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Caption: *Cyrtodactylus myntkyawthurai*, endemic to Myanmar. Medium: Water colours on watercolor sheet. © Aakanksha Komanduri



Trypanosoma evansi infection in a captive Indian Wolf *Canis lupus pallipes* – molecular diagnosis and therapy

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Abstract: A five-year old, apparently healthy male Indian Wolf *Canis lupus pallipes* of Nandankanan Zoological Park, Odisha became ill with acute signs of anorexia, lethargy, staggering gait, and was non-responsive to external stimuli. Microscopic examination of Giemsa stained blood smear revealed presence of extracellular flagellates having morphological similarity to *Trypanosoma* spp. Haematological parameters showed anaemia (Hb 6.0 g%), mild leucopenia (total leukocyte count $5 \times 10^3 / \text{mm}^3$) and thrombocytopenia ($180 \times 10^3 / \mu\text{l}$). Serum biochemistry revealed high aspartate aminotransferase (AST) (830 IU/L), blood urea nitrogen (BUN) (178.2 mg/dl), creatinine (4.44 mg/dl), and low glucose (25.7 mg/dl) levels. Polymerase chain reaction (PCR) analysis targeting internal transcribed spacer (ITS1) region followed by National Centre for Biotechnology Information blast confirmed *Trypanosoma evansi* infection in the captive Indian Wolf. The animal showed clinical recovery with the administration of single dose of quinapyramine sulphate and quinapyramine chloride @ 4.0 mg/kg b wt subcutaneously. The wolf started taking meat from the very next day with improved activity. No trypanosomes could be detected in the stained blood smears as well as through PCR carried 25 days post treatment. The occurrence became an eye opener for the zoo and henceforth, all canids were included under chemoprophylaxis protocol against trypanosomosis.

Keywords: Anemia, Canids, captivity stress, Chemoprophylaxis, PCR, Quinapyramine salts.

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Author contributions: SKS, SKG and DM have executed the treatment, collected samples and documented the clinical findings. MD and NS have carried out molecular screening, interpreted the results and guided the treatment. All authors formulated and revised the manuscript, and approved the final version.

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INTRODUCTION

Trypanosomosis, caused by an unicellular, eukaryotic haemoprotozoan of different *Trypanosoma* spp., is an important disease of domestic and wild animals (Aulakh et al. 2005; Gupta et al. 2009). A number of trypanosomes exist worldwide; however, *Trypanosoma evansi* is the only pathogenic species prevalent in India (Desquesnes et al. 2001; Kumar et al. 2021). Sengupta (1974), Ziauddin et al. (1992), and Shukla (2002) reported trypanosomosis in Indian Wolves in Indian zoos at Kolkata, Mysore, and Lucknow, respectively. This extra-cellular haemoparasite is transmitted by biting flies of genera *Tabanus*, *Stomoxys*, and *Haematobia* (Parashar et al. 2006, 2018). The disease is characterized by anaemia, anorexia, intermittent fever, generalised weakness, conjunctivitis, corneal opacity, oedema of head and throat, difficulty in swallowing, hoarse voice, and staggering gait (Chaudhuri et al. 2009). The disease can be diagnosed by direct demonstration of trypomastigote forms of the parasite in the stained blood smears, but the polymerase chain reaction (PCR) has an increased diagnostic potential with high sensitivity and specificity to detect parasite DNA (Eloy & Lucheis 2009). Trypanosomosis has been successfully treated with a single dose of diminazine aceturate @ 3.5 mg/kg body weight intramuscular (Rani & Suresh 2007) or sulphate and chloride salts of quinapyramine @ 4.0 mg/kg bw subcutaneous (Singh et al. 1993). The present case study documents molecular diagnosis through PCR and successful therapy of *Trypanosoma evansi* infection in a captive Indian Wolf at Nandankanan Zoological Park (NKZP), India.

CASE HISTORY AND OBSERVATION

The NKZP received a pair of wolves during September 2018 from Sri Chamarajendra Zoological Gardens, Mysuru under an animal exchange program. Both were housed in an open air enclosure of 28 sq meters attached to a feeding cell of 15 sq meters. Regular prophylactic measures included annual vaccination against rabies, parvo, distemper, parainfluenza, adenovirus type I and II, hepatitis and *Leptospira* spp., fecal sample examination followed by deworming with albendazole/ fenbendazole at three month intervals and ground spray of enclosure with ectoparasiticides deltamethrine/cypermethrine in alternate months. The female partner died on 07 March 2019 due to cardiac dysfunction leaving the male wolf alone.

On 24 September 2019, the 5-year old apparently healthy male partner (approximate body weight 20.0 kg) was noticed anorectic, debilitated, non-responsive to external stimuli, reduced activity levels with staggering gait. Close examination inside a squeeze cage revealed shallow breathing and pale conjunctiva. Body temperature was 103.2°F. Peripheral blood samples were collected on the same day from the left saphenous vein in ethylene diamine tetraacetic acid @ 1.5 mg/ml (EDTA) and clot activator vials for haemato-biochemical and parasitological examination. Faecal samples were collected for detection of gastrointestinal infection.

DIAGNOSIS AND TREATMENT

Coprological examination did not reveal the presence of any endoparasite ova or cyst. Blood smear stained with Giemsa stain and examined under oil immersion showed the presence of extracellular flagellated Trypanosomes (Image 1). Molecular test was performed for confirmation of the species. DNA was extracted from the EDTA blood sample using Qiamp DNA blood Mini kit (M/S Qiagen, Germany) according to the manufacturer's instructions. PCR was carried out in 50 µl reaction volumes containing 10X reaction buffer with KCl, 25 mM MgCl₂, 2 mM dNTPs, 3 units of Taq DNA polymerase, 2 µM of each primer (Njiru et al. 2005), nuclease free water and 2 µl of template DNA. PCR was programmed to perform a denaturation step at 95° C for 10 mins followed by 35 cycles consisting of 30 secs at 94°C, 30 secs at 55° C, and 30 secs at 72° C. The last extension step was 10 mins at 72° C. The PCR product was run in 2% agarose gel with ethidium bromide-stain using an electrophoresis system (M/S BIO-RAD, USA) along with one positive (1 µg of DNA) and one negative control (Image 2). After getting the desired band at 480 bp, the PCR product was sequenced and the data was compared in National Centre for Biotechnology Information (NCBI) database. The sequenced data matched with *T. evansi* with 93.6% identity and 97.0% query cover. The consensus sequence (generated in BIOEDIT software) was submitted in genbank (NCBI) and the assigned accession number was MZ321577.

Analysis results depicted in Table 1 revealed decrease in certain haemato-biochemical values like haemoglobin (6.0 g%), total leukocyte count (5.0 10³/mm³) neutrophil (56%), platelets count (180 × 10³/µl) and glucose (25.7 mg/dl). Increased values in both haematological and biochemical parameters included lymphocyte (41%), AST (830.4 IU/l), total protein (7.63 g/dl), urea (178.2

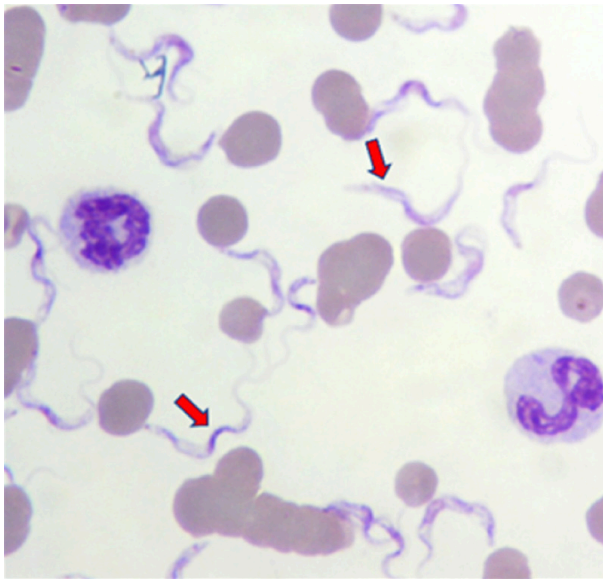


Image 1. Giemsa stained blood smear showing *Trypanosoma evansi* marked in red arrow (X1000).

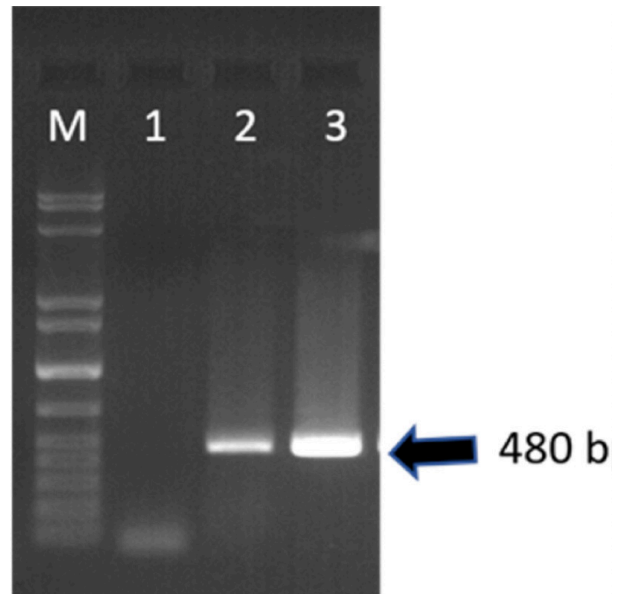


Image 2. Gel electrophoresis of PCR products (480bp). Lane M: 100 bp marker, lane 1: Negative Control, Lane 2: sample of interest, Lane 3: Positive Control.

mg/dl), creatinine (4.44 mg/dl), cholesterol 272.7 mg/dl), triglyceride (418.8 mg/dl), calcium (11.1 mg/dl), phosphorous (11.4 mg/dl), magnesium (2.7 mg/dl), and total bilirubin (0.80 mg/dl)

Quinapyramine sulphate and chloride @ 4.0mg/ kg b wt (Injection Triquin of M/S Vetoquinol India Animal Health Pvt Ltd., Thane) was administered subcutaneously. As supportive therapy, the Indian Wolf was administered with paracetamol inj (Injection Fevastin of M/S Tablets India Limited, Chennai) @ 2.0 ml intramuscular and electrolytes with 20% dextrose infusion @ 300 ml (Rintose of M/S Vetoquinol India Animal Health Pvt Ltd.). The Indian Wolf started responding to treatment from the very next day itself. Body temperature dropped to 101.4°F with signs of improvement in the activity and appetite.

DISCUSSION

NZKP had the earlier records of trypanosomosis among white Tigers *Panthera tigris*, Bengal Tigers *Panthera tigris tigris*, and Jungle Cat *Felis chaus* (Parija & Bhattacharya 2001; Sahoo et al. 2009). Hence, the NZKP is following a chemoprophylaxis protocol against trypanosomosis for all large felids (N= 46) and calculated doses of quinapyramine salts (Injection Triquin of M/S Vetoquinol India Animal Health Pvt Ltd, Maharashtra) are being administered subcutaneously at every four month intervals. But the canids were not included in this

chemoprophylaxis protocol, as there was no incidence of the said disease amongst canids at NKZP.

It is quite challenging to ascertain the species of *Trypanosoma* spp. from the blood smear. PCR is the ultimate diagnostic protocol to reveal the fact. PCR targeting internal transcribed spacer (ITS1) region is highly sensitive and reliable for the diagnosis of pathogenic *Trypanosoma* spp. such as *T. evansi*, *T. brucei brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. congolense*, *T. savannah*, *T. congolense kilifi*, *T. congolense forest*, *T. simiae*, *T. simiae tsavo*, *T. godfreyi*, and *T. vivax* (Njiru et al. 2005). Successful detection of *Trypanosoma* spp. has been reported using ITS1 CF and BR PCR primers in cattle, tsetse fly, sand fly, dogs, equids, monkeys, and camels (Thumbi et al. 2008; Alanazi et al. 2018; Gaithuma et al. 2019; Medkour et al. 2020). The current study unveiled incidence of *T. evansi* in a captive Indian Wolf at NKZP.

Wild animals often exhibit moderate levels of trypano-tolerance with their innate ability to co-exist with trypanosomes without showing overt disease (Sudan et al. 2017). The disease flares up when the animal gets exposed to physiological and somatic stress following concurrent infection, capture, translocation and captivity that often compromises their innate resistance (Fowler 1986; Singh et al. 2003).

The clinical signs in the present case were high rise of temperature (103.2°F), pale mucous membrane, bilateral lacrimation, and generalised debility. These observations were in agreement with the findings of Rani

Table 1. Pre- and post-treatment haemato-biochemical values of an Indian Wolf with *Trypanosoma evansi* infection.

Parameter	Days of blood collection		Reference range
	24.ix.2019 (Pre-treatment)	18.x.2019 (Post-treatment)	
Hematology			
Haemoglobin (g %)	6.0	13.0	10.5-15 ^a
Total leucocyte count (10 ³ /mm ³)	5.0	5.6	5-14.1 ^b
Neutrophil (%)	56.0	70.0	58-71 ^a
Eosinophil (%)	3.0	3.0	0-4 ^a
Lymphocyte (%)	41.0	26.0	28-39 ^a
Monocyte (%)	-	1.0	0-2 ^a
Basophil (%)	-	0	0 ^a
Platelet (×10 ³ /μl)	180.0	226.0	211-621 ^b
Biochemistry			
ALT(IU/L)	10.3	331.1	24-64 ^a
AST(IU/L)	830.4	159.8	23-66 ^b
ALP(IU/L)	96.1	26.3	20-156 ^b
BUN (mg/dl)	178.2	63.8	16-41 ^a
Creatinine (mg/dl)	4.4	2.18	0.5-1.5 ^b
Glucose (mg/dl)	25.7	117.2	58.2 - 91 ^a
Total protein(g/dl)	7.63	6.4	5.07- 6.49 ^a
Albumin (g/dl)	1.5	2.7	2.92-3.53 ^a
Globulin (g/dl)	5.0	3.6	2.03- 3.16 ^a
Cholesterol (mg/dl)	272.7	178.5	138-198 ^a
Triglyceride(mg/dl)	418.7	39.7	20-112 ^b
Calcium (mg/dl)	11.1	10.9	5.58-7.94 ^a
Phosphorous (mg/dl)	11.4	2.6	4 - 5.32 ^a
Magnesium (mg/dl)	2.7	2.4	1.8-2.4 ^b
Total Billirubin (mg/dl)	0.8	0.8	0.10-0.50 ^b

^a Sabapara & Vadalia(1999) | ^b Kaneko et al.(2008)

& Suresh (2007). The fever might be due to the effects of toxic metabolites produced by dying trypanosomes (Tizard et al. 1978).

Anemia was a consistent finding as reported earlier in different hosts including dogs infected with Trypanosomosis (Moreira et al. 1985; Monzon et al. 1991; Silva et al. 1995; Gurtler et al. 2007). The anaemia is attributable to extravascular destruction of RBC which may be through the process of erythrophagocytosis or metabolic product and toxins liberated from the parasites. Blood cellular changes revealed leucopenia along with reduced neutrophil count. Similar findings were recorded by Barr et al. (1991).

Increase in AST, ALT, ALP, urea, creatinine level as compared to reference level corroborated with findings of Barr et al. (1991) who reported a similar pattern

of changes in a dog during the acute phase. Marked elevation in the level of total protein values were recorded as compared to reference level. Hyperproteinemia found in this study could be associated with hypergammaglobulinemia due to antigenic stimulation provoked by the parasite, as seen in canines (Aquino et al. 2002). There was a decrease in the albumin and globulin ratio. The fall in albumin levels was secondary to hyperglobulinemia as a compensatory mechanism for maintenance of normal blood viscosity increased by globulin levels (Aquino et al. 2002). Hyperbilirubinemia has been reported in naturally infected dogs as a consequence of an increase in unconjugated bilirubin (Sandoval et al. 1994) and conjugated bilirubin. There was decrease in serum glucose (25.7 mg/dl) level. Hypoglycemia has been shown to be an important

clinical laboratory finding in naturally infected animals, and it is inversely proportional to blood trypanosome count.

Diminazine aceturate is a commonly used drug in the treatment of trypanosomiasis (Rani & Suresh 2007). However, a combination of quinapyramine sulphate and quinapyramine chloride (3:2 w/w) at dose rate 4.0 mg/kg b wt is also effective in achieving complete recovery (Singh et al 1993). Shukla (2002) did not get a complete cure with diminazine @ 0.8g/ 100 kg b. wt in case of an Indian Wolf, rather, quinapyramine sulphate @ 5.0mg/ kg b wt resulted in complete recovery. In a similar line, combination of quinapyramine sulphate and quinapyramine chloride @ 4.0mg/kg b wt administered subcutaneously as a single dose showed uneventful recovery in the present case.

The incidence of trypanosomiasis in an Indian Wolf became an eye opener for the zoo to extend the chemoprophylaxis to other hosts. As per the recommendation, the susceptible species, viz., Indian Wolf, Jackal, Dhole, and hyenids of NKZP are being included in the preventive protocol against trypanosomiasis now.

CONCLUSION

Molecular diagnosis of *Trypanosoma evansi* infection in an Indian Wolf followed by successful treatment with a single injection of quinapyramine sulphate and quinapyramine chloride @ 4.0 mg/kg b wt subcutaneously was recorded at Nandankanan Zoological Park.

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