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RESEARCH ARTICLE

PCR-Based Detection of *Trypanosoma evansi* Infection in Semi-Captive Asiatic Black Bears (*Ursus thibetanus*)

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ABSTRACT

Clinical signs, *viz* lethargy, increased heart rate and reduced appetite, making trypanosomiasis a possible differential diagnosis, were found in five out of twenty semi-captive Asiatic black bears (*Ursus thibetanus*) in a sanctuary, located in Kund, District Sawabi, KPK, Pakistan. Microscopic examination of blood samples of bears expressing clinical signs and symptoms revealed the presence of haemoflagellates, which was found to be trypanosomes. Subsequently, the PCR technique was exploited to screen for the presence of trypanosomal species in all bears' blood samples. Blood samples from 20 individual bears were screened using three sets of primers specific to *Trypanosoma evansi* species. Three primer pairs used are equally effective in successful detection of the parasite. Two out of five, diseased bears died prior to any trypanosoma specific medication while the rest were given an administered dose of Melarsomine (Immiticide). The treated bears survived and were assured to be aparasitemic on post-treatment examination after six weeks.

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INTRODUCTION

The genus *Trypanosoma* (Phylum: Sarcomastigophora, Class: Kinetoplastida, Order: Trypanosomatida) comprises of many species causing the disease trypanosomiasis in domestic and wild animals as well as in human populations. Epidemiological surveys of trypanosomiasis (humans as well as animal) reveal that the disease is endemic to the sub-Saharan regions and that the mode of transmittance of the parasite is through the Tsetse flies (*Glossina spp*). *T. evansi* is the causative agent of trypanosomiasis in Himalayan black bears' (*Selenarctos thibetanus*) population of Punjab, Pakistan (Muhammad *et al*., 2007).

Molecular detection provides means for discrimination between different species and strains of parasites by employing use of specific molecular markers. PCR based techniques, having much greater sensitivity and specificity have out-shone the conventional methods in medical diagnostics (Omanwar *et al*., 1999; Deborggraeve *et al*., 2008; Fernández *et al*., 2009; Tamarit *et al*., 2010). The present study involved the use of species specific markers for the timely identification of trypanosome infection in a semi-captive bears of Bioresource Research Centre's (BRC) bear sanctuary at Balkasar, Pakistan.

MATERIALS AND METHODS

Sample Collection: Twenty blood samples from bears showing clinical signs of infection as well as apparently healthy bears of Bioresource Research Centre's bear sanctuary at Balkasar, Pakistan were collected from lingual vein in heparinized vacutainers. Out of twenty, three bears were expressing clinical symptoms of trypanosomiasis and the remaining 17 were apparently healthy.

Identification of the organism: A drop of blood was dropped onto a clean slide, smeared and fixed using methanol. The fixed smear was stained with 10% Giemsa stain and rinsed with tap water to remove excess stain (Garcia, 2001). Slides were viewed under the microscope.

DNA from blood samples was extracted using standard phenol: choloform: isoamly protocol (Sambrook and Russell, 2001). Extracted DNA was stored in TE buffer at 4°C for further experimental procedures.

Three primer pairs; one genus-specific (Deborggraeve *et al*., 2008) and two specie-specific (Omanwar *et al*., 1999; Tamarit *et al*., 2010) were used for the detection of *T. Evansi* (Table 1). PCR amplifications were carried following the standard method.

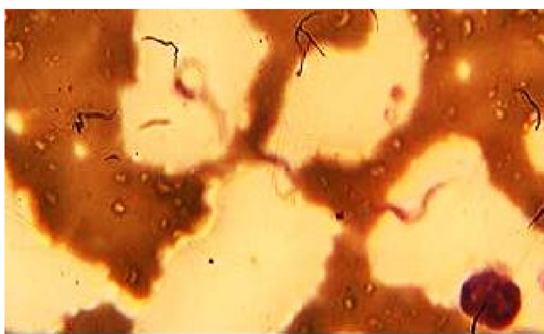

Fig. 1: Microscopic view (100 X) of slide of blood smear stained with 10% Giemsa stain showing *Trypanosoma evansi.*

PCR products were analyzed through agarose gel electrophoresis using 1.5% agarose gels stained with ethidium bromide (1 µg/mL). Gels were photographed on Alpha Innotech gel documentation system.

RESULTS

Based on clinical symptoms of trypanosomiasis in Asiatic black bear explained by Muhammad *et al.* (2007), three bears were found affected by *T. evansi*. Blood analysis revealed microcytic hypochromic anaemia, leucopenia and thrombocytopenia (Table 2). Microscopic examination of the blood samples of the three clinically sick bears revealed the presence of trypomastigote, form of trypanosome found in vertebrates in two samples (Fig.1).

PCR based screening for the parasite revealed that out of 17 apparently healthy bears two were the carriers of the *T. evansi*. Approximately 257 bp fragment was amplified using TR3/TR4 primer pair (Fig. 2), 227 bp and 205 bp fragment was amplified using pMUtec and RoTat 1.2 primers pair respectively (Fig. 3 and 4) for three clinically positive bears and two apparently healthy ones. Table 3 summarizes the detection of the parasite using three methods (clinical symptoms, microscopy and PCR), PCR based diagnostic has greater sensitivity (100%) in detection of parasites at an early stage of infection as compared to diagnostics through symptomology (60%) and microscopy (40%). Two bears (both positive for clinical symptoms, microscopic blood examination as well as PCR) were at such an advanced stage of infection that they died prior to initiation of medical treatment. Rest of three bears (all positive through PCR but only one positive through clinical signs and microscopy) were treated with melarsomine (Immiticide®) at 2.2 mg/kg in three doses over a period of 1 month. After treatment the detection methods were repeated after every two weeks, clinical symptoms disappeared during the course of medication, while microscopic examination remained positive after six weeks however PCR results took 2 months to give negative results.

DISCUSSION

Trypanosomes have wide range of hosts, and are responsible for variety of diseases in mammals, reptiles, rodents as well as in amphibians (Muhammad *et al.,* 2007; Ravindran *et al.*, 2008; Da Silva *et al.*, 2009, 2011a, 2011b; Fernández *et al.*, 2009; Rodrigues *et al.*, 2009; Umezawa *et al.,* 2009; Tamarit *et al.*, 2010). The

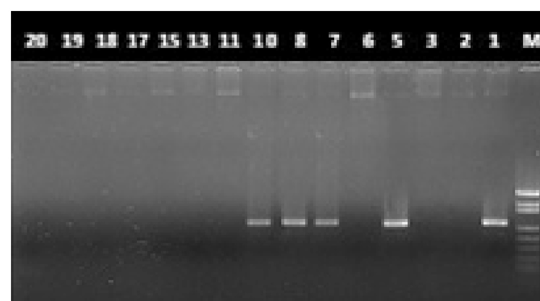

Fig. 2: 1.5% Agarose gel of PCR product of TR3/TR4 primer pair stained with ethidium bromide. Lane labeled M indicates the DNA ladder with eight fragments (in bp): 489, 404, 331, 242, 190, 147, 111 and 67. Lanes labeled 1,5,7,8 and 10 have PCR product of approximately 257 bp indicating the samples are positive for the parasite while rest of lanes have negative PCR results.

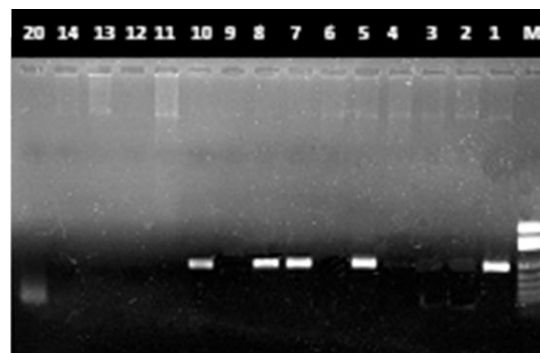

Fig. 3: Gel picture of 1.5% agarose gel of PCR product of pMUtec primer pair stained with ethidium bromide. Lane labeled M indicates the DNA ladder with eight fragments (in bp): 489, 404, 331, 242, 190, 147, 111 and 67. Lanes labeled 1,5,7,8 and 10 have amplicon of approximately 227 bp representing the presence of the parasite in samples while rest of lanes have negative PCR results.

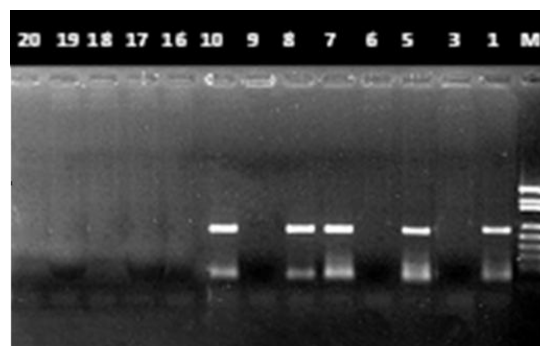

Fig. 4: 1.5% Agarose gel of PCR product of RoTat 1.2 primer pair stained with ethidium bromide. Lane labeled M indicates the DNA ladder with eight fragments (in bp): 489, 404, 331, 242, 190, 147, 111 and 67. Lanes labeled 1,5,7,8 and 10 have amplified product of about 205 bp which is indicative of the fact that parasite is present in these samples while rest of lanes have negative PCR results.

confirmation of *T. evansi* as a causative agent of trypanosomiasis, with clinical sign and symptoms as pyrexia, rapid pulse rate, depression, ataxia, weakness and difficulty in breathing, in black bear population of Punjab, Pakistan was done on the basis of detailed microscopic investigation of blood samples (Muhammad *et al.*, 2007). Several antibody-based biological assays have also been employed for the detection of the specific parasite (Wuyts *et al.,* 1995; Chansiri *et al.*, 2002; Desquesnes *et al.*,

Table 1: Primer sequences for PCR based detection of *Trypanosoma evansi* in blood samples of twenty semi captive Asiatic black bears (*Ursus thibetanus*). Ta, optimum annealing temperature for the primer pair is given in °C and amplicon size for specific primer set is given in base pairs (bp).

Primer Name	Primer Sequence	Ta(°C)	Amplicon Size (bp)	References
TR3	5'-GCGCGGATTCTTTGCAGACGA-3'	55	257	Tamarit *et al.* (2010)
TR4	5'-TGCAGACACTGGAATGTTACT-3'			
pMUtec F	5'-TGCAGACGACCTGACGCTACT-3'	60	227	Omanwar *et al.* (1999)
pMUtec R	5'-CTCCTAGAAGCTTCGGTGTCCT-3'			
RoTat1.2 F	5'-GCGGGGTGTTTAAAGCAATA-3'	59	205	Deborggraeve *et al.* (2008)
RoTat1.2 R	5'-ATTAGTGCTGCGTTCGGAGAGC-3'			

Table 2: Comparison of different types of blood cells count of diseased bears to the normal range

Test Name: Blood Complete Picture (CP)				
	Samples			Normal Range
	1	5	7	
WBC Count (no,/mm3)	2800	3,700	3000	4500 - 11000
RBC Count (mL/mm3)	3.29	3.03	4.72	3.2 - 7.0
Hemoglobin (g/dL)	7.2	6.53	10.7	6.5-18
Hematocrit (%)	24	21	34	30-50
MCV (fl)	72	69	72	65 - 90
MCH (pg)	22	22	23	20-28
MCHC (g/dL)	30	31	31	30 - 35
RDW-CV (%)	15	13	14	12 - 18
Platelets (No./ mm3)	33,000	39,000	50,000	222,000-500,000
Test Name: Differential Leukocyte Count (DLC)				
Types of Leukocyte	Samples			Normal Range
	1	5	7	
Neutrophils	51	46	41	35 - 65
Lymphocytes	47	42	54	25 - 50
Monocytes	0	5	2	2-11
Eosinophil	2	7	3	2 - 6
Basophil	0	0	0	0 - 1
Bands	0	0	0	0 - 5

MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Hemoglobin; MCHC = Mean Corpuscular Hemoglobin Concentration; RDW-CV = Red Cell Distribution Width-Coefficient Variation.

Table 3: Comparative summary of results of three diagnostics method *viz* symptomatic, microscopy and PCR, employed for detection of *T. evansi* (n=20).

Primers	Samples																			
	1*	2	3	4	5*	6	7**	8	9	10	11	12	13	14	15	16	17	18	19	20
TR3/ TR4		-	-	-					-		-	-	-	-	-	-	-	-	-	-
pMUtec		-	-	-					-		-	-	-	-	-	-	-	-	-	-
RoTat1.2		-	-	-					-		-	-	-	-	-	-	-	-	-	-

Indicate microscopically as well as PCR +ive samples
Indicate PCR +ive samples
\- Indicate -ive samples
*Depicted severe sign and symptoms of trypanosomiasis (clinically confirmed)
**Depicted mild sign and symptoms of trypanosomiasis (clinically doubted)

Table 4: Sensitivity of each test is given. PCR proved to be most sensitive detection technique compared to microscopic examination and clinical symptoms.

Methods	No. of Positive Bears	% Sensitivity
Symptomatic	3	60
Microscopy	2	40
PCR	5	100
Total No. of Diseased Bears	5	--

2009). However, the chances of false positive results are higher in most of serological methods (Ravindran *et al.,* 2008)**.** The PCR has solved the problem of sensitivity and is reported to be an efficient detection method for *T. evansi*, as it amplifies the parasite specific genome (Omanwar *et al.*, 1999; Deborggraeve *et al.*, 2008; Tamarit *et al.*, 2010). The sensitivity of a PCR based assay is stated as high as detecting a minimum of five trypanosomes per blood sample (Artama *et al.,* 1992; Ravindran *et al.,* 2008; Fernández *et al.,* 2009).

The combinations of methods were adapted to counter confirm the presence of parasite, in the group of 20 semi captive bears. PCR proved to be most sensitive technique as it gives positive results for all the infected bears unlike microscopic and symptoms based detection which failed to detect parasite in 34% of the infected bears. The study confirms the efficacy of three primer pairs for detection of *T. evansi* in *Ursus thibetanus*. Such study for the molecular identification of *T. evansi* in semi captive bears in Pakistan will lay a foundation for further epidemiological researches on this parasite and its vectors to improve the control of future infections by allowing timely treatment of the affected individuals and targeted efforts to reduce vector prevalence.

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