Molecular characterization of internal transcribed spacer 1 (ITS 1) region of different *Trypanosoma evansi* isolates of India

Souti Prasad Sarkhel¹ · Surender Kumar Gupta¹ · Jyoti Kaushik² · Jarnail Singh² · Deepak Kumar Gaur² · Sanjay Kumar² · Rajender Kumar²

Received: 26 October 2015 / Accepted: 21 September 2016 / Published online: 29 September 2016
© Indian Society for Parasitology 2016

Abstract Six *Trypanosoma evansi* isolates collected from ponies (PH1 and PK6), camel (CB2), donkeys (DJ3 and DH4) and cattle (CK5) from Haryana, Rajasthan, Uttar Pradesh and Gujarat states of India were used for molecular characterization of internal transcribed spacer 1 (ITS 1). The DNA was isolated from purified trypanosomes of these six isolates after propagation in mice model. ITS1-PCR of purified parasite DNA yielded an amplification product approximately 540 bp in size. Nucleotide sequence of ITS1 gene of CB2 isolate had 530 bp while CK5, DH4, DJ3, and PH1 isolates had 532 bp, whereas, PK6 isolates had 533 bp size. Blast data of the Indian isolates revealed 99 % homology with other available sequences of *T. evansi*. Multiple alignment of nucleotide sequence of ITS1 gene variants from Indian *T. evansi* isolates with selected homologous sequences from GenBank revealed that nucleotide substitution mostly occurred at the position of 101–103, 218–223, 243–244, 301–396 and 470–480. The isolates PH1, CK5, DH4 and DJ3 were found more associated with *T. evansi* isolates from the Philippines, Thailand, Iran, Egypt and China, whereas, PK6 and CB2 isolates were related to each other and were phylogenetically distant from rest of the Indian isolates used in this study. Based on the ITS1 rDNA sequence, the Neighbour-Joining consensus tree indicated clear evidence of

existence of genetic diversity among *T. evansi* isolates from India.

Keywords Molecular characterization · Internal transcribed spacer 1 · ITS 1 · *Trypanosoma evansi*

Introduction

Trypanosoma evansi infection is transmitted mechanically by several haematophagous biting flies, has a broad host range including a diverse variety of mammalian species, both domestic and wild. The parasite is widely prevalent in tropical and subtropical countries, including most parts of India (Pathak et al. 1993; Gupta et al. 2009; Kumar et al. 2013; Kundu et al. 2013; Sumbria et al. 2014). Variations in parasite strain virulence, host susceptibility, as also disease manifestations (acute, sub-acute and chronic) are commonly observed in the field. Genetically different *T. evansi* isolates are likely to differ in their pathogenicity and biology as indicated by parameters like drug sensitivity and virulence (Waitumbi et al. 1994; Zhou et al. 2001, 2007). The advent of DNA sequencing technology has provided more information on the genetic variation in most organisms and it has been found very useful in classifying protozoan parasites including trypanosome species with the molecular characterization of ribosomal DNA gene (Homan et al. 1997; Chansiri et al. 2002; Amer et al. 2011; Lollis et al. 2011).

Several genetic markers have been used in the past for phylogenetic analysis and/or characterization of polymorphism in *T. evansi* populations around the world. Ribosomal DNA (rDNA) has been studied extensively for understanding the evolutionary events occurring till date (Appels and Honeycutt 1986; Mindell and Honeycutt

✉ Rajender Kumar
rkg.nrce@mail.com

¹ Department of Veterinary Parasitology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana 125004, India

² ICAR-National Research Centre on Equines, Sirsa Road, Hisar, Haryana 125001, India

1990). The Different regions of the rDNA repeat unit evolve at different rates, thus making rDNA useful for phylogenetic analysis (Hillis and Dixon 1991). Analyses of the internal transcribed spacer (ITS) region (Khuchareontaworn et al. 2007; Areekit et al. 2008; Tian et al. 2011; Pourjafar et al. 2013; Villareal et al. 2013) have been utilized in molecular classification of trypanosomes.

The present study was carried out to know the genetic diversity and phylogeographical patterns based on ITS1 rRNA of *T. evansi* circulating in different livestock species across different geographical regions of India.

Materials and methods

T. evansi isolates

Six isolates of *T. evansi* were used in the study, collected from camel (Bikaner, Rajasthan; Lab ID- CB2), cattle (Karnal, Haryana; Lab ID- CK5), donkeys (Hardoi, Uttar Pradesh; Lab ID- DH4 and Junagarh, Gujarat; Lab ID- DJ-3) and ponies (Hisar, Haryana; Lab ID- PH1 and Karnal, Haryana; Lab ID- PK-6) inhabiting different geographical regions. These isolates were passaged in vivo in mice and cryo-preserved in liquid nitrogen at National Fellow Laboratory, National Research Centre on Equines, Hisar. Prior to use, in the present study, they were thawed and passaged afresh in mice.

Experimental animals

Swiss albino mice were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The mice were inoculated with 10^5 parasites intra-peritoneally per animal. At the peak of parasitaemia (10^8 trypanosomes/ml), the infected mice were sacrificed and blood was collected by heart puncture under appropriate anaesthesia. Trypanosomes were purified from mice whole blood by anion exchange column chromatography (pre swollen microgranular DEAE cellulose) as per the method of Lanham and Godfrey (1970).

DNA extraction and PCR

Genomic DNA was extracted from purified parasites using Pure Link™ Genomic DNA isolation kit (Invitrogen, USA) according to the manufacturer's protocol. PCR was standardized for amplification of the Internal Transcribed Spacer 1 (ITS1) region from *T. evansi* chromosomal DNA template of six field isolates using specific ITS1 primer pair 5'-CGCCCCGAAAGTTCACC-3' and 5'-GCGTTCAAA-GATTGGGCAAT-3' (Mc Laughlin et al. 1996). ITS1-PCR

using purified parasite DNA gave an amplification product of approximately 540 bp (Fig. 1). Based on these findings, PCR products were purified for each of the six isolates for cloning.

Cloning and sequencing of ITS-1 gene

The PCR products of each isolates were resolved in 1 % agarose gel and PCR products were visualized in Omega Lum G gel imaging system (Aplegen Inc., USA) and PCR amplicons of six different isolates were excised and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey–Nagel Inc., Germany) following the manufacturer's protocol. Approximate 540 bp amplicons of ITS1 gene region were ligated to the pTZ57R/T vector by using InsTAclone PCR cloning kit (Thermo Scientific, USA) as per manufacturer's protocol and competent DH5α *E. coli* cells were transformed with this insert. The white coloured transformants were screened by the colony PCR and one of the positive clones was grown in sufficient amount for plasmid DNA isolation by the QIAprep® Spin Miniprep Kit (Qiagen Group, Germany) and sequenced (SciGenom Labs Private Ltd, Kerala). The nucleotide sequence data was submitted to GenBank for accessioning.

Phylogenetic analysis

Nucleotide sequence data of six Indian isolates of *T. evansi* for ITS1 rDNA gene family were aligned against homologous sequences deposited in GenBank (Table 1) by using the ClustalW with default parameters in CLC workbench v.5.6.1 (<http://www.clcbio.com>) software. A neighbor-

Fig. 1 Agarose gel electrophoresis of ethidium bromide stained ITS1-PCR (on purified parasite DNA) product of approximately 540 bp. Lane # M: 100 bp DNA ladder (Fermentas, # SM0241); 1 Camel Bikaner (CB-2), 2 Pony Hisar (PH-1), 3 Donkey Hardoi (DH-4), 4 Donkey Junagarh (DJ-3), 5 Pony Karnal (PK-6), 6 Cattle Karnal (CK-5), N negative control

Table 1 Reference *Trypanosoma* spp. ITS1 region sequences retrieved from GenBank

Sr. No.	Accession number	Species/isolate	Host	Location	References
1.	AB551921	<i>T. evansi</i>	Camels	Egypt	Amer et al. 2011
2.	AB551922	<i>T. evansi</i>	Camels	Egypt	Amer et al. 2011
3.	AF306772	<i>T. brucei</i>	Pig	Nigeria	Agbo et al. 2001
4.	AF306775	<i>T. brucei</i>	Human	Sudan	Agbo et al. 2001
5.	AY912272	<i>T. evansi</i>	Buffalo	Thailand	Unknown
6.	AY912274	<i>T. evansi</i>	Buffalo	Thailand	Unknown
7.	AY912277	<i>T. evansi</i>	Cattle	Thailand	Unknown
8.	AY912278	<i>T. evansi</i>	Deer	Thailand	Unknown
9.	AY912279	<i>T. evansi</i>	Deer	Thailand	Unknown
10.	FJ712711	<i>T. evansi</i>	Buffalo	China	Tian et al. 2011
11.	FJ712712	<i>T. evansi</i>	Mule	China	Tian et al. 2011
12.	FJ712714	<i>T. evansi</i>	Horse	China	Tian et al. 2011
13.	FJ712715	<i>T. evansi</i>	Buffalo	China	Tian et al. 2011
14.	HQ593646	<i>T. evansi</i>	Buffalo	Philippines	Villareal et al. 2013
15.	JN896754	<i>T. evansi</i>	Camel	Iran	Pourjafar et al. 2013
16.	JN896755	<i>T. evansi</i>	Camel	Iran	Pourjafar et al. 2013
17.	U75507	<i>T. evansi</i>	–	Thailand	Unknown

joining (NJ) phylogenetic tree was constructed from nucleotide sequence data for ITS1 region by using MEGA version 5 (Tamura et al. 2011). The phylogenetic robustness was assessed by undertaking bootstrap re-sampling using 1000 replicate.

Results

Cloning, sequencing and phylogenetic analysis

Nucleotide sequence of ITS1 gene of CB2 isolate had 530 bp while CK5, DH4, DJ3 and PH1 isolates had 532 bp; PK6 isolate had 533 bp size. Blast data of the Indian isolates revealed 99 % homology with other available sequences of the *T. evansi*. Multiple alignment of nucleotide sequence of ITS1 gene variants from Indian *T. evansi* isolates with selected homologous sequences from Genbank revealed differences in nucleotides at certain position of various homologues sequences. The multiple sequence alignment of Indian isolates with the reference sequences from Genbank revealed that nucleotide substitution mostly occurred at the position of 101–103, 218–223, 243–244, 301–396 and 470–480 (Fig. 2). The sequence variation between *T. evansi* isolates from India was observed from 0.2 to 4.5 % (Table 2). In the NJ consensus tree, clear evidence was produced of existence of genetic diversity among *T. evansi* isolates from India, based on the ITS1 rDNA sequence. PH1 and CK5 isolates

were homologous with *T. evansi* isolates from the Philippines (HQ 593646, Thailand (AY912272, AY912275), Iran (JN 896754), and Egypt (AB551922) in decreasing order than with other Indian isolates. DH4 and DJ3 isolates were more related to *T. evansi* isolates from Thailand (AY912273), China (FJ712715) and with *T. brucei* (AF306772), whereas PK6 isolate had more similarity with *T. evansi* Thailand isolate (AY912274), than with CB2. CB2 isolate has a clear relatedness with Iranian isolates of *T. evansi* Genotype B. The data indicated PH1, CK5, DH4 and DJ3 are phylogenetically distant to CB2 and PK6 isolates (Fig. 3). This data may be helpful to know genetic variability among different strains and to correlate pathogenicity of different isolates. A Neighbour-Joining consensus tree was inferred from ITS1 regions of *Trypanosoma* spp. with 50 % bootstrap value cut off. Branches with less than 50 % bootstrap support were collapsed (Fig. 4).

Discussion

Molecular analysis based on rDNA sequences is essential for phylogenetic analysis and determination of taxonomic identities of trypanosomes. The present study was specifically aimed to investigate the genetic variability among Indian *T. evansi* isolates from four different hosts (camel, cattle, donkey and pony) based on ITS1 region of rDNA gene.

Fig. 2 continued

Although existence of genetic heterogeneity among *T. evansi* isolates derived from different hosts has been studied earlier (Kundu et al. 2010), this is probably the first report of ITS1 gene sequence of Indian isolates of *T. evansi*. Examination of its features revealed its closeness to several isolates of *T. evansi* from the Philippines, Thailand, Iran and China. Areekit et al. (2008) reported that ITS 1 region was not suitable for analyzing the genetic diversity of clonal population of *T. evansi* from single host. However, we observed clear evidence from the NJ-consensus tree that, a certain degree of genetic diversity existed among *T. evansi* isolates from India based on ITS 1. These differences might be due to the fact that present study

included *T. evansi* isolates from different regions and different hosts. The robustness of the tree based on ITS 1 region suffered from low bootstrap value indicating the high feasibility of tree collapse which has been reported recently (Tian et al. 2011; Areekit et al. 2008). According to Areekit et al. (2008), it might be due to the limited nucleotide sequence length as well as the high similarity at this region among the isolates and it could be improved by using the full ITS region (including ITS1, 5.8S and ITS2). Therefore, in the present study Neighbour-Joining consensus tree were inferred with 50 % bootstrap value cut off and less than 50 % bootstrap support were collapsed.

In conclusion, genetic diversity of *T. evansi* isolates from different geographical regions has been demonstrated. Phylogenetic trees can provide insights into the co-evolution of trypanosomes with their vertebrate hosts and vectors. The present study may be helpful in knowing virulence pattern and sub-typing of *T. evansi* isolates, disease dynamics and designing primers for diagnostics which can subsequently provide useful information for disease prevention and management in the country.

Fig. 2 Multiple alignment of ITS1 nucleotide sequences from Indian *T. evansi* isolates and those of other trypanosomes obtained from GenBank. Residues identical to the sequences of *T. evansi* CB2 isolate are indicated by dots (.). Spaces or dash (-) denotes gap generated during alignment, which are introduced for optimal comparison. The difference in nucleotide sequence with that of the majority are coloured as red

Table 2 Homology comparison of ITS1 nucleotide sequences from *Trypanosoma evansi* Indian isolates

Isolate ID (Accession number)	Pairwise distance					
	PH1	CK5	DJ3	DH4	CB2	PK6
Pairwise identity						
PH1 (KR858272)	ID	0.013	0.013	0.016	0.024	0.035
CK5 (KR858270)	0.986	ID	0.005	0.003	0.016	0.032
DJ3 (KR858269)	0.986	0.996	ID	0.008	0.022	0.038
DH4 (KR858271)	0.984	0.998	0.994	ID	0.019	0.035
CB2 (KR858268)	0.973	0.984	0.981	0.983	ID	0.016
PK6 (KR858267)	0.955	0.962	0.958	0.96	0.977	ID

Fig. 3 Neighbour-Joining consensus tree with 1000 bootstraps. The bootstrap confidence values at the *nodes* represent the percentage of times the group occurred out of 1000 trees and the *bar* represents the genetic distance **Fig. 4** A Neighbour-Joining consensus tree inferred from ITS1 regions of *Trypanosoma* spp. with 50 % bootstrap value cut off. The bootstrap confidence values at the *nodes* represent the percentage of times the group occurred out of 1000 trees. Branches with <50 % bootstrap support were collapsed

Acknowledgments The authors are thankful to Director ICAR-National Research Centre on Equines, Hisar for providing research facilities and Indian Council of Agricultural Research, New Delhi for financial support under National Fellow scheme for this study.

Compliance with ethical standards

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).

References

- Agbo EC, Majiwa PA, Claassen EJ, Roos MH (2001) Measure of molecular diversity within the *Trypanosoma brucei* subspecies *Trypanosoma brucei brucei* and *Trypanosoma brucei gambiense* as revealed by genotypic characterization. *Exp Parasitol* 99:123–131
- Amer S, Ryu O, Tada C, Fukuda Y, Inoue N, Nakai Y (2011) Molecular identification and phylogenetic analysis of *Trypanosoma evansi* from dromedary camels (*Camelus dromedarius*) in Egypt, a pilot study. *Acta Trop* 117:39–46
- Appels R, Honeycutt RL (1986) rDNA evolution over a billion years. In: Dutta SK (ed) DNA systematics. CRC Press, USA, pp 81–135
- Areekit S, Singhaphan P, Kanjanavas P, Khuchareontaworn S, Sriyapai T, Pakpitcharoen A, Chansiri K (2008) Genetic diversity of *Trypanosoma evansi* in beef cattle based on internal transcribed spacer region. *Infect Genet Evol* 8:484–488
- Chansiri K, Khuchareontaworn S, Sarataphan N (2002) PCR-ELISA for diagnosis of *Trypanosoma evansi* in animals and vectors. *Mol Cell Probes* 16:173–177
- Gupta MP, Kumar H, Singla LD (2009) Trypanosomosis concurrent to tuberculosis in black bucks. *Indian Vet J* 86:727–728
- Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453
- Homan WL, Limper L, Verlaan M, Borst A, Vercammen M, van Knapen F (1997) Comparison of internal transcribed spacer, ITS1, from *Toxoplasma gondii* isolates and *Neospora caninum*. *Parasitol Res* 83:285–289
- Khuchareontaworn S, Singhaphan P, Viseshakul N, Chansiri K (2007) Genetic diversity of *Trypanosoma evansi* in buffalo based on internal transcribed spacer (ITS) regions. *J Vet Med Sci* 69:1215–1217
- Kumar R, Kumar S, Khurana SK, Yadav SC (2013) Development of an antibody-ELISA for seroprevalence of *Trypanosoma evansi* in equids of North and North-western regions of India. *Vet Parasitol* 196:251–257
- Kundu K, Rao JR, Tewari AK, Baidya S, Mishra AK (2010) Existence of genetic variability among Indian isolates of *Trypanosoma evansi*. *Indian J Anim Sci* 80:3–6
- Kundu K, Tewari AK, Kurup SP, Baidya S, Rao JR, Joshi P (2013) Sero-surveillance for surra in cattle using native surface glycoprotein antigen from *Trypanosoma evansi*. *Vet Parasitol* 196:258–264
- Lanham SM, Godfrey DG (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exp Parasitol* 28:521–534
- Lollis L, Gerhold R, Mc Dougald L, Beckstead R (2011) Molecular characterization of *Histomonas meleagridis* and other parabasalids in the United States using the 5.8S, ITS-1, and ITS-2 rRNA regions. *J Parasitol* 97:610–615
- Mc Laughlin GL, Ssenyonga SS, Nanteza E, Rubaire-Akiki WO, Hansen RD, Vodkin MH, Novak RJ, Gordon VR, Montenegro-James S, James M, Armijos R, Santrich C, Weigle K, Santrich C, Saravia N, Wozniak E, Gaye O, Mdachi R, Shapiro SZ, Chang KP, Santrich C, Kakoma I (1996) PCR based detection and typing of parasites. In: Zcel MA, Alkan MZ (eds) Parasitology for the 20th century. CAB International, Wallingford, pp 261–287
- Mindell DP, Honeycutt RL (1990) Variability in transcribed regions of ribosomal DNA and early divergences in birds. *AUK* 106:539–548
- Pathak KML, Arora JK, Kapoor M (1993) Camel trypanosomosis in Rajasthan. *India Vet Parasitol* 49:319–323
- Pourjafar M, Badieli K, Sharifiyazdi H, Chalmeh A, Naghib M, Babazadeh-Dezfouli M, Moutbi-Alavi A (2013) Genetic characterization and phylogenetic analysis of *Trypanosoma evansi* in Iranian dromedary camels. *Parasitol Res* 112:899–903
- Sumbria D, Singla LD, Sharma A, Moudgil AD, Bal MS (2014) Equine trypanosomosis in central and western Punjab: prevalence, haemato-biochemical response and associated risk factors. *Acta Trop* 138:44–50
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Tian Z, Liu G, Xie J, Shen H, Zhang L, Zhang P, Luo J (2011) The internal transcribed spacer 1 (ITS-1), a controversial marker for the genetic diversity of *Trypanosoma evansi*. *Exp Parasitol* 129:303–306
- Villareal MV, Mingala CN, Rivera WL (2013) Molecular characterization of *Trypanosoma evansi* isolates from water buffaloes (*Bubalus bubalis*) in the Philippines. *Acta Parasitol* 58:6–12
- Waitumbi JN, Murphy NB, Peregrine AS (1994) Genotype and drug resistance phenotype of *T. evansi* isolated from camels in northern Kenya. *Annls Trop Med Parasitol* 88:677–683
- Zhou JL, Zhou YZ, Jin FH (2001) Sensitivities for Suramin of 12 *Trypanosoma evansi* isolates from China. *Chi J Vet Sci* 1:60–62
- Zhou YZ, Shen J, He GS, Zhou JL (2007) Virulence of 12 Chinese *Trypanosoma evansi* strains in mice. *Chi J Prev Vet Med* 6:432–434