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Associative Diversity of ITS 1 gene in Different *Trypanosoma evansi* Isolates of Equine Origin from Semi Arid India

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ABSTRACT

Trypanosoma evansi is an important haemoprotozoan parasite from the Indian context. A wide range of variations in the parasite genotype and subsequently, in disease pathogenesis have been described in the literature. The present study was designed to find out the associative genetic diversity, within the various equine isolates of *T. evansi* across India and the globe, based on the ITS 1 gene. A total of five equine isolates were characterized. PCR products were sequenced. A phylogenetic tree was constructed based on the maximum parsimony (MP) method with the tree–bisection– regrafting (TBR) algorithm. Indian isolates formed three haplotypes. The present isolates showed 99.06-100.00% nucleotide homology within themselves. In broader terms, present isolates were found to be phylogenetically closer isolates from Haryana. Broadly, Indian isolates of *T. evansi* were closely related to isolates reported from Thailand and China than those from Iran, Nigeria, and Egypt. Another remarkable finding is the close association of equine isolates from India with other isolates of family equidae and their clear divergence from isolates of *T. evansi* affecting other hosts from India and abroad. Vast genetic divergence was seen between the isolates suggesting of multiple distinct lineages of *T. evansi* amongst the Indian livestock.

HIGHLIGHTS

- Study comprised of characterization of equine isolates of *Trypanosoma evansi* from semi arid India based on ITS gene.
- Appreciable genetic diversity was noticed based on ITS gene locus.
- Marked host specific closeness was noticed between equine isolates on the phylogenetic tree.

Keywords*:* Phylogenetic characterization, ITS 1 gene, *Trypanosoma evansi*

Trypanosoma evansi infects a wide range of domestic animals alongside wild life and causes the disease- Surra (Sudan *et al.,* 2017a). The haemoflagellate parasite is a serious threat to the optimum health and productivity of animals throughout the world (Desquesnes *et al.,* 2013; Jaiswal *et al.,* 2015; Pandey *et al.,* 2015). There are ample reports of existence of genetic diversity within the various isolates of *T. evansi* across the globe (Desquesnes *et al.,* 2013). A large number of target molecules have been explored to assess the phenotypic characterization *T. evansi* like 18S rRNA (Hughes and Piontkivska, 2003), ITS 1 (Villareal *et al.,* 2013), oligopeptidase B (Sudan *et al.,* 2018), variable surface glycoprotein (VSG) (Devi *et al.,* 2017a; 2018; Sudan *et al.,* 2017b), heat shock protein (HSP 70) (Sudan *et al.,* 2017c), beta-tubulin (Li *et al.,* 2007) and actin (Li *et al.,* 2009) genes.

The present study was designed with the objective of characterization of five equine isolates of *T. evansi* based on ITS gene. Alongside, the evolutionary phylogenetics of various isolates of *T. evansi,* affecting different host species

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across the world, was also delineated. Finally, nucleotide homology within these isolates was comminuted.

MATERIALS AND METHODS

Topography, *T. evansi* isolates, ethical compliance and DNA extraction

The studied area is located at 27.49ºN latitude and 77.67ºE longitude axis, respectively, in semi-arid zone of India. The area is considered to be endemic for haemoprotozoan diseases due to warm temperature and high humidity with a little of rainfall in the area that favours the abundance of vector population (Patel *et al.,* 2013).

The blood was collected in compliance with the ethical standards of the Institutional Animal Ethics Committee (IAEC) *via.,* voucher no. 125/IAEC/19/12 dated 22th January, 2019. Blood samples were collected from equines as a part of routine epidemiological survey for surra. A total of five samples that were found positive for *T. evansi* infection [through microscopic examination and confirmation by PCR (Devi *et al.,* 2017b) were processed for DNA extraction using commercial blood DNA isolation kit (Fermentas, Germany) following manufacturer's protocol.

Primer selection, PCR conditions and visualization of PCR amplification

The primers specific for ITS 1 gene of *T. evansi* (Mc Laughlin *et al.,* 1996) were custom synthesized from Imperial Life Sciences Pvt. Ltd., Gurugram, India. The primer sequences consisted of ITS1-F: 5′-CGCCCGAAAGTTCACC-3′ and ITS1-R: 5′-GCGTTCAAAGATTGGGCAAT-3′. These primers encoded for a 540 bp product. The PCR reaction were set up into 50 µl volume containing 25 µl of Green PCR Master Mix (0.05/µlTaq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 3 µl of each primer (10 pmol/µl of each primer) and 5 µl of the extracted DNA template. The total volume of the PCR mix was made up to 50 µl using nuclease-free water. The PCR conditions consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 58 °C for 40 sec and extension at 72 °C for 90 sec. A final extension at 72 °C for 5 min was given before termination. The amplified products were visualized in ethidium bromide incorporated 1.5% agarose gel.

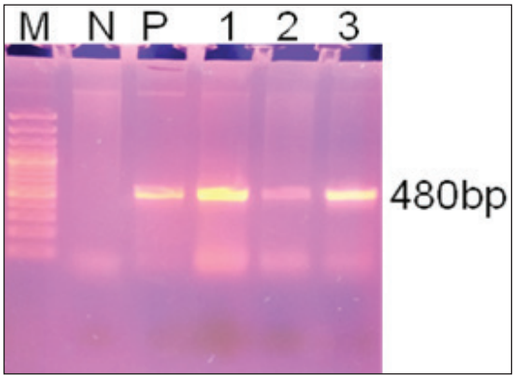
Sequencing, sequence analysis and construction of phylogenetic tree

The PCR products were purified using Gel purification and DNA clean up kit (Promega, USA) following the manufacturer's protocol and sent for outsourced DNA sequencing (both forward and reverse sides) to Invitrogen Bio Sciences Pvt. Ltd., Gurugram, India. The obtained sequences were submitted into NCBI and corresponding accession numbers were obtained. A multiple sequence alignment was generated with the Clustal W programme within MEGA 6 software (Tamura *et al.,* 2011) using both the pair-wise and multiple alignments. The phylogenetic relationship was constructed employing maximum parsimony (MP) method with the tree–bisection–regrafting (TBR) algorithm. The sequences were truncated at both ends, so that nearly all sequences started and ended at the homologous nucleotide position. The phylogeny was tested with bootstrap method using 1000 bootstrap replications and all sites were used in the analysis. Alongside, nucleotide homology between the sequences was also calculated.

RESULTS AND DISCUSSION

PCR amplification of ITS 1 gene

A single band of 540 bp was seen upon agarose gel electrophoresis in all the five equine isolates of *T. evansi* (Fig. 1).



Lane M: 100bp plus ladder (Fermentas, Germany); Lane N: Known negative control; Lane P: Known positive control (DNA of *T. evansi*); Lane1-3: Equine isolates of *T. evansi* (480 bp amplification)

Fig. 1: PCR based amplification of ITS 1 gene of *T. evansi*

Sequence and phylogenetic analysis

The five sequences were submitted to NCBI and accession numbers (MT605803- MT605807) were obtained. The phylogenetic analysis revealed three haplotypes with multiple nucleotide changes (Fig. 2). Sequences MT605803-05 represented one haplotype while sequences MT605804 and MT605805 represented two separate haplotypes (Fig. 3). In general, the present sequences showed 99.06-100.00% nucleotide homology within themselves wherein the sequences in the first haplotype (MT605803-05) showed 100% nucleotide homology within themselves while they showed 99.06 and 99.25% nucleotide homologies with the rest two haplotypes (MT605804 and MT605805, respectively). On broader terms, all the five equine isolates were found to be phylogenitically closer to the pony isolate from Haryana, India. Wide variation was noticed among the various Indian isolates and overall the Indian isolates were found scattered all over the phylogenetic tree (Fig. 3) with variable similarities to respective *T. evansi* isolates reported worldwide. Broadly, Indian isolates of *T. evansi* were closely related to isolates reported from Thailand and China than those from Iran, Nigeria and Egypt.

Fig. 2: Nucleotide changes observed in the various isolates of *T. evansi* based on ITS 1 gene (changes are depicted by yellow colour)

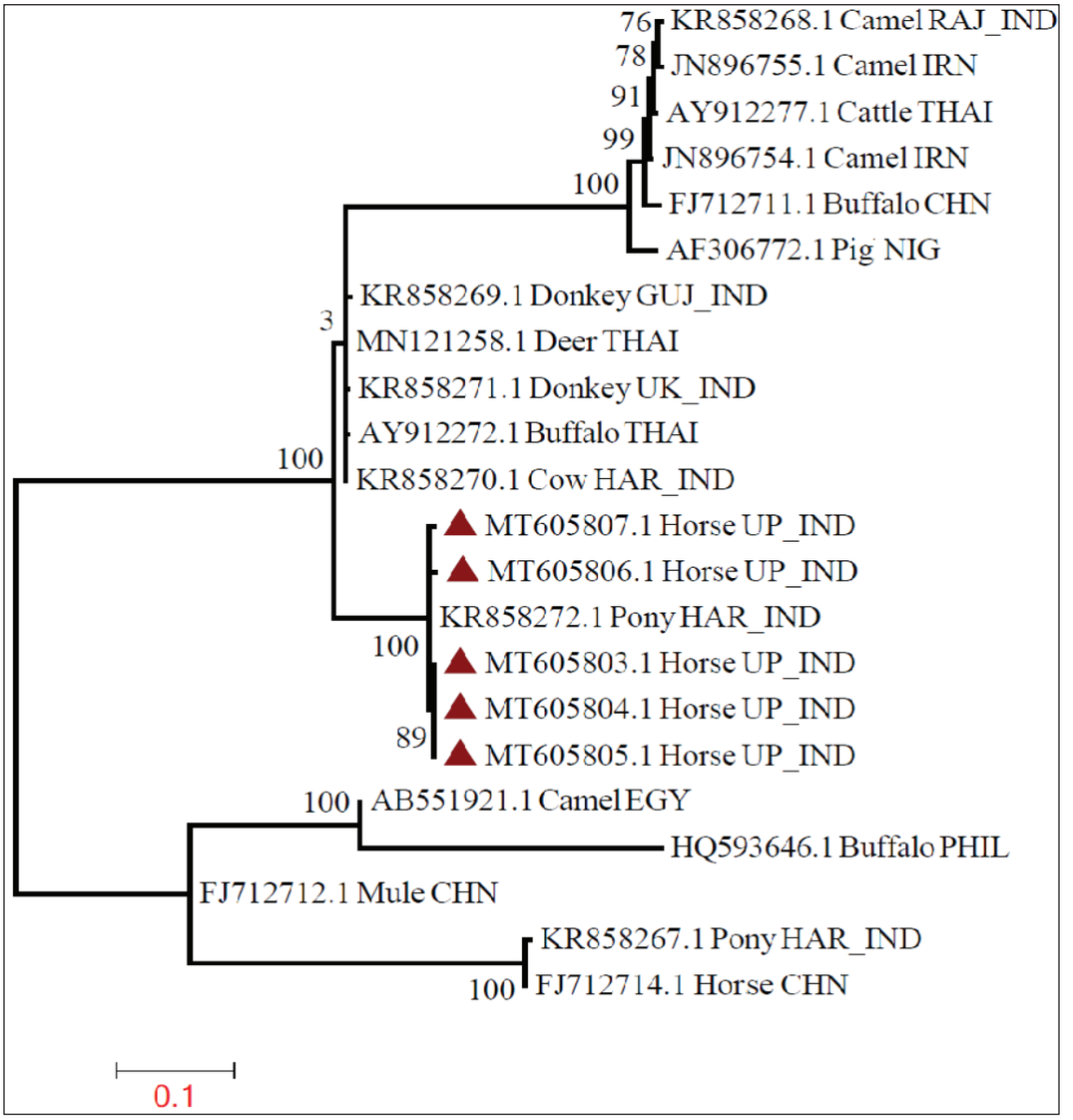


Fig. 3: Phylogenetic relationship of *T. evansi* isolates across the world based ITS 1 gene. All accession numbers corresponds to different *T. evansi* isolates followed by their country of origin and the host from which it was isolated. The sequences generated in the present study are marked as red triangle. {CHN: China; NIG: Nigeria; PHIL: Philippines; THAI: Thailand; IRN: Iran; IND: India (GUJ: Gujarat; HAR: Haryana; RAJ: Rajasthan; UP: Uttar Pradesh)}

The ribosomal DNA (rDNA) genes have been very much significant in molecular and phylogenetic analysis of trypanosomes alongside their taxonomic identity. The present communication aimed at phylogenetic characterization of equine isolates of *T. evansi* from semi arid India based on ITS1 region of rDNA gene. In the present study, the various stocks of *T. evansi* affecting different hosts, across different countries, were phylogenetically compared on ITS 1 locus. Characterization of five equine *T. evansi* isolates showed distribution into three haplotypes with 99.06-100.00% nucleotide homology within themselves. In comparison with the isolates across the globe, they were phylogenetically closer to *T. evansi* isolates reported from Haryana than to the isolates

reported from rest of India. So far as global scenario is concerned, the Indian isolates of *T. evansi* were closely related to isolates from Thailand and China than to those reported from Iran, Nigeria and Egypt. Wide distribution of Indian isolates on the phylogenetic tree and their marked resemblance with specific yet selective isolates across the globe have raised questions about presence of multiple distinct lineages of *T. evansi* amongst the Indian livestock.

Sarkhel *et al.* (2017) reported genetic variability in *T. evansi* isolates, from different regions of India and affecting different hosts, based on ITS 1 gene. However, Areetik *et al.* (2008), in their preliminary work, described ITS 1 region as an unsuitable marker for analyzing genetic diversity within a clonal population of *T. evansi* originating from a single host. This was attributed to the robustness of the phylogenetic tree based on ITS 1 gene that suffers from lower bootstrap values indicating a higher feasibility of tree collapse (Tian *et al.,* 2011). Selection of a comparatively larger nucleotide sequence length often helps to nullify such scenario (Sarkhel *et al.,* 2017). Another remarkable finding in the present study is the close association of equine isolates from India with other isolates of *T. evansi* affecting members of family equidae as their hosts (pony, mule and donkey) and their clear divergence from isolates of *T. evansi* affecting other hosts (buffalo, cattle, deer, camel and pig). Although, this is a very preliminary observation yet the fact cannot be nullified that isolates of *T. evansi* have got markedly closeness among the related hosts. Similar genetic heterogeneity among *T. evansi* isolates derived from different hosts has earlier been also reported using AP-PCR (Kundu *et al.,* 2010) alongside, using RoTat 1.2 VSG (Gaur *et al.,* 2021) and ITS 1(Sarkhel *et al.,* 2017) genes in sequence based studies.

CONCLUSION

The findings of the present study are very much significant from molecular evolutionary point of view. Well planned studies using different molecular targets and covering a large number of isolates of *T. evansi* affecting different hosts need to be done for actual delineation of evolutionary phylogenetics. Moreover, the effect of host species and phylogenetics of distribution of *T. evansi* needs to be thoroughly analyzed.

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