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Genetic differentiation amongst different *Trypanosoma evansi* isolates using random primer DNA typing

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Trypanosomosis (surra) caused by *Trypanosoma evansi* is one of the important diseases of livestock including equines. The disease has the widest geographical range of all the pathogenic trypanosome species and is a major constraint to livestock productivity on the three continents, Asia, Africa and South America. *T. evansi* infects a wide range of domestic and wild animals in India, viz. camels, horses, donkeys, mules, cattle, buffaloes, dogs, pigs, goats, tigers and elephants. It is hypothesized that *T. evansi* has evolved from *T. brucei* when infected camels moved to tse tse free areas and adapted to survive without natural biological vector and transformed to mechanical transmission through haematophagus biting flies and thus existence of sexual recombination in this species is highly unlikely and follow clonal propagation population structure. The variations in host susceptibility, virulence of different isolates are commonly observed conditions in animals suffering from surra. Therefore, study of genetic variability among different *T. evansi* isolates is very important to know molecular epidemiology, and formulation of prevention and control strategies of the disease. For specific detection and molecular typing of blood protozoan, random amplification of polymorphic DNA (RAPD) technique has gained importance in recent years. The present study was carried out to know genetic variability among Indian *T. evansi* isolates of different hosts and agro-ecological regions using RAPD- PCR approach with new and already reported informative primers.

Six isolates of *T. evansi* were used in the study, collected from different livestock species (horse, donkey, camel and cattle) inhabiting different agro-ecological regions of India (Table 1), were propagated *in vivo* in Swiss albino mice. Initial inoculation to mice was done with 1×10^5 trypanosomes by intra-peritoneal route. Thereafter, the parasitaemia was monitored regularly. At peak parasitaemia, mice were bled by cardiac puncture for isolation and purification of parasites. The trypanosomes from infected mice blood were purified by DEAE-cellulose chromatography. The genomic DNA was extracted from purified trypanosomes. Seventeen decamer primers including already published informative primers were chosen for the present study for typing of different *T. evansi* isolates (Table 2). Amplification reactions were performed in a 25µl containing 10 ng of DNA from each *T. evansi*

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Table 1. Detail of *T. evansi* isolates collected from different hosts and geographical regions

Host	Lab ID	Place of isolation
Horse	PH	Hisar, Haryana
Horse	PK	Karnal, Haryana
Donkey	DJ	Junagarh, Gujarat
Donkey	DH	Hardoi, Uttar Pradesh
Cattle	CK	Karnal, Haryana
Camel	CB	Bikaner, Rajasthan

Table 2. List of decamer arbitrary primers used for randomly amplified polymorphic DNA analysis

Code	Sequence	Length	GC (%)	Reference
PR1	GCCGTCCGAG	10	80	Basagoudanavar (1998)
PR2	GTGATCGCAG	10	60	Basagoudanavar (1998)
PR3	CGCCCCACGT	10	80	Basagoudanavar (1998)
PR4	TGCATCGTAC	10	50	Basagoudanavar (1998)
PR5	GGTGCGGGAA	10	70	Tibayrenc *et al.* (*1*993)
PR6	GTTTCGCTCC	10	60	Tibayrenc *et al.* (*1*993)
PR7	GTAGACCCGT	10	60	Tibayrenc *et al.* (*1*993)
PR8	AAGAGCCCGT	10	60	Tibayrenc *et al.* (*1*993)
PR9	AACGCGCAAC	10	60	Tibayrenc *et al.* (*1*993)
PR10	CCCGTCAGCA	10	70	Tibayrenc *et al.* (*1*993)
PR11	GGGCGGTACT	10	70	Lun *et al.* (2004)
PR12	CGGACGTCGC	10	80	Waitumbi and Murphy (*1*993)
PR13	GAGTGGTGAC	10	70	Lun *et al.* (2004)
PR14	GCCTGAAAAC	10	50	Ventura *et al.* (2000)
PR15	ATCCGAGCCT	10	60	Unpublished
PR16	GCCGTGACCG	10	80	Unpublished
PR 17	CCCCGGTAAC	10	70	Kundu *et al*. (2010)

Table 3. Randomly amplified polymorphic DNA fragments generated with different *T. evansi* isolates using decamer arbitrary primers

T .evansi isolates	DNA fragment size (bp)												
	PR1	PR2	PR3	PR4	PR5	PR6	PR7	PR8	PR9	PR10	PR12	PR13	PR17
PK	1000	1000	750	490	1400	750	400	1000	750	NA	NA	NA	600
	800	700	500		900	600		600	600				
	700				800	300		400	500				
	n=3	n=2	n=2	n=1	n=3	n=3	n=1	n=3	n=3	n=0	n=0	n=0	n=1
CK	1000	700	750	490	900	1600	600	1000	1300	700	1000	750	800
	700		500		800	1000	400		1000		850	400	600
						750		600	900		750		400
						600			750				300
						300			500				
									400				
									350				
	n=2	n=1	n=2	n=1	n=2	n=5	n=2	n=2	n=7	n=1	n=3	n=2	n=4
DH	1100	2500	1400	490	3100	4000	600	1250	1300	700	1000	850	800
	1000	1300	750		1600	1600		1000	1000		750	400	
	750	1200	500		1500	1000		600	900				
	700	700			1400	300			750				
					900				500				
					800								
	n=4	n=4	n=3	n=1	n=6	n=4	n=1	n=3	n=5	n=1	n=2	n=2	n=1
PH	1000	1500	750	490	1400	1000	400	1000	900	NA	1000	400	600
	700	800	500			750		600	750				400
		700				600		450	600				
	n=2	n=3	n=2	n=1	n=1	n=3	n=1	n=3	n=3	n=0	n=1	n=1	n=2
DJ	1000	1300	750	490	900	1600	600	1000	1000	700	1000	850	300
	700	1200	500		800	750	400	600	900			400	
		700				300			750				
									400				
	n=2	n=3	n=2	n=1	n=2	n=3	n=2	n=2	n=4	n=1	n=1	n=2	n=1
CB	1000	700	1400	490	3100	4000	1000	1500	1300	700	1000	1400	600
	700		750		1600	1600	750	1250	1000			850	400
			500		1500	1000	600	1000	900			400	300
					1400			600	750				
					900				500				
					800								
	n=2	n=1	n=3	n=1	n=6	n=3	n=3	n=4	n=5	n=1	n=1	n=3	n=3
GC content of primer (%)	80	60	80	50	70	60	60	60	60	60	80	70	70

*n, number of scorable DNA bands.

isolate, 0.15mmol/L of a single primer and Taq master mix for PCR reaction. The samples were pre-incubated for 5 min at 94°C in a thermocycler. This was followed by 45 cycles each of 1 min denaturation at 94°C, 45 sec annealing at 36°C and 1 min elongation at 72°C. There was then a final extension for 5 min at 72°C. After the reactions, the tubes containing the PCR products were held at 4°C prior to analysis. The amplified product would be analyzed using agarose gel electrophoresis and would be visualized in UV light in gel documentation system.

The results of the RAPD reaction are shown in Fig. 1 (A-M). Of the 17 primers analysed, 13 were able to amplify polymorphic DNA fragments from the genome of *T. evansi* and produced 179 scorable bands, 38 of which were polymorphic. The amplicons showing similarity in three and more *T.evansi* isolates were considered as monomorphic amplicon. The pattern of RAPD disclosed high polymorphism among different isolates of *T evansi*. Depending upon the *T. evansi* isolate primer combination, DNA fingerprints of 300 to 4000 bp were amplified, suggesting minor and major differences in the RAPD profiles. In all the assays performed, a few fragments were amplified more efficiently, giving more intense bands than other reproducible bands in the same reaction. The RAPD-PCR revealed strong reaction products of 4000, 2500, 1800, 1500, 1400, 1300, 1250, 1200, 1100, 1000, 950, 900, 800, 750, 700, 600, 500, 490, 400 and 300 bp size (Table 3). The primer PR2 (Fig.1 A) showed different polymorphic DNA fragments of different isolates of *T. evansi* which were unique for particular isolate and all these band were scorable

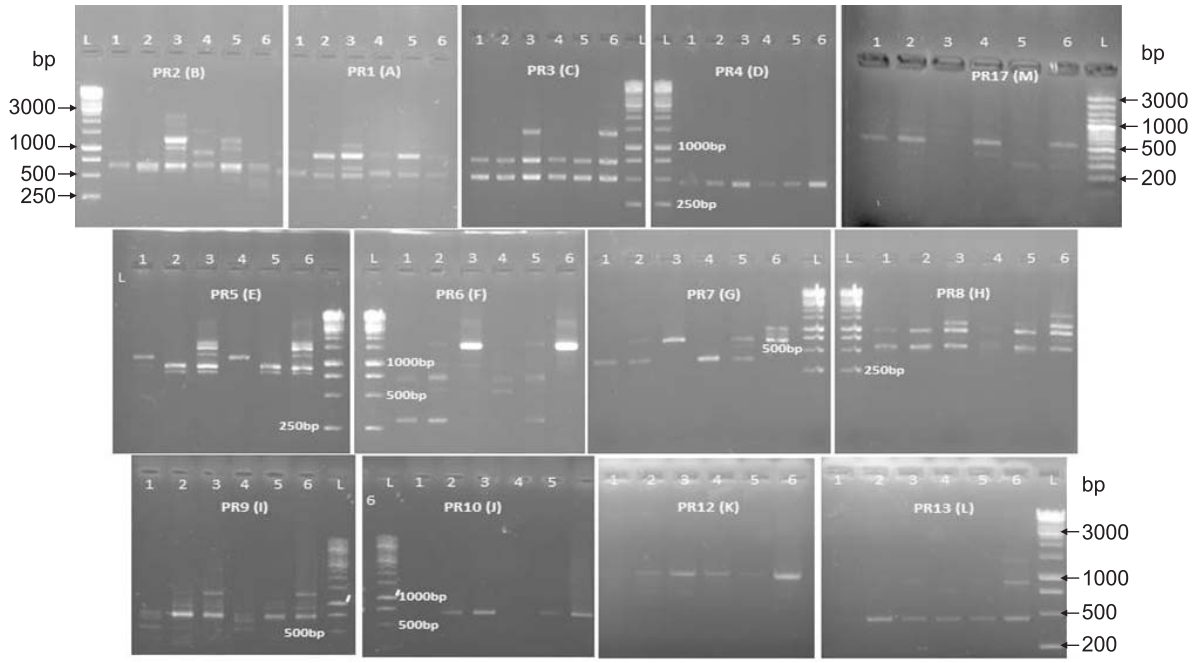

Fig. 1 (A-M) RAPD amplicons obtained using different primers with 6 isolates of *Trypanosoma evansi*. Lane 1, pony (Karnal, Haryana); lane 2, cattle (Karnal, Haryana); lane 3, donkey (Hardoi, Uttar Pradesh); lane 4, pony (Hisar, Haryana); lane 5, donkey (Junagarh, Gujarat); lane 6, camel (Bikaner, Rajasthan); lane L, ladder; PR, primer code. Different types of primers shows different patterns of polymorphism.

as either distinct or likely distinct. Primer PR1 can separate DH from the remaining isolates. Two groups of monomorphic bands amplified with primer PR3 that distinguish one group i.e. PK, CK, PH, DJ with another group i.e. DH and CB. The amplified DNA segment from PR4 and PR10 primers generated only monomorphic DNA fragments (Fig. 1C, 1J). The remaining primers i.e. PR5, PR6, PR7, PR8, PR9 (Fig. 1E-I) showed various polymorphic bands which demonstrated genetic variation among protozoa obtained from diversified isolates. The RAPD profile obtained with PR13 primer mainly showed monomorphic band except PK and CB, the primer amplified a large number segments with DNA of CB isolate (Fig. 1K). PR12 primer revealed monomorphic bands with DNA of all *T. evansi* isolates except PK (Fig. 1L). PR 17 primer also showed monomorphic as well as polymorphic bands with 5 isolates expect DH which did not show any amplification (Fig. 1M). Total number of polymorphic and monomorphic bands with percentage of polymorphism and monomorphism are presented in Table 4.

The primer PR2 appeared to be the most suitable for targeting genome variability owing to the reproducible production of profiles probably distinct amplification products with *T. evansi* DNA template of different isolates.

Table 4. Amplicon polymorphism observed among *T. evansi* isolates using different decamer arbitrary primers

Primer code	Total amplicon	Polymorphic amplicon	Monomorphic amplicon	% polymorphism	% monomorphism
PR1	15	3	12	25	75
PR2	14	8	6	57	43
PR3	14	2	12	14	86
PR4	6	0	6	0	100
PR5	20	6	14	30	70
PR6	21	2	19	10	90
PR7	10	2	8	20	80
PR8	18	3	15	16	84
PR9	27	5	22	19	81
PR10	4	0	4	0	100
PR12	8	3	5	38	62
PR13	10	2	8	20	80
PR17	12	2	10	17	83
Total	179	38	141	21	79

The genetic variability and its relationship to host pathology has been reported for other trypanosomatids (Macedo *et al*. 2002, Morrison *et al.* 2009, Duarte *et al.* 2014). A pronounced feature of the DNA fingerprints was the variation in the intensity of the bands. The more intense bands were probably due to priming within the repeated regions of the trypanosome genome, which would result in more copies being produced during PCR. The typing of *T. evansi* isolates is very important due to variation in disease manifestation and drug susceptibility in response to different *T. evansi* isolates. The polymorphism in *T. evansi* strains and difference in drug resistance and virulence for mice among different T. *evansi* isolates from China had also been described by earlier researchers (Zhou *et al*. 2007). In the present study, we have used potential informative RAPD primers reported by earlier workers, but we observed different polymorphism pattern with our *T. evansi* isolates. The variation in polymorphism observed in the present study might be due to variation in *T. evansi* isolates, hosts and geographical distribution. The present study indicated that RAPD approach is an easy technique and may be used for preliminary typing and differentiation of different *T. evansi* isolates, and disease epidemiological study.

SUMMARY

In order to know the genetic variability amongst the 6 isolates of *Trypanosoma evansi* obtained from different livestock species (horse, donkey, camel and cattle) of different geographical regions, genomic DNA was amplified by polymerase chain reaction using 17 decamer arbitrary primers. In PCR profiles, 13 out of the 17 primers generated differences. The primer PR2 showed different polymorphic DNA fragments among different isolates of *T. evansi* which were unique for particular isolate and all these band were scorable as either distinct or likely distinct. Various informative bands ranging from 250 to 4,000 bp were observed through PCR amplified DNA profile of different isolates and marked the minor and major difference among the isolates. Randomly amplified polymorphic DNA (RAPD) analysis revealed heterogeneity among *T. evansi* isolates of different livestock hosts and geographical regions.

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