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Successful Clinical Management and Molecular Investigation of Trypanosomosis in a Wild Captured Leopard

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ABSTRACT

Background: *Trypanosoma evansi* is a widely prevalent trypanosomes of the Indian subcontinent and is widely documented in species like cattle, buffalo, horses, camel etc. In the wild, an array of animals like deers, jaguar, tigers, leopards, lions, foxes etc can be infected by the trypanosome. Trypanotolerance is an innate mechanism by which the wild animals harbour moderate infection without the development of clinical signs.

Methods: The current report highlights the clinical management and molecular investigation of Trypanosomosis in a wild captured leopard. An adult male leopard, was found to exhibit partial inappetence with no other deviation from normal. Blood, serum and faecal investigations were carried out. A Polymerase Chain Reaction was carried out to gain molecular insights. The amplicon was purified and sequenced. The sequenced was submitted to NCBI after BLASTn analysis.

Result: The blood smear revealed trypanosomosis while faecal examination revealed heavy infestation with *Spirometra* which was treated rationally. The animal showed an uneventful recovery after the treatment. The causative agent was identified to *Trypanosoma evansi* and was found to be identical to similar sequences reported from India and abroad by the phylogenetic analysis.

Key words: Co-infection, Leopard, Quinapyramine salts, Sparganosis, *Spirometra*, *T. evansi*, Trypanosomosis, Wildlife.

INTRODUCTION

Trypanosoma evansi is a widely prevalent trypanosome of the Indian subcontinent and is widely documented in species like cattle, buffaloes, horses, camel *etc* (Luckins, 1988). In the wild, majority of the reports from India have surfaced from captive wildlife. A limited number of reports on Trypanosomosis have emerged from the country in elephant (*Elephas maximus*) (Evans, 1910), Spotted deer (*Axis axis*) (Pathak *et al*., 1988), Jaguar (*Panthera onca*) (Sinha *et al*., 1971), Tiger (*Panthera tigris*) (Sengupta, 1974), (Ziauddin *et al*., 1992), (Upadhye and Dhoot 2000), (Samantray and Monoj-Mohapatra, 2003), (Devasena and Shobhamani, 2006), fox (*Vulpes vulpes*) (Kinge *et al*., 2010), wolves (*Canis lupus*) (Dash *et al*., 2022), jungle cat (*Felis chaus*) (Dakshinkar *et al*., 2002). Except for a case report from Himalayan Zoological Park Darjeeling (Dasgupta *et al*., 1979), no report of trypanosomosis has been reported in leopards from the country. Transmission of trypanosomosis is majorly mechanical or vector borne. India is a tropical country and the climate is conducive to the proliferation of haematophagous arthropod vectors of families Hypoboscidae, Tabanidae and Stomoxyinae (Parashar *et al*., 2006). World over, trypanosomosis can affect both livestock and wildlife, thus carrying a significant risk of cross-transmission and interspecific spill over. Livestock plays an important role as a maintenance host of the infection. In the wild Trypanosomosis has been documented in Bovidae, Equidae, Elephantidae, Suidae, Pantherinae, Hyaenidae, Mustelidae, Canidae, Cervidae, Muridae, Crocodilinae, Alcephinae *etc* (Kasozi *et al*., 2021). There are many species of *Trypanosoma* infecting the wildlife including *T. evansi, T. theileri, T. godfreyi. T. brucei, T. vivax and T. congolense*.

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The life cycle is generally heteroxenous requiring multiple hosts to complete their life cycle. In the sylvatic cycle, biting insects vectors like Tsetse flies, Tabanids, Hippobosca, Stomoxys (Silva *et al*., 1995); (Musinguzi *et al*., 2016) are credited for the transmission of trypanosomosis to wild animals. Prey and carrion have also been identified as a possible source of infection via peroral route, particularly in felids and canids (Diall *et al*., 2017).

In felids, trypanosomosis is a sub-clinical, chronic progressive disease with muscle wasting, pyrexia and anaemia as important clinical manifestations. Wildlife infected with the protozoan at times does not progress to clinical manifestation. The ability of the wildlife to tolerate the infection is due to an innate mechanism called as trypanotolerance. It is widely documented in wildlife on

account of innate resistance due to the presence of serum xanthine oxidase and catalase which are trypanolytic molecules (Sarabian *et al*., 2018), (Wang *et al*., 2002). Biotic and abiotic stress play a vital role in the clinical outcome of trypanosomosis both in livestock and wild animals.

MATERIALS AND METHODS

Case signalment

An adult male leopard (*Panthera pardus*), approximately 45 kgs, was presented to Wildlife Research and Training Centre, Nagpur on account of proving dangerous to Human life in 2022. The leopard was responsible for two human deaths in Allapalli Division, Gadchiroli district of Maharashtra, India. During the 30-day quarantine period the leopard was found to be active and exhibited good appetite for carabeef. However, on day 15 of the quarantine it was noticed that the leopard did not consume the complete feed offered and often consumed 2 kg of the 4 kg offered. Blood was collected from the lateral coccygeal vein after physical restraint in a squeeze cage taking aseptic precautions. Thin blood smears were examined by Giemsa stain as per the standard protocol. The wet mount study was carried out to study the motility pattern of the blood protozoans if any. The serum was separated after centrifugation at 3000 rpm for 20 minutes. The serum biochemistry was carried out using

Table 1: Sequences used for phylogenetic analysis along with attributes such as species of origin, country and accession number.

Accession no.	Organism	Host	Country	Author
MK696289	*T. evansi*	Cattle	India	Unpublished
KY114581	*T. evansi*	Cattle	India	Unpublished
MZ321577	*T. evansi*	Indian grey wolf	India	Unpublished
MT225786	*T. evansi*	Lion	India	(Krishnamoorthy *et al*., 2020)
MT233332	*T. evansi*	Leopard	India	(Krishnamoorthy *et al*., 2020)
MT225627	*T. evansi*	Dog	India	(Krishnamoorthy *et al*., 2020)
MT225591	*T. evansi*	Buffalo	India	(Krishnamoorthy *et al*., 2020)
MN197603	*T. evansi*	Dog	India	Unpublished
MK761177	*T. evansi*	*Rhipicephalus* sp.	India	Unpublished
MG050164	*T. evansi*	*Rhipicephalus* sp.	India	Unpublished
KR858272	*T. evansi*	Horse	India	Unpublished
KR858267	*T. evansi*	Horse	India	Unpublished
AY912272	*T. evansi*	buffalo	Thailand	Unpublished
AY912279	*T. evansi*	deer	Thailand	Unpublished
AB551922	*T. evansi*	Camel (*Camelus dromedarius*)	Egypt	(Amer *et al*., 2011)
AB551920	*T. evansi*	*Camelus dromedarius* (dromedary camels)	Egypt	(Amer *et al*., 2011)
D89527	*T. evansi*	-	Tansui-Taiwan	Unpublished
MN446740	T. evansi	-	China	Unpublished
ON533622	*T. evansi*	*Panthera pardus*	India	Unpublished
KR858268	*T. evansi*	camel	India	Unpublished
AF362831	*T. cruzi*	*Holochilus brasilensis*	Brasil	(Mendonca *et al*., 2002).
AF362828	*T. cruzi*	Human	Brasil	Unpublished
AF362824	*T. cruzi*	Human	Brasil	Unpublished
AF362821	*T. cruzi*	*Rhodinus*	Brasil	Unpublished
FJ555672	*T. cruzi*	*Dasypus novemcintus*	Paraguay	Unpublished
FJ555664	*T. cruzi*	*Triatoma rubrovaria*	Brasil	Unpublished
KT305831	*T. cruzi*	*Myotis nigricans*	Brasil	Unpublished
GU991790	*T. cruzi*	Human	Colombia	Unpublished
KX584875	*T. vivax*	*Glossina* sp.	Mozambique	(Garcia *et al*., 2018)
KX584871	*T. vivax*	*Glossina morsitans*	Mozambique	(Garcia *et al*., 2018)
KX584859	*T. vivax*	*Glossina morsitans*	Mozambique	(Garcia *et al*., 2018)
EU482080	*T. vivax*	*Glossina morsitans*	Mozambique	(Garcia *et al*., 2018)
KX766453	*T. vivax*	*Cattle*	Mozambique	Unpublished
KC196695	*T. vivax*	*Tsetse Fly*	Mozambique	Unpublished
AF306775	Trypanosoma brucei	*Homo sapiens*	Sudan	(Agbo *et al*., 2001)
AF316893	Plasmodium vivax	*Homo sapiens*	Russia	Unpublished

Abaxis Biochemical Analyser. The freshly voided faecal sample was used for the endoparasitic investigation using direct mount method. The Egg Per Gram (EPG) was calculated using the standard procedure as in the reference (Hendrix and Robinson, 1998).

DNA isolation and molecular investigation

To draw molecular insights, DNA was isolated from 100 µl of whole blood following the manufacturer's instructions (DNeasy® Blood and Tissue Kit; Mfg. Qiagen Inc, MD, USA). Using the protocol mentioned in the reference, Polymerase Chain Reaction (PCR) targeting the ITS (Internal Transcribed Spacer) region was performed and an amplification of approximately 550 bp was obtained on 1% agarose gel (Desquesnes *et al*., 2001). The positive control was obtained from DNA isolated from positive sample of cattle provided by Dr. Dhoot V.M. from Teaching Veterinary Clinical Complex (TVCC), Nagpur. The negative control was devoid of DNA. The amplicon was purified (QIAquick PCR Purification Kit; Mfg. Qiagen Inc, MD, USA) and the purified amplicon was sequenced (ABI 3130 automated DNA Sequencer, Mfg. Applied Biosystems, CA, USA) using both the primers. A systematic phylogenetic investigation was carried out preferentially involving *Trypanosoma* sequences reported in wild animals from India and abroad (Table 1). Neighbour-joining phylogenetic tree using the bootstrap method with bootstrap values of 1000 replications were analysed using Mega X software (Kumar *et al*., 2018).

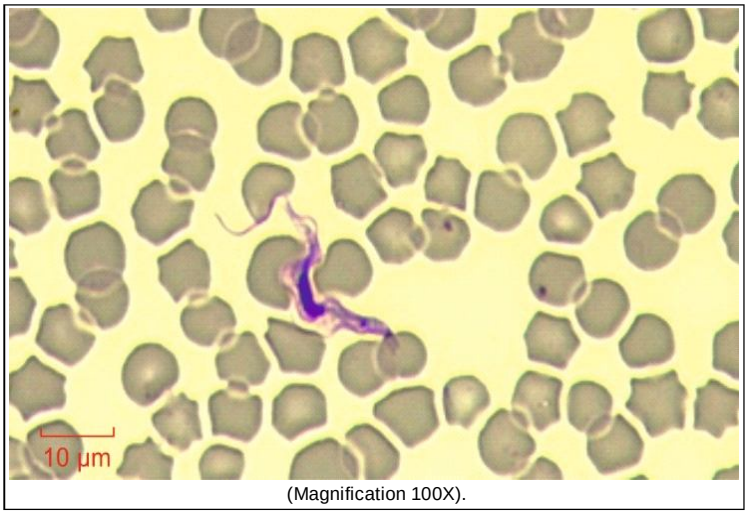
Treatment

The animal was treated with injection Dextrose 10%, injection Neurobion® (Vitamin B_1, B_6 and B_{12}) by intravenous route. Injection Triquin® (Quinapyramine Sulphate and Quinapyramine Chloride) was subcutaneously injected lateral aspect of the base of the tail at the dose rate of 4.4 mg/ kg body weight. Supportive therapy in the form of Tab. Silybion (Silymarin) and Tab. Supradyn (Multi Vitamin and Mineral) were administered orally for ten days. The animal was observed for any local adverse reaction at the site of injection for a week. The animal was also treated with Tab. Kiwoff XL (Praziquantel 175 mg, Pyrantel Pamoate 504 mg and Febental 525 mg) total dose to treat *Spirometra* infection. The animal was monitored for feeding and behaviour by Closed Circuit Television (CCTV) and disturbance was kept to a minimum. Blood and serum samples were monitored after every three days to monitor the condition of the leopard. Post 20 days of the administration of injection Quinapyramine a local tissue sluffing was noticed which was rationally treated.

RESULTS AND DISCUSSION

The peripheral blood smear examination clearly indicated a high quantum of *Trypanosoma* infection (Fig 1). The wet blood film examination revealed random and irregularly swimming trypanosomes. The flagellate protozoan measured 25.5±0.6 µm with a subterminal kinetoplast. The undulating membrane was well developed and prominent free flagellum. The leopard showed no signs of illness except for irregular partial inappetence. The blood values indicated leukocytopenia, thrombocytopenia, altered blood indices and hypoglycemia, while serum values were elevated for Blood Urea Nitrogen (BUN), Alanine Transaminase (ALT) and Aspartate Transaminase (AST) indicative of liver involvement (Table 2). Faecal sample was examined for ova and worms by direct smear method (Hendrix and Robinson, 1998). The sample was found positive for ova of *Spirometra* species (Fig 2). The EPG value of the faecal sample was estimated to be 14200 per gram indicating high quantum of infection. The faecal samples were re-examined 10 days after the deworming and were found negative for the ova of tapeworms and other parasites.

The Polymerase Chain Reaction (PCR) with primers Kin1 and Kin2 produced and amplification of approximately 550 bp indicative of either *T. equiperdum*, *T. evansi* and *T. brucei* (Fig 3). The amplicon was sequenced and the



(Magnification 100X).

Fig 1: Blood smear examination revealed presence of flagellated trypomastigotes indicative of Trypanosomosis.

sequence was accepted by National Center for Biotechnology Information (NCBI) and assigned accession No. ON533622. In the Blastn analysis, current sequence was found to be 99.02% identical to *T. evansi* (Accession No. AB551922) reported in camels from Egypt and 99.22% identical to *T. evansi* (Accession No. U75507.1) reported in from Thailand. Also, in phylogenetic analysis by neighbour joining method, the query sequence formed a distinct clade with other sequences of T. *evansi*, while *T. vivax and T. cruzi* formed distinct clades (Fig 4). *Plasmodium vivax* formed a distinct outgroup.

India is a biodiverse country and is home to a significant population of large wild felids. Among the large cats, the country is home to Lions (*Panthera leo*), Tigers (*Panthera tigris*), Leopard (*Panthera pardus*) and Clouded leopards (*Neofelis nebulosa*). Trypanosomosis is a vector-borne illness that is widely documented in production animals in the country. The losses of account of the infection are tremendous and account to losses of 671.1 million USD (United States Dollar) annually (Kumar *et al*., 2017). In wild animals majority of the reports from India are from captive wildlife and the number of such reports is sparse. In the current case report, the leopard was found to be apparently healthy with no outward manifestation of the infection. The wild animals infected with trypanosomosis may fail to exhibit any clinical signs due to moderate levels of trypanotolerance

Table 2: Hemato-biochemical blood and serum values of the ailing leopard recorded on days 0, 2 and 8.

Parameter	On reception	Day 0	Day 2	Day 8	Reference value
Lymphocytes (%)	26.3	29.1	27.5	22.4	20-55
Monocytes (%)	1.6	1.9	2.3	2.3	1-4
Neutrophils (%)	68.1	66.0	67.8	73.3	35-75
Eosinophils (%)	4.0	3.0	2.4	2.0	2-12
Basophils (%)	0.0	0.0	0.0	0.0	0-1
WBC	5800	14600	20000	16300	5500-195000
RBC (10^{12}/L)	8.15×10^6	10.97×10^6	8.78×10^6	8.42×10^6	$4.5\text{-}9.5 \times 10^6$
Platelets (10^9/L)	332000	145000	173000	317000	300000-700000
Haemoglobin (g/dL)	14.5	18.6	15.2	15.2	8-15
Mean corpuscular volume (fL)	54	58	57.0	58.0	39-55
Mean concentration haemoglobin (MCH) (pg)	16.2	16.9	17.3	18.1	13-17
MCHC (g/dl)	28.6	29.4	30.2	31.3	30-36
RDW	15.4	15.6	15	16.4	13.2 - 17.5
MPV	13.6	12.4	11.5	12.3	9.1 - 24.3
BUN (mg/dL)	14.6	39.2	65.1	40.3	15-30
Creatinine (mg/dL)	1.5	1.9	1.39	1.43	0.5-1.9
ALT (U/L)	43.1	174	98.4	64.7	8.3-52.5
AST (U/L)	36.7	93	26.2	22.8	9.2-39.5
ALP (U/L)	23.5	38.8	24.7	36.3	7-70
Gamma glutamyl transferase (GGT) (U/L)	1.7	2.5	2.1	1.8	0-2
Total Bilirubin (mg/dl)	0.28	0.4	0.3	0.2	0-0.8
Direct Bilirubin	0.2	0.2	0.1	0.1	0-0.4
Glucose (mg/dl)	89.6	68	87.0	99.2	60-125
Calcium (mg/dl)	12.1	11.9	12.2	11.0	7.9-10.9
Phosphorus (Inorganic)	5.9	5.8	5.6	5.3	4-7.3
Total Protein (g/dL)	7.4	7.3	6.9	6.8	5.5-7.5
Albumin (g/dL)	3.6	3.4	3.2	3.7	2.5-3.75
Globulin (g/dL)	3.8	3.9	3.7	3.1	3-3.75
Na^+ (mmol/ L)	144	152.1	149.6	148.9	147-156
K^+ (mmol/ L)	4.6	6.1	4.65	3.93	4-4.5
Cl^- (mmol/ L)	103.4	97.4	101.2	101.0	114-124
Peripheral smear	Negative	Trypanosoma found in the smear	Negative	Negative	-
Clinical presentation	Appetite Normal	Reduced appetite,	Appetite Normal activity dull	Appetite Normal	-

Sequences used for phylogenetic studies.

(Wulari Mbaya *et al*., 2008); wherein the excessive proliferation of the infection is limited alongside the pathogenic effects (d'leteren *et al*., 1998). However, the quantification of what level of parasitaemia be considered moderate and the cut-off level when it is to be considered severe is faint and indistinct.

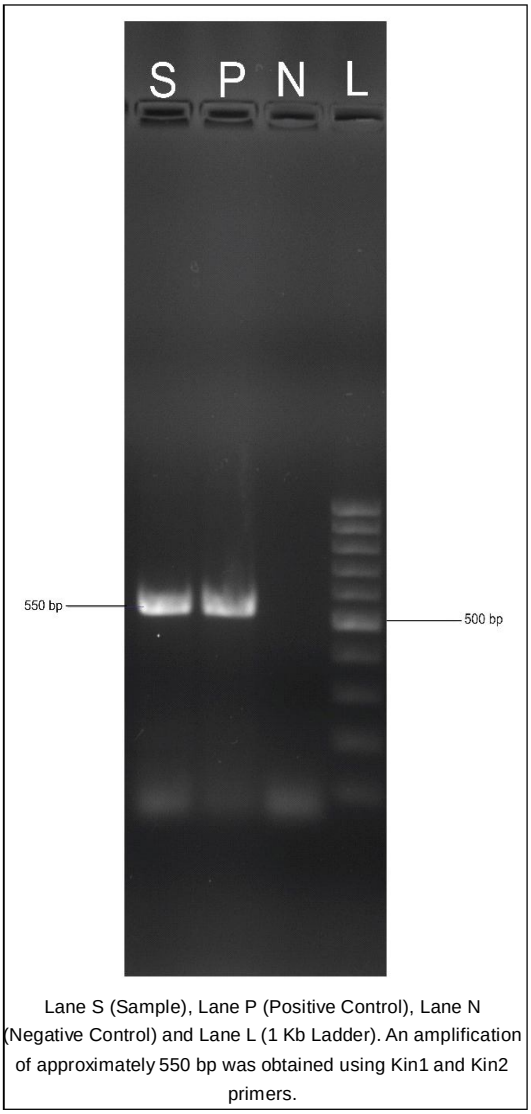
Trypanosomosis has been well documented since 1907 in many parts of India and the Indian subcontinent in particular. The disease has been reported in rural and urban areas of India, though the number of cases has declined in recent years (Shegokar *et al*., 2006). *Trypanosoma evansi* has been identified as the causative agent for the infection in humans (Joshi *et al*., 2005). There are evidences of the infection in the wild animals, however the reports have majorly emerged from captive wildlife (Sudan *et al*., 2017). The ruminants and equines are important reservoirs of the infection, ensuring the spread of the infection through mechanical transmission by biting flies (Kumar *et al*., 2013). The upsurge of the infection in livestock and humans coincides with the season of fly abundance, generally after the monsoons (Desquesnes *et al*., 2013).

Trypanosomosis has been well studied in companion animals and is characterised by presence of thrombocytopenia, anaemia, leucocytosis, elevation of liver function. The animals often exhibit hypoglycemia, hyperkalemia and elevated ALT and AST levels indicating liver involvement (Shukla, 2002). The leopard was found to be normal on reception and blood investigation at reception did not reveal any signs of infection; however, the flaring up of the infection during captivity can be attributed to captivity stress and resulting immunosuppression (Parija and Bhattacharya, 2001). The clinical findings are similar to the reports in the reference; exhibiting, elevated AST, ALT, hyperkalaemia and hypoglycaemia which is characteristic of the disease (Upadhye and Dhoot, 2000). Quinapyramine sulfate and chloride combination is considered therapeutic and preventive in action against trypanosomosis. There are many reports of successful therapeutic management of trypanosomosis using quinapyramine salts from all over the world (Ranjithkumar *et al*., 2014), (Manuja *et al*., 2014). In the current study, the protozoan was eliminated in the peripheral blood smear after 36 hours of the treatment. A major concern in the use of Quinapyramine salts is local tissue reaction that can be complicated by secondary bacterial infection. The site of the subcutaneous injection was selected in such a way that it can be administered during physical restrain and local tissue reactions can be easily identified and treated if needed.

Detection of trypanosomosis has been reported in a wide range of species by PCR. PCR is a molecular tool that provides sensitivity and accuracy in the detection of trypanosomosis; however, the assay fails to differentiate live and dead life forms of the disease. Kin primers are designed to target the internal transcribed spacers (ITSs) of ribosomal DNA and produce species-specific amplification of target DNA. When supplemented with sequencing and

(Hendrix and Robinson, 1998).

Fig 2: Direct mount method of faecal sample examination revealed the presence of egg of *Spirometra* under 40× magnification.



Lane S (Sample), Lane P (Positive Control), Lane N (Negative Control) and Lane L (1 Kb Ladder). An amplification of approximately 550 bp was obtained using Kin1 and Kin2 primers.

Fig 3: Gel electrophoresis on 1% agarose gel.

phylogenetic analysis qualitative data regarding epidemiology can be generated. Insights regarding the circulating species of trypanosomes in wildlife can be obtained. The query sequence was found to be identical to other sequences of *T. evansi* reported in deer (Accession No. AY912279) and buffalo (*Bubalus bubalus*) (Accession No. FJ712713) from China; also, it was found to be similar to other reported sequences from cattle from India (Accession No. KR858270) and abroad (Accession No. AY912275). The dense cattle population in the neighbourhood of the protected areas and the wild herbivores play a crucial role in the maintenance and transmission of the infection to the wild felids.

Spirometra sp. is known to be widely prevalent among wild felids of India and is known to be transmitted to wild felids through fishes and crustaceans. The prevalence of sparganosis was reported to be 20 % in white tigers (n=5) from Rajiv Gandhi Zoological Park, Katraj, Pune, Maharashtra, India (Shrikhande *et al*., 2008), (Dhoot *et al*., 2010). The studies on prevalence of sparganosis from the protected areas highlight the quantum of infection circulating among the wild felids of India (Marathe *et al*., 2002), (Thawait and Maiti, 2016). In Africa, the prevalence of sparganosis has been reported to be 100% among the wild lions of Tarangire National Park (Kavana *et al*., 2015) and 63% in Serengeti National Park and Ngorongoro Crater respectively (Muller-Graf, 1995). Praziquantel was found to be 100% efficient in the treatment of sparganosis in wild cats (Dhoot *et al*., 2010). In the present case, combination of Praziquantel 175 mg, Pyrantel Pamoate 504 mg and Febental 525 mg was found to be 100% efficient in the treatment of sparganosis in leopards. Though sparganosis is widely prevalent in wild felids, very little is known about the clinical outcome in wildlife. Also, the implications in the case of co-infection with other diseases are also understudied

Fig 4: Phylogenetic analysis of sequence by Neighbour Joining phylogenetic tree using Bootstrap Method (1000 replications) to ensure tree consistency.

There are a few reports of treatment of trypanosomosis in wild felids, Diminazene aceturate has been identified and used as a therapeutic agent to treat trypanosomosis with caution due to drug-related toxicity (Upadhye and Dhoot 2000). Quinapyramine prosalts have been utilised with utmost success in the management of trypanosomiasis in wildlife without any side effects (Gupta *et al*., 2009). However, local tissue reactions due to quinapyramine have been reported in many species of domestic and wild animals (Manuja *et al*., 2018). In the current study, a novel administration site was selected at the base of the tail where a skin fold can be easily grasped for subcutaneous injection. The local tissue reaction was mild and was managed rationally using antiseptic sprays and dressing. The drug eliminated blood forms of trypanosomosis within 36 hours of administration, thus, can limit the need for prolonged hospitalization and associated stress in wild felids.

CONCLUSION

Though the origin of the infection is uncertain, the report highlights the utility of quinapyramine prosalts in the management of trypanosomosis in wild leopards without any significant side effects. The tenure of hospitalization can be significantly reduced resulting in minimization of captive stress. Also, collateral management of sparganosis with trypanosomosis highlights the high prevalence of common endoparasites in free range. The study also highlights the need to synchronise the study in leopards and cattle in the vicinity of the protected area to understand the spill over of trypanosomosis in the sylvatic cycle. Finally, molecular tools like sequencing have provided important insights into the prevalence and molecular understanding of the infection in wild animals.

Conflict of interest statement

The authors have no conflict of interest.

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