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Short Communication

Molecular characterization and phylogenetic analysis of *Trypanosoma evansi* from Northern India based on 18S ribosomal gene

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ABSTRACT

Six *Trypanosoma evansi* isolates were collected from ponies (PH1 and PK6), camel (CB2), donkeys (DJ3 and DH4) and cattle (CK5) from different States of Northern India (Haryana, Rajasthan, Uttar Pradesh and Gujarat) for molecular characterization based on 18S rRNA gene. The 18S rRNA gene (2251 bp) of different isolates was amplified, cloned and custom sequenced separately. Based on sequence and phylogenetic analysis of all six isolates, collected from different hosts as well as geographical areas, were having high identity among Indian *T. evansi* strains (99.7%) and with other strains of *T. evansi* (99.2%) distributed worldwide. There is less genetic diversity among different salivarian strains of *T. evansi* except few nucleotide changes at significant locations in one Indian isolate of camel origin (CB2). All Indian *T. evansi* isolates were grouped in salivarian clade with high bootstrap values and remained far away from stercorarian clade having 88–90% nucleotide identity. The study will be helpful in understanding the evolutionary relationship, molecular epidemiology and variation in disease pathogenesis among different *T. evansi* strains. Further, more studies are required on large number of isolates collected from diverse host and geographical areas to reaffirm the present finding.

1. Introduction

Animal trypanosomosis, caused by *Trypanosoma evansi*, is an important disease of livestock leading to severe morbidity and mortality. *T. evansi* is one of the most pathogenic trypanosomes transmitted mechanically by biting of blood sucking arthropods especially by tabanid flies. The disease has a detrimental effect on livestock health and leads to tremendous economic losses on livestock productivity globally (Kristjanson et al., 1999; Dargantes et al., 2009; Kumar et al., 2017). It has wide host range viz. camels, horses, donkeys, mules, cattle, buffaloes, dogs, pigs, sheep, goats and wild animals (Losos, 1980; Tuntasuvan et al., 1997; Adrian et al., 2009). A couple of years ago the first instance of *T. evansi* infection in humans was reported from India (Joshi et al., 2005) and recently from Vietnam (Van Vinh Chau et al., 2016). The parasite shows an across the board geographic distribution, a wide range of mammalian hosts and a variety of clinical symptoms. Virulence of the different *T. evansi* strains also varies to an extent suggesting genetic variability within *T. evansi* species (Luckins, 1988; Uche et al., 1992).The genetic variability among *Trypanosoma* strains may be one of the important factors responsible for variation in virulence. Genetic variability in *T. evansi* has been demonstrated by variety of molecular methods like isoenzymes (Gibson et al., 1983), RAPD (Lun et al., 2004), AFLP (Masiga et al., 2006) and most recently by microsatellite analysis (Biteau et al., 2000; Njiru et al., 2006; Saini et al., 2014). Most of these techniques do not provide earlier accessibility to molecular level information, due to which they didn't able to indicate high heterogeneity among strains having few base pair differences among genotypes (Masiga et al., 2006; Ventura et al., 1996). So, in present context studies shifted to DNA cloning and sequencing of target genes. The DNA sequencing technology has provided more realistic information on the genetic diversity in most organisms and it has been found very useful in classifying parasites including trypanosome species.

Previous studies on the 18S rDNA gene has proven that it is exceptionally helpful in analyzing ancient evolutionary events as it is among the slowest evolving element discovered broadly all through living life form (Haag et al., 1998; Lukes et al., 1997; Stevens and Gibson, 1999). In present study, we investigated the befitting of 18S rRNA full length gene in analyzing the genetic diversity of Indian *T. evansi* isolates collected from different hosts and geographical area. This data could be applicable for the survey of parasite dynamics, epidemiological studies as well as prevention and control of the disease

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caused by this parasite.

2. Materials and methods

2.1. T. evansi isolates and isolation of genomic DNA

Six isolates of *T. evansi* were collected from naturally infected camel (CB2), cattle (CK5), donkeys (DJ3 and DH4) and ponies (PH1 and PK6) inhabiting Northern (Haryana), Western (Gujarat and Rajasthan) and Central (Uttar Pradesh) India and cryo-preserved in liquid nitrogen were propagated in Swiss albino mice and purified using DEAE column chromatography technique. Genomic DNA was extracted from purified parasites using Genomic DNA isolation kit (Invitrogen, USA) according to the manufacturer's protocol and stored at −40 °C for future use.

2.2. PCR amplification and purification

The PCR was standardized for amplification of the 18S rDNA gene (2251 bp) of *T. evansi* from complete DNA of the six field isolates utilizing forward primer *A*: 5′-AACCTGGTTGATCCTGCCAGT-3′ and reverse primer B: 5′-TGATCCTTCTGCAGGTTCACCTAC-3′ (Sogin, 1990). The PCR reaction was carried out in 25 μl reaction mixture according to the manufacturer's protocol using Hi Fidelity Platinum Taq (Invitrogen, USA), using 50 ng genomic DNA. Amplification of target gene was performed using a gradient thermal cycler (Peqlab, UK) under following conditions: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles (94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min) and a final extension step at 72 °C for 15 min.

The amplified PCR product was checked by electrophoresis on 1.5% agarose gel and purified using commercial Gel and PCR Clean-up kit (Macherey-Nagel Inc., Germany) following manufacturer's protocol.

2.3. Cloning and sequencing

For cloning of full length of 18S rRNA gene, the purified PCR products of six different isolate were independently ligated into pTZ57R/T TA cloning vector (Thermo Scientific, USA) and incubated at 4 °C for overnight. The plasmid DNA constructs were transformed into competent DH5α *Escherichia coli* cells using PCR cloning kit (InsTAclone™, Thermo Scientific, USA) in accordance with manufacturer's protocol. The transformed cells were then plated immediately on pre-warmed LB agar plates supplemented with ampicillin (50 mg/ml), X-gal (30 mg/ml) and IPTG (0.5 mM) for the development of blue and white colonies. The positive clones were confirmed by colony PCR using gene specific primers and restriction enzyme digestion of extracted plasmid with *Bam*HI and *Hind*III. The positive plasmid clones were sequenced at least twice to rule out the possibility of sequencing errors.

2.4. Sequence analysis

All the newly generated sequences of 18S rRNA gene of *T. evansi* isolates were compared with each other and with selected published sequences available in GenBank by BLAST program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov). Multiple and Pairwise alignment was carried out using nucleic acid analysis software (MegAlign™, DNASTAR) so as to examine sequence variations.

2.5. Phylogenetic analyses

Phylogenetic analyses of aligned sequences of six *T. evansi* isolates were done independently with the sequences of the 18S rRNA gene available in GenBank database by utilizing the MEGA6 (Tamura et al., 2011) with the Maximum Likelihood method. The Neighbourhood Joining method was used for clustering of Indian isolates among themselves. The nucleotide substitution model for best fit to the data set was evaluated in MEGA6. Tamura-Nei (TN93) with gamma distribution (TN93 + G + I) was found to be the model of choice for phylogenetic analysis of 18S rRNA gene. The phylogeny was analyzed using this model with 1000 bootstrap replications. Bootstrap values ≥ 60 were considered significant and indicated the confidence with which the same cluster would be formed, thus, maintaining the topology (Hillis and Bull, 1993; Park et al., 2010).

3. Results

3.1. PCR amplification and cloning of 18S rRNA

PCR amplification of 18S rRNA gene showed single band of ~2251 bp size on agarose gel (1.5%) electrophoresis. The PCR products were purified, cloned into pTZ57R/T cloning vector and confirmed by colony PCR and by the release of specific fragments on RE digestion. Subsequently, 18S rRNA gene of each of the six isolates was sequenced.

3.2. Sequence analysis and phylogenetic relationships of 18S rRNA gene

Six sequences of the 18S rRNA gene (2251 bp) generated in this investigation were submitted to GenBank. Sequence similarity searches in BLAST revealed that newly generated sequences of 18S rRNA gene were highly similar (99% identity) with the published sequences of *T. evansi* available in GenBank. The phylogenetic tree was constructed using Maximum Likelihood method with Tamura-Nei model for distance calculation based on the 2251 bp region of 18S rRNA gene of *Trypanosoma* spp. is presented in Fig. 1. A closer comparison of these sequences after alignment revealed that they shared 99.4–99.8% identity among themselves by Neighbourhood Joining method and 99.7% with different Indian strains of *T. evansi*. All these newly generated sequences of *T. evansi* isolates differed at 10th nucleotide position and it replaced with 'C' instead of 'T' with other isolates of *T. evansi* used in present study. Sequence alignment report also showed various nucleotide changes at different positions such as changes in DH4 (7 positions), PK6 (5 positions), PH1 (4 positions), CB2 (3 changes), DJ3 (6 positions) and CK5 (4 positions). In the present study, one *T. evansi* isolate of camel origin (CB2) was found distinct to other salivarian trypanosomes due to nucleotide changes at significant places, but remained within subgenus *Trypanozoon* salivarian group.

These sequences revealed 99.2–99.4% identity with the sequences of *T. evansi* from Thailand (U75507, AY904050, AY912268 and AY912269.1 strains), Taiwan (D89527), Japan (AB301940) and Egypt (AB551921). It forms a separate clade and remain far away from salivarian strains Kenya (U22315.1, U22320.1 and U22316), Brazilian strains (AJ012416.1), Germany strains (AJ223566.1), Japan strains (AB007814) and France strains (AJ009148.1).

4. Discussion

The present study was aimed to investigate genetic variability among Indian *T. evansi* isolates from different host species and geographical places. Surra outbreaks are responsible for thousands of deaths in affected animals, hence affecting world's livestock industry (Yutuc, 1935; Manuel, 1998; Reid, 2002). It needs evolutionary relationship study to elucidate variation in pathogenesis and for formulation of proper prevention and control strategies. The phylogenetic relationships based on the complete 18S rRNA gene sequences was reported only among the 3 species of subgenus *Herpetosoma* (*T. lewisi*, *T. musculi* and *T. microti*) (Haag et al., 1998; Stevens and Gibson, 1999). Additionally, some have reported relationship based on partial 18S rDNA sequences from *T. microti*, *T. evotomys*, *T. grosi* and an unidentified trypanosome species from wood mice *Apodemus sylvaticus* (Noyes et al., 2002). A limited work has been carried out on genetic diversity among *Trypanosoma* spp. worldwide including India. Therefore, the present study was carried out using 18S rRNA gene to

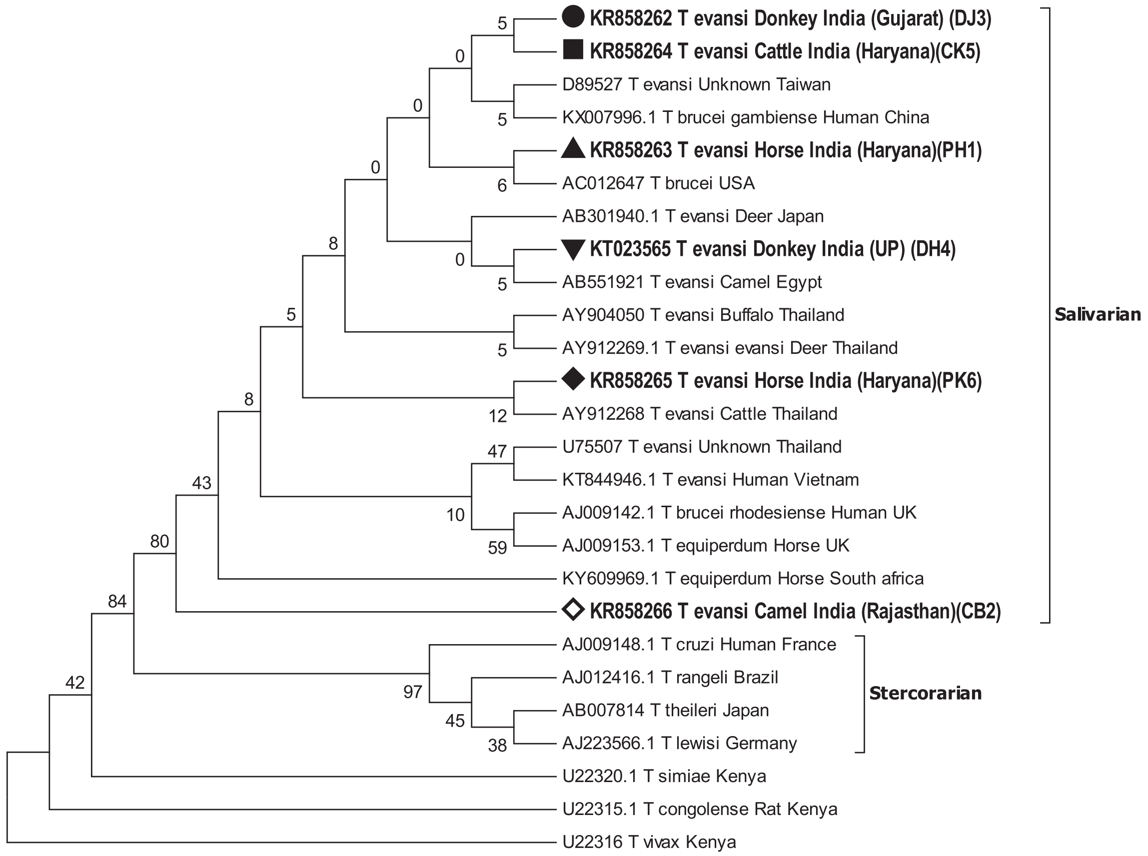

Fig. 1. Phylogenetic tree based on 18S rRNA gene of *Trypanosoma evansi* isolates using Maximum Likelihood method (Tamura-Nei model). The percentage of replicate trees in which the associated species clustered together in the bootstrap method (1000 replicates) is shown next to the branches.

determine the heterogeneity within the Indian *T. evansi* isolates from different hosts and geographical areas.

The sequences comparison of each of the six *T. evansi* isolates from diverse hosts and geographical regions of India revealed that the 18S rRNA gene revealed minimal intra-specific variation (99.4–99.8% identity). Analogous results have been previously reported for *T. evansi* (Amer et al., 2011; Areekit et al., 2008; Khuchareontaworn et al., 2007; Liu et al., 2008), *B. gibsoni* and also for *Toxoplasma gondii* (Criado-Fornelio et al., 2003; Homan et al., 1997; Mandal et al., 2014).

Neighbourhood joining tree grouped PH1 and PK6 strains into one cluster because of isolation from same horse species. Similarly, donkey isolates DJ3 and DH4 were also grouped in other cluster due to identical in nature. Other species isolates such as CB2 and CK5 isolated from camel and cattle remain outgroup but still having 99.5% nucleotide identity with *T. evansi* of other species.

Phylogenetic analysis by Maximum Likelihood method with other worldwide strains revealed isolates from Haryana pony (PH1 and PK6) are more closely related to Thailand and Japan *T. evansi* strains having nucleotide identity > 99%. Isolates from western part of country such as Gujarat and Rajasthan are more identical with Thailand and Egypt *T. evansi* strains having > 99 nucleotide identity than other isolates. Further, these Indian strains formed a major cluster which revealed 99% nucleotide identities with different strains of *T. brucei and T. equiperdum*.

There were different nucleotides changes present in different isolates of India such as five and seven in DH4 and PK6 isolates of *T. evansi*. However, these nucleotide changes in all isolates except CB2 are similar with other strains of *T. evansi* making them interspersed between Indian isolates. The changes present in CB2 are only three in number but these are distinct from other salivarian strains which lead to separation of this isolate from other Indian and world strains. It indicates that mutations would have continued after the original speciation event.

The genus *Trypanosoma* can be divided into two major groups that infect vertebrates, the salivaria and the stercoraria (Hoare, 1972). In phylogenetic tree, all salivarian and stercorarian trypanosomes (*T. rangeli*, *T. theileri*, and *T. lewisi*) formed a separate major clade in the tree with high bootstrap support and 88–90% nucleotide identity. Further, within the salivarian group, both *Trypanozoon* (*T. brucei*, *T. evansi*, *T. equiperdum*) and *Nanomonas* subgenera (*T. simiae* and *T. congolense*) occupied two separate clades in the phylogenetic tree with high (84%) bootstrap value. Two species of *Nanomonas* and *Duttonella* (*T. vivax*) subgenus were placed separately with low bootstrap value (42%). In the present study, representing phylogenetic tree of all the isolates of *T. evansi* formed a different major clade including *T. brucei* and *T. equiperdum* with low bootstrap value (< 60%) showing the monophyletic nature of all the salivarian isolates. Within this clade, the Indian *T. evansi* isolates were found interspersed with isolates from Egypt, Thailand, China and Japan. Other salivarian strains including *T. brucei brucei*, *T. brucei gambiense* and *T. brucei rhodesiense* formed a separate group but remain near to Indian isolates with 99% nucleotide identity. It indicates a divergent evolution of these isolates from other strains of *T. evansi*.

This analysis supports previous studies suggesting that *T. evansi* displays high degree of homogeneity regardless of wide geographic distribution and host range (Lun et al., 2004, Masiga et al., 2006, Nijru et al., 2007, Perrone et al., 2009). However, emerging genetic variability in *T. evansi* may be responsible for variation in pathogenicity of parasite and affecting diverse host species including human.

5. Conclusion

In conclusion, the characterization of *T. evansi* at the molecular level will be helpful for better species classification and assessment of evolutionary relationships with other organisms. This 18S region can be used in assessing genetic variation in a number of protozoans including *T. evansi* and distinguish it inter-specifically and intra-specifically. The molecular approach has the potential to identify the distinct species of *T. evansi*, dynamics of the parasites and epidemiology. This study will provide recommendations for future work, and indicates a need to consider the risk of horizontal transfer of epidemiologically relevant traits among different *Trypanosoma* species in any control campaign.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2018.100259.

Animal ethics

The animal experimentations carried out in the present work after approval of Institute Animal Ethic Committee (IAEC) and as per guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Government of India (IAEC approval was accorded vide F. No. NRCE/CPCSEA/2012-13 dated 22.12.2012).

Conflict of interest statement

All authors disclose that they have no financial and personal relationships with other people or organization that could inappropriately influence their work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registration, and grants or other funding. The authors declare no conflict of interest.

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