MOLECULAR EVIDENCE OF PATHOGENIC TRYPANOSOMA EVANSI IN DOGS OF KERALA

Hitaishi V N
MVSc Scholar, Department of Veterinary Parasitology, CVAS, Mannuthy
Jain Jose K
Department of Parasitology,CVAS, Mannuthy
Bindu Lakshmanan
Assistant Professor, Department of Veterinary Parasitology,CVAS, Mannuthy

Abstract: Canine trypanosomosis is a haemoprotozoan disease reported to be of serious concern in Asian elephants. This article focuses on the molecular detection of *T. evansi* using Polymerase Chain Reaction (PCR), since trypanosomes cannot always be detected by conventional diagnostic techniques, especially in subclinical conditions. The PCR protocol for *T. evansi* was standardised using canine, blood that were found positive by wet film and blood smear examination.

Keywords: Trypanosoma Evansi, Dogs, PCR.

Introduction: Trypanosomosis in India is caused by the intercellular haemoprotozoan *Trypanosoma evansi*, which is transmitted by biting flies of *Tabanus* spp. Trypanosomes are zoonotically significant and also have a wide host range including several domestic and wild animals alike. Dogs too, have been reported to be victims of this disease. Trypanosomosis is manifested by a wide variety of clinical signs and hence clinical signs alone cannot be considered as reliable diagnostic criteria. The infection maybe subclinical, and is often difficult to diagnose even by conventional techniques such as wet film and blood smear examination. In such situations, molecular methods such as polymerase chain reaction (PCR) can play an important role in the detection of causative agents, since they are capable of detecting very low levels of parasitaemia. The present study envisages to provide molecular evidence of *Trypanosoma evansi* in dogs, to analyse the evolutionary relationship of the isolate using phylogenetic tools and to correlate the infection with haematobiochemical parameters.

Materials and Methods: Whole blood was collected from a dog in Thrissur district which exhibited different combinations of clinical signs that included anorexia, anaemia, corneal opacity and lymphadenopathy. The dog tested positive for *T. evansi* by blood smear and wet film examination. The complete blood count and serum analysis was carried out before proceeding.

Isolation of DNA: Isolation of DNA from whole blood was performed using a DNeasy® Blood & Tissue Kit, Qiagen, as per the Quick-Start protocol recommended by the manufacturer.

Polymerase Chain Reaction: PCR reactions were performed using the MJ MiniTMPersonal Thermal cycler, Bio-Rad, according to the protocol followed by Ravindran *et al.* (2008), with minor modifications.
The primers employed for the PCR reactions were synthesized by Sigma Aldrich Chemicals Pvt. Ltd. and amplified a 227 base pair region of the Variable Surface Glycoprotein (VSG) gene specific to *Trypanosoma evansi*. Sequences of the forward and reverse primers used were 5'TGCAGACGACCTGACGCTACT-3' and 5'CTCCTAGAAGCTTCGGTGTCCT-3', respectively. Primers were selected based on the study conducted by Ravindran *et al*. (2008).

Gradient PCR was performed in a 25 µL reaction volume containing 2.5µL of buffer (10X) without $MgCl_2$, 200 µM each of dNTP, 25 pmol each of forward and reverse primers, 2.0 mM of $MgCl_2$, 1.5U of *Taq* DNA polymerase and 5.0 µL of template DNA. All the reagents were procured from Sigma Aldrich (USA). A no template control (NTC) was included in each run. A gradient thermal cycling program ((Bio-Rad T100, USA) with initial denaturation at 90°C for 7 min followed by 35 cycles of denaturation (90°C, 30s), annealing (72°C, 30s) and extension (72°C, 1 min) and a final extension at 72° C for 7 min was adopted. The amplified PCR products were

subjected to electrophoresis in 1.5% agarose gel (Hoefer, USA) and visualized in Gel-documentation system (Bio-Rad Laboratories, USA).

Sequencing and Phylogenetic Analysis: The amplicons were purified using silica gel purification columns (GeneJET, Thermoscientific), sequenced using Sangers dideoxy chain termination method and the sequences aligned using Sequencher Version 5.0 (SciGenom Labs Pvt Ltd,Cochin). Bidirectional sequencing was done with both forward and reverse primers. The sequences were aligned using EMBOSS (www.bioinformatics.nl>cgi-bin>merger) and blasted using NCBI BLAST tool (www.blast.ncbi.nlm.nih.gov >blast) to analyse their similarity with other published sequences available in online databases. Sequences corresponding to partial 18S rRNA gene of *T.evansi* were downloaded from online databases and aligned with the corresponding partial sequence of *T. evansi* obtained in this study and were further utilised for the construction of phylogenetic tree using Maximum likelihood method. The bootstrap consensus tree was inferred from 1000 replicates, in Dambe 5.5.24.

Haemato-Biochemical Parameters: The haematological parameters were analysed using autoanalyzer (Master T, Hospitex international, Italy) and compared with the normal range. Parameters like red blood cell (RBC) count , white blood cell (WBC) count , haemoglobin (Hb), haematocrit, (HCT), mean corpuscular volume (MCV), red cell distribution width (RDW), platelet count (Plt) were estimated.The biochemical parameters like Creatinine, Total protein, ALT, Albumin, A: G ratios were estimated.

Results and Discussion: Polymerase chain reaction was standardised for amplifying partial region of VSP gene of *T. evansi* by gradient protocol. A bright band of size 227 bp was observed in annealing temperatures of 72^{O}C which was in accordance with Ravindran *et al.*, 2008. Alignment of the bidirectional nucleotide sequences of the product yielded a 227 bp sequence which revealed 99 per cent similarity with corresponding published sequences of *T.evansi*. There was no significant similarity with any other species of *Trypanosoma*, thus confirming the identity of the haemoflagellate of this genus among dogs in Kerala as *T. evansi*. The specificity of the designed primers was also well ascertained by the absence of cross amplification with DNA of common haemoparasites in south India *viz*., *B.canis*, *Ehrlichia canis,* or *Dirofilaria spp.* available in the Department of Veterinary Parasitology of this institution.

The phylogenetic tree based on maximum likelihood method with Tamura -Nei model using 18S r RNA gene sequences with *P. falciparum* as outgroup species is presented in Fig. 1. Phylogenetic analyses revealed that Kerala isolate of *T. evansi* was closest with other Indian isolates. This clade was closest and could be observed as a sister clade Punjab isolate (LC 008133). However, the Kerala isolate was farthest from the Brazil isolate (AF 397194)

The analysis of haemato- biochemical parameters suggested that dog exhibited a near normal leukogram while the erythrogram values were below the normal range. The haematological alterations of decreased RBC, haemoglobin and haematocrit suggested that all animals were anaemic. Anisocytosis was suggested by the high values of RDW. Severe thrombocytopenia was also observed. The analysis of biochemical parameters showed a slight increase in creatinine and total protein.

Anaemia is the consistant finding in case of animals affected with trypanosomosis Proposed mechanisms include hemolysis as a result of erythrophagocytosis, hemodilution and depression of erythropoiesis (Silva et al., 1995). Thrombocytocaemia is due to haemolysis and hypoglycaemia due to consumption of energy from the host (Jaktar et al., 1973). Lymphadenopathy is caused due to infiltration of macrophages in the lymph nodes.
A significant increase in serum protein levels, as consequence of globulin rise, and a parallel decrease in albumin concentrations were observed in infected dogs. The decrease in albumin:globulin ratio has been frequently reported in *T. evansi* infection in various studied hosts (Jaktar et al., 1973; Boid et al., 1980; Moreira & Machado, 1985; Galhorta et al., 1986; Monzon et al., 1991). It is suggestive that fall in albumin levels was secondary to hyperglobulinemia as a compensatory mechanism for the maintenance of normal blood viscosity increased by high globulin levels.

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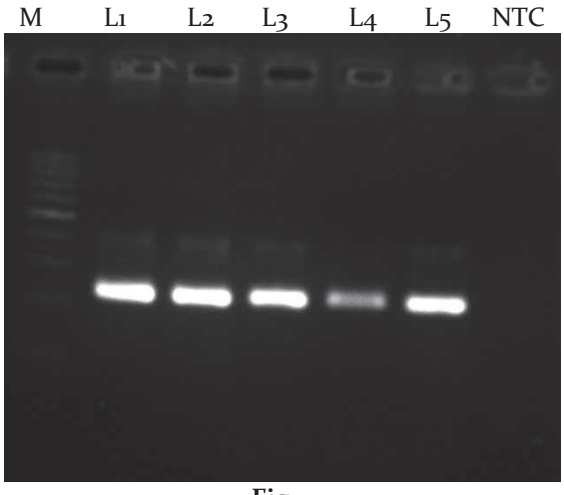

Fig 1:
M: 100 bp ladder
L1- L5: Positive Amplicons
NTC: No Template Control

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