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ITS-1 Gene based PCR as a Diagnostic Tool for Phylogeographic Characterization of *Trypanosoma evansi* in Naturally Infected Equines in Agro-climatic Zones of Uttar Pradesh, India

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ABSTRACT

Background: Six representative ITS1-PCR amplified products were used for the amplifications of conserved regions of 18S and 5.8S rDNA to amplify Internal Transcribed Spacer1 (ITS1) gene of 540 bp of *Typanosoma evansi* isolates in equine from three agro-climatic zones of Eastern Uttar Pradesh, India.

Methods: Multiple sequence alignment was done by MAFFT online multiple sequence alignment tools by selecting the necessary parameters. The phylogenetic study of ITS1 gene revealed all the three isolates (*T. evansi* horse India Uttar Pradesh- North Eastern Plane Zone, *T. evansi* mule India Uttar Pradesh- Eastern Plane Zone and *T. evansi* horse India Uttar Pradesh- Vindhyan Zone) of *T. evansi* were clustered in single group.

Result: Multiple alignment of nucleotide sequence of ITS1 genes showed that *T. evansi* isolates from Eastern region of Uttar Pradesh, Indian had > 99% nucleotide homology with isolates of camel Iran (KX898420), camel Egypt (MW603779.1) and buffalo China (FJ712715.1).

Key words: ITS1-PCR, Multiple sequence alignment, *Typanosoma evansi*.

INTRODUCTION

Equine trypanosomosis caused by *Trypanosoma evansi*, is an endemic disease in India, reported from different states of country comparatively higher in Northern India (Singh *et al*., 2010; Chaudhry *et al*., 2000; Sumbria *et al*., 2014). The parasite is transmitted mechanically through the blood sucking dipteran flies and can also be vertical, horizontaland iatrogenic (Desquesnes *et al*., 2001). The effective control of the disease requires molecular- based techniques which are more reliable than microscopy test (Desquesnes *et al*., 2013; Bhagwan *et al*., 2015; Sumbria *et al*., 2015; Diallo *et al*., 2018; Agrawal *et al*., 2024). The isolation of several genes from *T. evansi* such as invariable surface glycoprotein (ISG) genes, nuclear DNA (Deoxyribonucleic acid), kinetoplastic DNA, ribosomal DNA (Ijaz *et al*., 1998 and Maharana *et al*., 2019) and a region from rRNA (Ribosomal Ribonucleic acid) internal transcribed spacer 1 (ITS-1) (Agbo *et al*., 2001) are contributed major role in molecular diagnosis. The 18S rRNA gene has been found as superior target for phylogenetic study due to low substitution rate, conserved region, five different genotypes (A, B, C, D and E) based on the presence of different clades (Qablan *et al*., 2013; Liu *et al*., 2016; Ketter-Ratzon *et al*., 2017) and occurrence in several copies (Qablan *et al*., 2013).

The detailed and systematic information is insufficient about to the phylogenetic characterization of *Trypanosoma evansi* infection in equine population in the Eastern Uttar Pradesh, India. Therefore the present study was undertaken to know the phylogeographic characterization of *Trypanosoma evansi* in naturally infected horses in agro-climatic zones of Eastern Uttar Pradesh, India.

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MATERIALS AND METHODS

Study area

The present study was conducted in North Eastern Plain Zone, Eastern Plain Zone and Vindhyan Zone of Eastern region of Uttar Pradesh, India. It was carried out at Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, Kumarganj, Ayodhya, Uttar Pradesh and ICAR-National Research Centre on Equines (NRCE), Sirsa Road, Hisar, Haryana, India from February,

2020 to February, 2021. The climate of study area is defined as humid sub-tropical and has three major seasons *viz*. winter, summer and rainy season (Table 1).

Extraction of genomic DNA

Blood samples were collected randomly from 524 equines from study area across different seasons. Total genomic DNA was extracted from microscopically positive whole blood using DNeasy Blood and Tissue Kit-50 (Qiagen India Pvt. Ltd., New Delhi). Out of positive samples, six representative DNA samples; two from each agro-climatic zone of study area were stored at -40°C for phylogenetic study (Fig 1).

Optimization and amplification of Internal transcribed spacer1 (ITS1) gene of *T. evansi*

Kin 1 (ITS1-R, 20 bp length) and Kin 2 (ITS1-F, 16 bp length) set of primers (540 bp) used for the amplifications of conserved regions of 18S and 5.8S rDNA to amplify Internal Transcribed Spacer1 (ITS1) gene of 540 bp of *T. evansi* (McLaughlin *et al*., 1996). ITS1-PCR reaction was manipulated in 25 µl reaction mixture containing more than 40 nanogram (ng)/µl of genomic DNA samples. Each PCR cycle was consisted of denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 30 sec. PCR products were purified from gel extraction (Fig 2) procedure by QIAquick gel extraction kit (Qiagen) and were eluted in 20 µl of NFW (Nuclease-free water) and stored at -40°C until further use.

Sequence and phylogenetic analysis of ITS1-*T. evansi* gene

PCR products were sequenced by outsource sequencing agency (Genosys Informatics, Azadpur, Delhi, India). Best homologous sequences were selected by searching NCBI BLAST (National Centre for Biotechnology, Basic Local Alignment search tool) database on the basis of E- value and pasted in separate file in FASTA format. Multiple sequence alignment was done by MAFFT (Multiple alignments using fast Fourier transform). Phylogenetic analyses of aligned sequences of six isolates were done independently with the sequences of the ITS-1 gene available in GenBank database by utilizing the MEGA soft software version 6.0. Phylogenetic tree was constructed by

Table 1: Salient features of three agro-climatic zones of Eastern region, Uttar Pradesh, India.

Features	Eastern region of Uttar Pradesh		
	North Eastern Plain Zone	Eastern Plain Zone	Vindhyan Zone
Mini. Temp. (°C)	4.9	5.7	5.0
Max. Temp. (°C)	44.2	41.4	45.2
Avg. Annual Rain Fall (mm)	1240	803	1134
Climate	Moist sub humid to dry Sub humid	Dry sub humid to moist sub humid	Dry sub humid to moist sub humid
Total geographical area in hectare	2955485	3808718	1381840
No. of districts	11	13	03

Source: (https://dolr.gov.in/sites/default/files/SPSP_Uttar%20Pradesh.pdf).

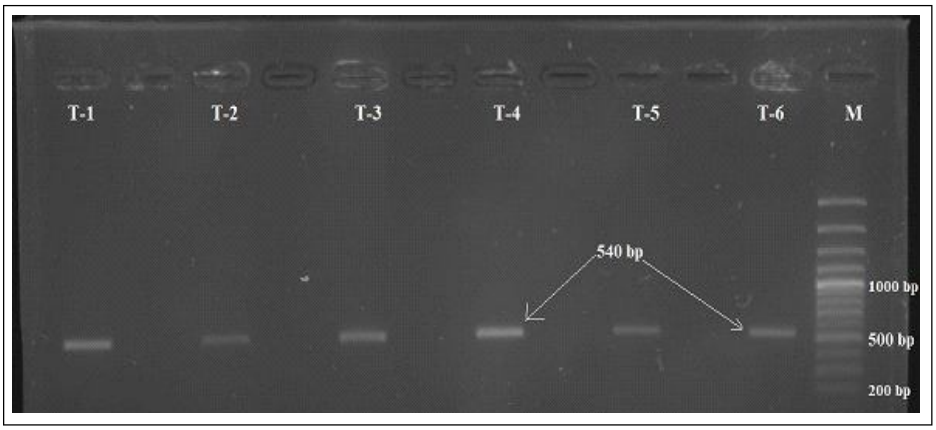


Fig 1: Agarose gel electrophoresis of 540 bp of PCR product for phylogenetic analysis of six representative *Trypanosoma evansi* isolates.

maximum likelihood method by setting the boot strap value and algorithm for *T. evansi*-Tamura-Nei-TN93 model (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

The phylogenetic study of ITS1 gene revealed all the three isolates (*T. evansi* horse India Uttar Pradesh- North Eastern Plane Zone, *T. evansi* mule India Uttar Pradesh- Eastern Plane Zone and *T. evansi* horse India Uttar Pradesh-Vindhyan Zone) of *T. evansi* were clustered in single group (Fig 3). ITS 1 *T. evansi* horse India Uttar Pradesh -North Eastern Plane Zone and ITS 1 *T. evansi* horse India Uttar Pradesh-Vindhyan Zone clone were more associated with *T. evansi* isolates cattle from India- Assam (KY114581.1), *T. evansi* isolates donkey India- Gujarat (KR858269.1), *T. evansi* camel Iran (KX898420) and *T. evansi* camel Egypt (MW603779.1) whereas ITS 1 *T. evansi* mule India Uttar Pradesh- Eastern Plane Zone clone was more related to *T. evansi* isolates from *T. evansi* camel Iran (KX898420.1), *T. evansi* camel Egypt (MW603779.1), *T. evansi* camel Egypt

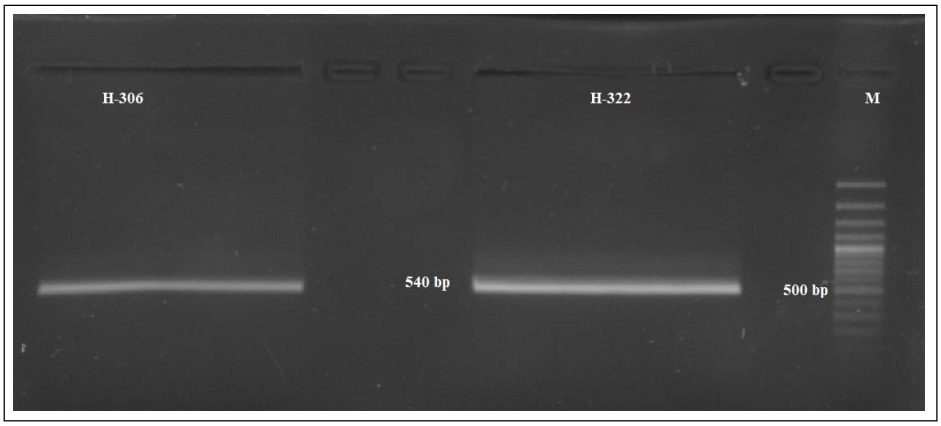


Fig 2: Agarose gel slice containing 540 bp of PCR product of *Trypanosoma evansi* isolates for extraction of genomic DNA.

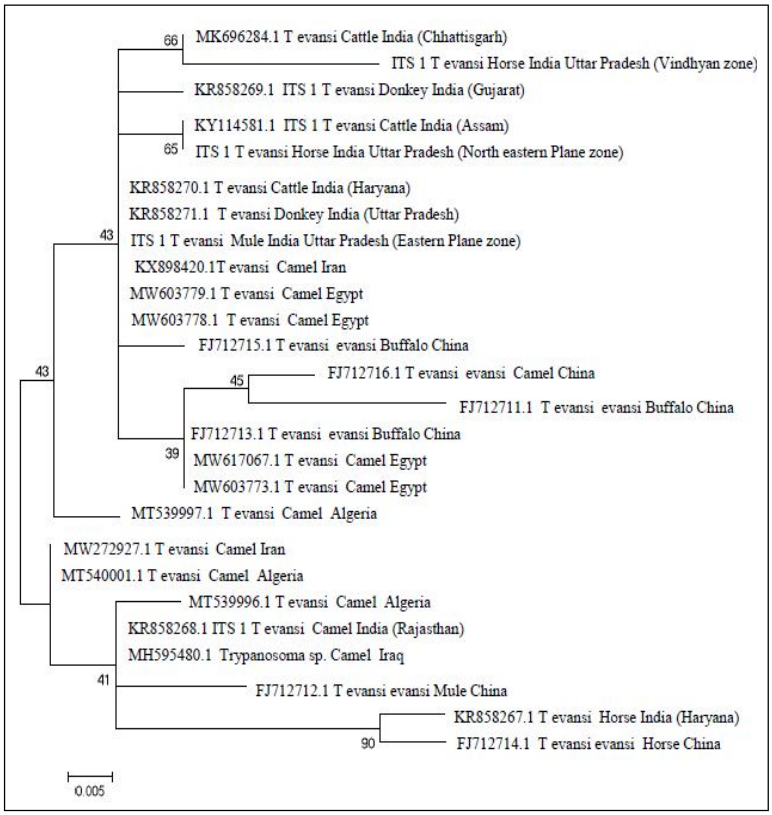


Fig 3: Phylogenetic tree based on Internal Transcribed Spacer1 (ITS1) gene of *trypanosoma evansi* isolates.

(MW603778.1) and *T. evansi* buffalo China (FJ712715.1). This result may helpful to know about genetic variability among different isolates of *T. evansi* from other parts of India and PCR primers may be designed from conserved regions for development of sensitive molecular diagnostic.

ITS of the rDNA are most appreciated target for molecular charecterization and resolution of taxonomic identities of trypanosome belonging to subgenus, species and type (Cupolillo *et al*., 1995; McLaughlin *et al*., 1996). Each transcribed unit of the rDNA locus is composed of 18S, 5.8S and 28S rRNA genes, as well as various ITS (ITS1 and ITS2) flanked by non-transcribed spacers (Hernandez *et al*., 1993). McLaughlin *et al*. (1996) were designed a pair of Kin primer (Kin 1 and Kin 2) especially for kinetoplastid species. These primers especially target to the conserved domain of the 18S and 5.8S rDNA to amplify the ITS1 gene amplify which is situated between 18S and 5.8S rRNA genes. The present investigation of sequencing and molecular characterization based on the ITS1 region of rDNA was carried out to evaluate genetic mutability among Indian equine *T. evansi* isolates from three agro-climatic zone of Eastern region of Uttar Pradesh, India. The phylogenetic tree shows also the mutative relationship of the sequences in which the length of the branch was corresponding to the assessed genetic distance between the sequences. The length of the branch is very small between ITS 1 *T. evansi* horse India Uttar Pradesh -North eastern plane zone and ITS 1 *T. evansi* horse India Uttar Pradesh -Vindhyan zone from *T. evansi* camel Iran (KX898420) and *T. evansi* camel Egypt (MW603779.1) having nucleotide identity > 99%. ITS 1 mule isolate from Eastern Plain Zone of Eastern region, Uttar Pradesh was more closely related to *T. evansi* camel Iran (KX898420.1), *T. evansi* camel Egypt (MW603779.1 and MW603778.1) and *T. evansi* buffalo China (FJ712715.1) having nucleotide identity > 99%. Similar finding was reported by Kumar *et al*. (2019).

CONCLUSION

T. evansi isolates from Eastern region of Uttar Pradesh, Indian had >99% nucleotide homology with isolates of camel Iran (KX898420), camel Egypt (MW603779.1) and buffalo China (FJ712715.1).

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Conflict of interest

The authors declare that there is no conflict of interest.

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