal fields consistent with *Ehrlichia canis* morulae within the cytoplasm of the monocytes which was further confirmed with acridine orange staining method. Nested Polymerase chain reaction was done on CSF to confirm the Ehrlichiosis induced meningoencephalitis in the present case. The dog was treated with respective antibiotics. Later euthanized under humanitarian grounds upon owner's request.

Keywords: *Ehrlichia canis*, cerebral form, cerebrospinal fluid, cytology, paraplegia, nested PCR, Acridine orange staining

1. Introduction
Ehrlichiosis is a tick transmitted disease of dogs which can cause a wide range of clinical signs. *Ehrlichia canis* (a small, Gram-negative, coccoid bacterium), which parasitizes cytoplasm of the circulating monocytes in form of distinct clusters termed as "Morulae" [1]. The disease is transmitted by the brown tick *Rhipicephalus sanguineus* which passes the organism into the blood following the bite and is characterized by high fever (104-105°F), anorexia, weakness, lymphadenopathy and epistaxis and edema of dependent parts especially in chronic cases [2].

The disease is highly endemic in dogs in India [3] which may be attributed to the presence of high tick population as one of the reasons. Once the dog is infected the course of ehrlichiosis can be divided into three phases: acute, subclinical, and chronic [4]. Acute disease usually develops within 2 to 4 weeks following tick transmission [5]. Despite the severe nature of some cases, many dogs appear to tolerate infection without developing overt clinical disease which enters a subclinical phase of infection in which they remain chronically infected for months to years [4]. Chronic cases develop fever, anorexia, and weight loss accompanied by myalgia, bleeding tendencies, ocular lesions, and neurologic abnormalities [6]. Ataxia, head tilt, nystagmus, and seizures have been reported but are present in a minority of dogs with clinical Ehrlichiosis [1].

This case mainly emphasizes the cerebral involvement in Ehrlichiosis which should be ruled out whenever cases with neurological entity are presented. However, cerebral involvement in Ehrlichiosis has been much less reported [7], but one such case with cerebral signs with normal thrombocyte count has been reported in Thailand. Blood smear examination can be helpful in the initial diagnosis but only with 4% sensitivity [8]. Hence, subsequent rapid diagnosis can be made with cerebrospinal fluid examination accompanied by acridine orange method of staining to rule out the concurrence of bacterial meningoencephalitis which may dismay the differentials arising in cases.

2. Materials and Methods

2.1 Detailed physical and neurological examination

A 3 year old intact female German shepherd dog with history of inappetance, paraplegia of both the hind limbs and ocular signs was presented to the small animal outpatient unit of the Department of Clinics, Madras Veterinary College, Chennai. A thorough physical examination was performed. The dog had congested mucous membranes, unilateral uveitis, pyrexia (40ºC), dehydration, tense abdomen, paraplegia and laterally recumbent.

General and system wise examination was done as per guidelines given by Defarges (2015) [9]. Initial Neurological assessment was done as per guidelines of Chrisman (2006) [10]. The animal was stabilized initially and the complete history of the case was obtained. Based on the clinical history and symptoms, a set of differentials for the case was noted down.

2.2 Haematology and serum biochemistry

Two millilitres of whole blood was collected aseptically from the dog by venepuncture of cephalic vein in vacutainers coated with 10% Ethylene Diamine Tetra Acetic Acid (EDTA) as anticoagulant. Haematological analysis was done using the automated haematology analyzer (Mindray - BC-2800 VET). Peripheral blood smears and buffy coat smears were prepared and examined for changes in blood picture. The collected blood samples were then kept at-20 °C until DNA extraction for PCR. Commercial biochemical kits were used for quantitative estimation of BUN, creatinine, total protein, albumin, globulin, ALT, ALP, total and direct bilirubin, calcium, phosphorus, glucose (Agappe ® Diagnostics, India).

2.3 Cerebrospinal fluid examination

Cerebrospinal fluid collection procedure was performed by administration of general anaesthesia. CSF was collected via subarachnoid space paracentesis at the cisterna magna (cerebellomedullary cistern) stated as per Di Terlizzi and Platt (2009) [11]. CSF was collected in empty sterile containers and made into different aliquots for cytology, biochemical tests and in EDTA tubes for performing PCR. Cytological evaluation of CSF samples were preformed within 30 minutes from collection and was performed by the sedimentation chamber technique by modified Wamsley method. CSF sample swabs were screened for bacterial and fungal aetiology by means of appropriate culture methods. A streak was made over MacConkey agar and EMB agar (for bacterial aetiology) and Sabrouds dextrose agar for fungal aetiology. The smear from the cerebrospinal fluid was placed for acridine orange staining.

2.4 Nested Polymerase chain reaction

DNA isolation kit (QIAamp DNA Mini Kit®, Qiagen, Shriram Enterprises) was used for the parasite DNA extraction from 200µl of blood collected in EDTA vacutainers according to the manufacturer's instructions. Genomic DNA was also isolated from the whole blood of infection-free dog and used as a negative control along with nuclease-free water.

Nested PCR for the amplification of the 16s rRNA gene fragment of *E. canis* was employed following the procedure of Rajagopal *et al*. (2009) [12] and Birkenheuer *et al*. (2003) [13] respectively. The primers used in this study are mentioned in the Table 1.

Table 1: Primer sequence of the *Ehrlichia* spp. and its product size

a. *Ehrlichia* spp. (16s rRNA gene)			
Primer name	Sequence		Size
ECC	5' AGA ACG GCT GGC GGC MG C 3''	1st cycle	387 bp
ECB	5' CGT ATT ACC GCG GCT GCT GGC A 3'		
ECAN5	5' CAA TTA TTT ATA GCC TCT GGC TAT AGG A 3'	2nd cycle	
HE3	(5' TAT AGG TAC CGT CAT TAT CTT CCC TAT 3'		

Thermal cycling for PCR amplification was as follows:

$$\underset{3\text{min}}{94^{\circ}\text{C}} \rightarrow \left[\underset{1\text{min}}{94^{\circ}\text{C}} \rightarrow \underset{2\text{min}}{65^{\circ}\text{C}} \rightarrow \underset{2\text{min}}{72^{\circ}\text{C}}\right] \text{X30 cycles} \rightarrow \underset{5\text{ min}}{72^{\circ}\text{C}} \rightarrow \underset{\infty}{4^{\circ}\text{C}}$$

3. Results and Discussion

Neurological examination revealed conscious proprioceptive and deep pain deficits with sluggish patellar reflex. Mentation was disoriented with lack of pupillary light reflex. Ophthalmic examination revealed unilateral uveitis. Radiographic imaging of the lateral abdomen revealed mild splenomegaly. Blood smear examination revealed *Ehrlichia spp*. morulae within the cytoplasm of few monocytes (Fig. 1). Hemato-biochemical alterations were significant towards relative thrombocytopenia, low red blood cell and white blood cell count, low hemoglobin levels, hypoalbuminemia and moderate elevation in the blood urea nitrogen. The dog was initially stabilized with anti-inflammatory and antibiotic drugs. To rule out the cause of neurological signs, cerebrospinal fluid examination was performed to rule out meningoencephalitis. CSF glucose reached upto 55 mg/dl and protein level was 97 mg/dl (reference range, 0-30 mg/dl). A total nucleated cell count of 47 cells/µL (reference range, 0-5/µL) was calculated with 95% of the cellularity contributed by monocytes and remaining 5% by lymphocytes denoting a high monocytic pleiocytosis (Fig. 2). In multiple fields, few monocytes revealed single compact solid round to oval, basophilic intracytoplasmic inclusion (Fig. 3) and almost 99% of the monocytes revealed a compact round to oval pale hollow space in the cytoplasm (those were devoid of inclusions) which are pathologically significant and needs further investigation.

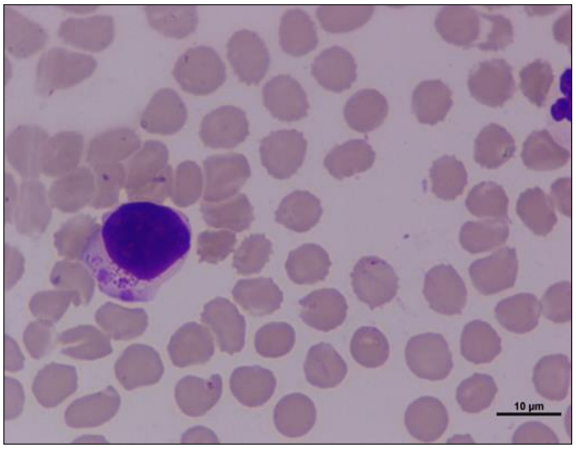

Fig 1: Dog - Peripheral blood smear - *Ehrichia canis* morula in the cytoplasm of monocyte (Leishman Giemsa stain) (100 x

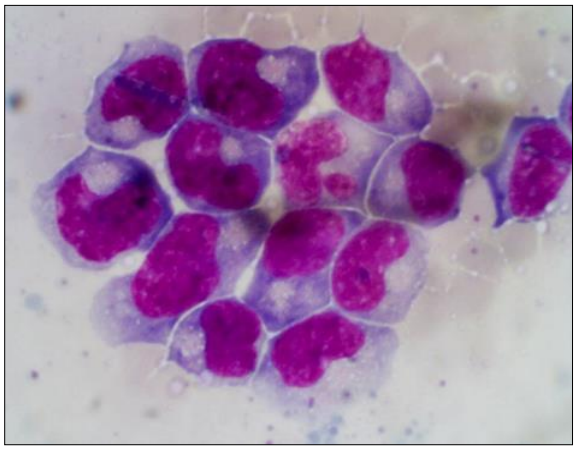

Fig 2: Dog - Cerebrospinal fluid-Cytology - Marked monocytic pleocytosis (Leishman Giemsa stain) (100 x)

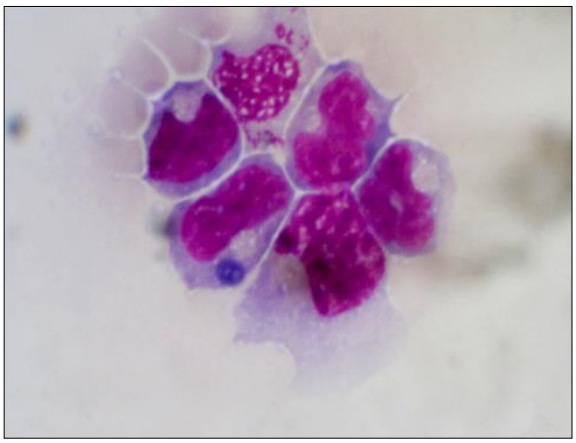

Fig 3: Dog – Cerebrospinal fluid - A single compact solid round shaped basophilic intracytoplasmic inclusion in the monocyte (Leishman Giemsa stain) (100 x)

To further confirm, acridine orange staining was performed to rule out the conflict of Ehrlichia morulae with apoptotic debris and nuclear fragments. Acridine orange stock solution was prepared as per the procedure of Neer and Harrus 2006 [14]. The peripheral blood smear and the smear from CSF was flooded with 0.01% acridine orange and allowed to act for two minutes. Then, it was slowly washed in tap water, cover slip placed over it when wet and viewed under a fluorescent microscope. Under green fluorescence, bright orange colored clearly distinct round to oval morulae were noticed within the cytoplasm of the monocytes (Fig. 4). The results of CSF culture were negative.

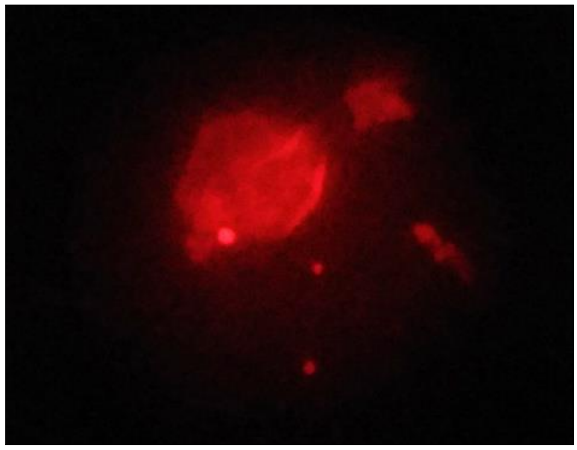

Fig 4: Dog – Cerebrospinal fluid - Acridine orange staining of the cerebrospinal fluid revealing bright orange fluorescence of the intracytoplasmic inclusion within the mononuclear cell (40 x) in green fluorescence

Further, a broad range nested PCR targeting 16s rRNA of *Ehrlichia canis* was performed to confirm the meningoencephalitis caused by the same. DNA was extracted both from the blood and CSF sample using DNA extraction kit as per manufacturer's instructions. Ehrlichia genus specific primers ECC (5'AGA ACG GCT GGC GGC M G C-3') and ECB (5'CGT ATT ACC GCG GCT GCT GGC A-3') (Alpha DNA, Canada) were used in the first round of amplification. Five µl of the amplicons of the first round of PCR was used as the template in the second round of amplification using the E. canis species specific primers ECAN5 (5'CAA TTA TTT ATA GCC TCT GGC TAT AGG A-3') and HE3 (5' TAT AGG TAC CGT CAT TAT CTT CCC TAT 3'). Temperature settings in both the rounds included an initial denaturation step at 94ºC for 3 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 65ºC for 2 min and extension at 72ºC for 2 min. A final chain elongation step was performed at 72ºC for 5 min. The PCR amplicons were electrophoresed in 2% agarose gel containing 0.5 pg of ethidium bromide per ml of the gel and visualized in a UV transilluminator (Fotodyne, USA) and photographed using video gel documentation system (Pharmacia, Biotech). Amplification of a 387-bp product in the nested reaction in the blood and CSF samples as well as in the known positive control was confirmatory for E. canis infection (Fig. 5).

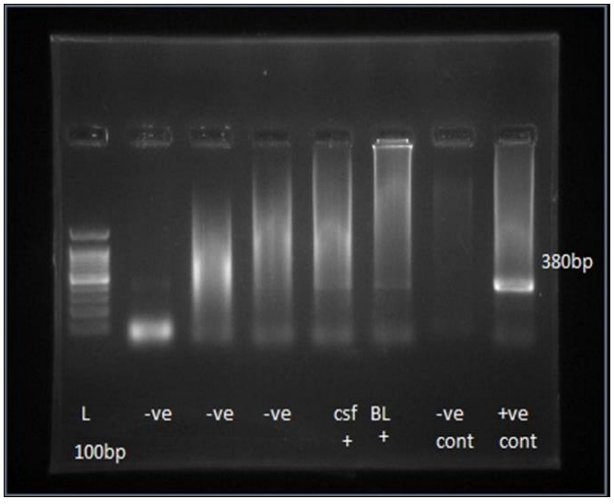

Fig 5: Dog – E. canis - Nested PCR on blood and CSF sample (Lane 1- 100bp ladder, Lanes 2-4- negative, Lane 4 - Positive CSF sample, Lane 5-Positive blood sample, Lane 6- Negative control, Lane 7- Positive control)

From the above results of PCR, the meningoencephalitis caused by *Ehrlichia canis* was confirmed. Later, the dog was prescribed doxycycline @ 3mg/kg/bwt P/O q24hrs for 3 weeks. Since the dog did not reveal any further improvement and the signs got worse, it was euthanized under humanitarian grounds.

The above findings were similar to that of cases with cerebral form of Ehrlichiosis wherein the CSF resembles that of viral diseases (i.e., the WBC count and protein may be normal or slightly to moderately elevated with a predominantly mononuclear pleocytosis which was clearly stated in previous reports [15, 16]. The above findings of nested PCR were in agreement with the previous findings [12, 7].

Hence, the present study revealed the cerebral involvement of *Ehrlichia canis* and its further evidence through cerebrospinal fluid cytology and utilization of acridine orange staining in CSF and molecular characterization of the same.

4. Conclusion

The significant findings of the present study involve the diagnosis and clinico-pathological evidence of the cerebral form of Ehrlichiosis in dogs. Cerebral form of ehrlichiosis is rarely reported in dogs. This report emphasizes the clinico-pathological and molecular detection of cerebral form of ehrlichiosis in cerebrospinal fluid through cytology and molecular methods and the novel use of acridine orange staining method in establishing the organism in cerebrospinal fluid in ehrlichiosis cases in dogs.

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