Indian Journal of Animal Sciences **84** (7): 732–735, July 2014/Article

Microsatellite genotyping of *Trypanosoma evansi* isolates from livestock of different agro-ecological zones of India

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Received: 21 January 2014; Accepted: 17 February 2014

ABSTRACT

The present study deals with genetic diversity analysis by microsatellite genotyping of *Trypanosoma evansi* isolates collected from different livestock hosts (horse, donkey, camel and cattle) inhabiting different geographical location in North and North-western parts of India. Out of 12 microsatellite loci used in the study 10 successfully genotyped 6 *T. evansi* isolates, and 6 of them were found polymorphic. At 6 microsatellite loci (TB8/11, TB11/29, TB10/1, TB1/8, TB3/3 and TB4/2) observed number of alleles per locus ranged from 3 (TB8/11) to 6 (TB4/2) with a mean of 4.83±1.75 and effective number of alleles ranged from 2.11 (TB8/1) to 5.00 (TB4/2) with a mean value of 3.32±1.16. At 4 microsatellite loci (TB11/13, TB2/19, TB11/1 and TB8/1) observed (na=2) as well as effective (ne=2) number of alleles were the same. The observed heterozygosity varied from 0.16 (TB8/11) to 1.00 (TB11/13, TB2/19, TB10/1, TB1/8 and TB4/2) with a mean of 0.76±0.37. The expected value of heterozygosity (Levene's) varied from 0.48 (TB8/1) to 0.88 (TB4/2) with a mean of 0.59±0.23. Polymorphism information content (PIC) value ranged from 0.34 (TB8/1) to 0.77 (TB4/2), indicating that TB11/29, TB10/1, TB1/8, TB3/3 and TB4/2 microsatellite loci were highly informative (PIC>0.5). Neighbour-joining tree analysis indicated different clustering of isolates based on allelic sharing. In the present study, 6 microsatellite markers were found useful to determine genetic diversity among Indian *T. evansi* isolates.

Key words: Genotyping, India, Livestock, Microsatellite loci, Polymorphism, Surra, *Trypanosoma evansi*

Surra disease caused by *Trypanosoma evansi* has a major economic impact world-wide in terms of mortality and production losses in livestock. The economic losses in Asia may be higher than those caused by the African trypanosomosis, which are estimated to be $ 1.3 billion annually (Kristjanson *et al.* 1999). In India, surra is widely prevalent in almost all the states, with greater incidence in the arid and semi-arid zones (Pathak *et al.* 1993, Kumar *et al*. 2013, Kundu *et al.* 2013). The variations in parasite strain virulence, host susceptibility and disease manifestation are routine observations in different hosts inhabiting in different agro-climatic zones. The one of the main factors responsible for these variations might be genetic diversity among *T. evansi* isolates. The study of genetic diversity of different isolates of *T. evansi* from different geographical areas is important for studying molecular epidemiology of disease, clinical and evolutionary research, vaccine and drug design, and prevention and control of the disease. Microsatellite profiling of isolates may be a useful tool for studying genetic diversity among different isolates owing to their random and abundant availability in eukaryotic genome (Hamada *et al.* 1982). Therefore, the present study was undertaken to know genetic diversity among various *T. evansi* isolates from livestock of different agro-ecological zones by microsatellite genotyping for understanding the molecular epidemiology of surra in the country.

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MATERIALS AND METHODS

Study area and source of T. evansi *isolate:* The present study was carried out at National Research Centre on Equines, Hisar, India. Isolates (6) of *T. evansi* were collected from livestock hosts from different geographical locations of India (Table 1), and were propagated in mice model and cryopreserved in LN_2 for further use.

Propagation of T. evansi isolates in mice: Swiss albino mice were used for experimental infection with different *T. evansi* cryopreserved isolates. Initial inoculation to mice was done with 1×10^5 trypanosomes by intra-peritoneal route. Thereafter, the parasitaemia was monitored daily in mice by microscopic examination of wet blood film. At the peak of peripheral parasitaemia, mice were bled by cardiac puncture and trypanosomes were purified by DEAE-

Table 1. Details of *Trypanosoma evansi* isolates collected from different agro-climatic zones of India

T. evansi isolates	Host	Lab ID	Agro-climatic zones*
T.ev-India-NRCE-Horse1 /Hisar/Haryana	Horse	PH-1	Trans Gangatic Plains Region
T.ev-India-NRCE-Camel 1/Bikaner/ Rajasthan	Camel	CB-2	Western Dry region
T.ev-India-NRCE-Donkey 2/Junagarh/Gujarat	Donkey	DJ-3	Gujarat Plains and Hill Regions
T.ev-India-NRCE-Donkey1/Hardoi/Uttar Pradesh	Donkey	DH-4	Upper Gangatic Plains Region
T.ev-India- NRCE-Cattle1/ Karnal/Haryana	Cattle	CK-5	Trans Gangatic Plains Region
T.ev-India-NRCE-Horse 2 /Karnal/Haryana	Horse	PK-6	Trans Gangatic Plains Region

* Broad agro-climatic zones defined by Planning Commission of India (Khanna 1989).

cellulose chromatography, followed by centrifugation (Lanham and Godfrey 1970). Purified parasites were used for DNA extraction.

Extraction of DNA: The genomic DNA was extracted from the purified *T. evansi* parasites as per Sambrook and Russell (2001) with modifications. Briefly, purified *T. evansi* (10^8 parasites) were treated with 5.0 ml cell lysis buffer (10 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 10% SDS) and incubated for 1 h at 37°C. After incubation, 50 µl of proteinase K (100 µg/ml) was added and further incubated at 50°C for 3 h with intermittent shaking. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and mixed for 1 min. The tube was centrifuged at 3000 × g for 10 min and aqueous phase was collected. Equal volume of chloroform: isoamyl alcohol (24: 1) was added to the aqueous phase, mixed it and upper aqueous phase was finally collected after centrifugation at 3000 × g for 10 min. To the aqueous phase one-tenth volume of 3M sodium acetate (pH 5.2) was added and DNA was precipitated by adding equal volume of chilled absolute alcohol. The DNA pellet was obtained by centrifugation at 4000 × g for 15 min and finally dissolved in 50µl of nuclease free water and stored at –20°C till further use

Microsatellite genotyping: Initially, 15 microsatellite primers pair were selected from the published reports, which were designed from the flanking regions of selected microsatellites chosen on the basis of perfect repeating motifs and from different chromosomes to minimise the chance of linkage, using data of *T. brucei* genome project release 4 (Balmer *et al.* 2006, Salim *et al.* 2011). Out of 15, 12 microsatellite loci were found useful after evaluation on different *T. evansi* isolates (Table 1) and 3 loci were not included in the study due to difficulty in PCR amplification. The reverse primers specific for 12 microsatellite loci were labelled with fluorescent dye, viz. fluorescein amidite (FAM), hexachloro-fluorescein (HEX), carboxyl-x-rhodamine (ROX). Six reverse primers (TB11/13, TB2/19, TB10/19, TB10/1, TB1/8 and TB3/3) were labelled with FAM, 5 reverse primers (TB11/1, TB8/11, TB11/29, TB7/12 and TB4/2) were labelled with HEX and 1 reverse primer (TB8/1) was labelled with ROX at their 5´ termini. All fluorescently labelled PCR amplicons were analysed on an ABI 3730xl genetic analyzer in conjunction with a GeneScan 500 LIZ size standard. Allele identification was performed using the ABI program GeneMapper V4.0.

Statistical analysis: Allele frequencies, observed and expected heterozygosity estimates along with observed and effective numbers of alleles were calculated using Cervus 3.0.3 and POPGENE software (Yeh *et al.* 1999). Closely related diversity measure, polymorphism information contents (PIC) was estimated (Botsein *et al.* 1980). The parameters F_{IT} (total inbreeding estimate), F_{ST} (measurement of population differentiation), and F_{IS} (within-population inbreeding estimate) were calculated using Fstat version 2.9.3 (Goudet 1995) and the variance-based method of Weir and Cockerham (1984). A Neighbor-joining phylogenetic tree derived from genetic distance based on the proportion of shared alleles among the 10

Table 2. List of selected primer sequences used for amplification of microsatellite loci of *Trypanosoma evansi*

Locus	Primer sequence 5´ - 3´	Chromosome number	References
TB11/13	F: CAAGAACTCTGCATTGAGC R: ATCTGTTGGCGATGGTGA	11	Balmer *et al*. (2006)
TB2/19	F: CTGGTGCGTGTAACTGTG R: GAAGTGAGGACATGCACG	2	Balmer *et al*. (2006)
TB11/1	F: AGTATGTGTGGACAGTTAAGA R: ACCTTTGAGTCTTTCCTGTT	11	Salim *et al.* (2011)
TB8/1	F: CCAAATATGCGATTAGTTTCC R: TGTTTATGTGGAAGGAAATGAA	8	Salim *et al*. (2011)
TB8/11	F: TGTAGCAGTGGTACGCAC R: CACCCAACGCATGTAAGC	8	Balmer *et al*. (2006)
TB11/29	F: AATGAGTGATACTATGAAAGTGT R: CACCATCACTGCTCTTATCA	11	Salim *et al*. (2011)
TB7/12	F: CATGGCGTACGTTGCTTCGGTTTC R: GGTCGGTGTTGGCAGTGTGCATAG	7	Salim *et al.* (2011)
TB10/19	F: CTGTTCGTTCTGAATTGTGTGCG R: GTGCACTTCCTTCTCTCATCCTTTTC	10	Salim *et al*. (2011)
TB10/1	F: GCTCTACGCACCCACACAATCCGT R: CTCACTTGAGTAACCTCTCATTGC	10	Salim *et al*. (2011)
TB1/8	F: AGGTTTAGTGCATGTCGGA R: CCTGTTGTACGGAGGTCA	1	Balmer *et al*. (2006)
TB3/3	F: CATTCGAAGTAAATGCGCGTATAAC R: GGTTGGAGCTTTCGACACAAGCG	3	Salim *et al*. (2011)
TB4/2	F: GCCGCTTGATCATTAGGTAACCAC R: CCGCCTCACTTTAAGGATGGTGCC	4	Salim *et al.* (2011)

microsatellite loci (Jin and Chakraborty 1994).

The animal experimentations carried out in the present work was after approval of Institute Animal Ethic Committee 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.

RESULTS AND DISCUSSION

Out of 12 microsatellite markers used in the study, successful amplification was observed at 10 microsatellite loci in 6 isolates of *T. evansi* and 6 of them were found polymorphic and useful to characterise and determine polymorphism among *T. evansi* isolates collected from different hosts in varied agro-ecological conditions of India. The observed number of alleles per locus ranged from 2 (TB11/13, TB2/19, TB11/1 and TB8/1) to 6 (TB4/2) with a mean value of 3.70±1.75 and effective number of allele ranged from 2 (TB11/13, TB2/19, TB11/1 and TB8/1) to 5 (TB4/2) with a mean value of 2.77±1.16. All the loci had lower value of effective number of alleles in comparison to observed number of alleles. In our study no significant data were obtained for allele sizes at 2 loci (TB7/12 and TB10/19). McInnes *et al.* (2012) also observed unscorable results on 11 microsatellite loci due to monomorphism and poor PCR amplification.

The observed value of heterozygosity was lowest at TB8/11 locus and highest at locus (TB11/13, TB2/19, TB10/1, TB1/8, and TB4/2). The expected value of heterozygosity (Levene) has a mean value of 0.6525±0.2369 whereas the expected value of heterozygosity (Nei's) has a mean value of 0.5966±0.2153 (Table 3). Shannon's information index was like observed number of alleles with minimum value in TB8/1 and maximum in TB4/2 with the mean value of 1.06±0.49. PIC value ranged from 0.34 (TB8/1) to 0.77 (TB3/3). Microsatellites loci (TB11/29, TB10/1, TB1/8, TB3/3 and TB4/2, were highly informative (PIC>0.5). Mean inbreeding coefficient (F_{is}) was negative at all 10 loci (–1.00). *T. evansi* isolates are generally considered homogenous with low heterogeneity due to absence of recombination because *T. evansi* does not undergo any development cycle in the vector unlike other members of subgenus Trypanozoon that are transmitted by the tsetse fly where genetic exchange is thought to occur (Jenni *et al.* 1986). We observed negative F_{IS} value at all 10 microsatellite loci and excess of heterozygotes. This indicated low genetic variability and predominant clonal reproduction (Simo *et al.* 2013). Even in more recent asexual lineages, strong excess of heterozygotes is expected, and this will translate into strongly negative F_{IS} estimates (Balloux *et al.* 2003, Bengtsson 2003). The impact of clonality may slightly vary from one life-cycle to another, thus leading to different patterns of genetic structure at individual and population levels (Tibayrenc 1999). However, in the near absence of theoretical models providing clear expectations under controlled conditions, estimating the rate of clonal reproduction in natural population appears problematic (Anderson and Kohn 1998) and even documenting strict clonal reproduction is often controversial (Tibayrenc 1997). We also observed 100% heterozygosity at 5 loci and at 8 loci higher value of observed heterozygosity than expected value of heterozygosity. Higher heterozygosity occurs in clonal organism might be due to accumulation of random mutation

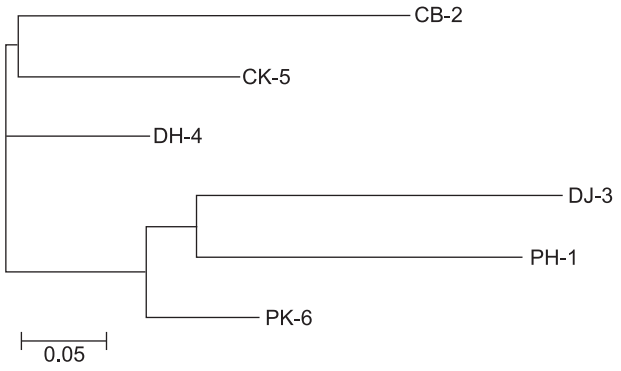

Fig. 1. N-J tree based on share allele (D_{AS}) genetic distances showing relationship between different isolates of *Trypanosoma evansi*.

Table 3. Allele size range, number of allele (observed n_a, effective n_e), heterozygosity (observed Ho, expected He), inbreeding coefficient (F_{IS}) and polymorphism information content (PIC) at 10 microsatellite loci in different *Trypanosoma evansi* isolates

Locus	Allele size range (bp)	na	ne	Ho	He		F_{IS}	PIC
					Levene	Nei's		
TB11/13	151–163	2.0000	2.0000	1.0000	0.5455	0.5000	–1.000	0.375
TB2/19	100–102	2.0000	2.0000	1.0000	0.5455	0.5000	–1.000	0.375
TB11/1	133–135	2.0000	2.0000	0.6667	0.5455	0.5000	–1.000	0.375
TB8/1	143–145	2.0000	1.8000	0.6667	0.4848	0.4444	–1.000	0.346
TB8/11	105–109	3.0000	2.3226	0.1667	0.6212	0.5694	–1.000	0.477
TB11/29	146–162	5.0000	2.1176	0.3333	0.5758	0.5278	–1.000	0.503
TB10/1	141–153	5.0000	3.1304	1.0000	0.7424	0.6806	–1.000	0.643
TB1/8	111–127	5.0000	4.2353	1.0000	0.8333	0.7639	–1.000	0.726
TB3/3	99–107	5.0000	3.1304	0.8333	0.7424	0.6806	–1.000	0.643
TB4/2	87–89	6.0000	5.0000	1.0000	0.8889	0.8000	–1.000	0.772
Mean	-	3.7000	2.7736	0.7666	0.6525	0.5966	–1.000	0.523
SD	-	1.7529	1.1655	0.3713	0.2369	0.2153	-	-

events (Halkett *et al*. 2005, De Meeus *et al.* 2006, McInnes *et al*. 2012).

A Neighbour-joining phylogenetic tree was constructed from share allele genetic distances calculated for the microsatellite data of 6 *T. evansi* isolates at 10 loci. The tree topology displayed the short distance between the DJ-3 and PH–1 isolate of *T. evansi*. The CB-2 isolate of *T. evansi* was found closer to CK-5 isolate. The DH-4 and PK-6 *T. evansi* isolates were found different from each other as well as from other *T. evansi* isolates (Fig. 1).

In the present study 6 microsatellite markers were found useful to determine genetic diversity among Indian *T. evansi* isolates. For evaluating appropriate genetic polymorphism mapping and molecular epidemiology of the disease, more number of isolates is required from different endemic agro-climatic zones. To search more number of informative microsatellite markers, genome sequencing data of *T. evansi* is needed. There is also a need to evolve improved statistical software for population genetic study of diploid clonal pathogenic organisms.

ACKNOWLEDGEMENTS

The authors are thankful to Director 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. We also thank Dr R.K. Vijh, Principal Scientist and Head, Animal Genetics Division, NBAGR, Karnal for valuable support in data analysis.

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