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Existence of genetic variability among Indian isolates of *Trypanosoma evansi*

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ABSTRACT

Genetic heterogeneity within *Trypanosoma evansi* isolates derived from buffalo, dog, horse and camel was studied by polymerase chain reaction (PCR). PCR was carried out using 17 arbitrary decamers with a GC content ranging from 60 to 70% and potentially informative primers on the genome of *T. evansi* were identified. The data on percentage difference between each pair of parasite isolates and the average percentage difference value for each of the isolate pairs for a given random oligonucleotide primer were elucidated. Depending upon the *T. evansi* isolate-primer combination between 3 and 15 reproducible DNA fingerprints of 179 bp to 4039 bp were amplified suggesting minor and major differences in their random amplified polymorphic DNA (RAPD) profiles. One arbitrary primer, 5′-CCCCGGTAAC–3′ was identified as potentially informative for intra-species differentiation of *T. evansi*.

Key words: AP-PCR, DNA polymorphism, Genetic variability, *Trypanosoma evansi*

Trypanosoma evansi, a haemocytozoic parasite that affects a wide range of hosts in India with marked differences in the pathogenesis and clinical course of the disease, exhibits polymorphism in morphology and biological characteristics. RAPD-PCR also known as arbitrary primer PCR (AP-PCR) was used to study DNA polymorphism and intra-species differentiation of *T. evansi* (Desquesnes and Davila 2002). Taking advantage of the usefulness of AP-PCR technique, the present study analyzes differences in genome of Indian isolates of *T. evansi* derived from bubaline, cameline, canine and equine origin. The paper also describes a potentially informative primers that can be used in PCR analysis to detect and differentiate the isolates.

MATERIALS AND METHODS

Parasite isolates: *T. evansi* isolates (4) of bubaline, cameline, canine and equine origin from Indian states of West Bengal, Rajasthan, Uttar Pradesh and Punjab, respectively, were passaged in laboratory rodents (rats and mice), giving short-term fulminating infections. The parasite strains from camel, buffalo and horse were isolated during non-febrile clinical course of infection from their respective hosts using mouse passage. Whereas the parasites from the dog were isolated during febrile infection showing teeming parasitaemia and showed greater mouse virulence (data not shown). The trypanosomes were separated from host blood components by DEAE-cellulose chromatography using phosphate saline glucose (PSG), pH 8.0, as eluting buffer. DNA was extracted from the trypanosomes (Sambrook and Russell 2001).

Arbitrary primed polymerase chain reaction (AP-PCR): The AP-PCR was set up in 25µl reaction volume in 0.2 ml thin walled PCR tubes. Seventeen different commercially synthesized oligonucleotide primers were chosen arbitrarily (Table 1). The reaction mixture contained 1 µl (10 ng) of genomic DNA of *T. evansi*, 2.5 µl of 10× *Taq* DNA polymerase buffer containing 1.5 mM $MgCl_2$, 1 µl (3U) of *Taq* DNA polymerase, 0.5µl of 10 mM dNTP mixture and 1.0µl of primer (15pmol or 50 ng). The volume was made up to 25 µl with autoclaved triple distilled water. The contents were mixed by flicking the tubes and flash spun to collect the constituents at the bottom. As a practice, master mix containing all the constituents except the template DNA was prepared. The DNA of the 4 parasite isolates was added to the individually marked PCR tubes after the distribution of the master mix. PCR was performed in a thermal cycler with heated lid. The cycling conditions included initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 45 sec

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Table 1. Details of arbitrary (random) primers

Primer code	Primer sequence	Nucleotide length	% GC
AP1	ACC TGG ACA C	10	60
AP2	GGA GGA GAG G	10	70
AP3	CAG AAG CCC A	10	60
AP4	CCG CCT AGT C	10	70
AP5	TGT TCC ACG G	10	60
AP6	AAG GCG GCA G	10	70
AP7	CAG CGA CAA G	10	60
AP8	TTT GCC CGG T	10	60
AP9	ACA ACG CCT C	10	60
AP10	GGG AAC GTG T	10	60
AP11	CTG GGC AAC T	10	60
AP12	CCG TGA CTC A	10	60
AP13	GCA TGC GAT C	10	60
AP14	GAC CGC TTG T	10	60
AP15	ACC CCC GAA G	10	70
AP16	CCC CGG TAA C	10	70
AP17	TCC CGC CTA C	10	70

and elongation at 72°C for 1 min. This was followed by final extension at 72°C for 5 min. On completion of reaction, tubes were stored at 4°C until analysis by electrophoresis.

Five microlitres of 6× loading dye was added to each tube containing 25 µl of PCR product and 15 µl of the product was run on ethidium bromide stained 1.5% agarose gels. Standard molecular weight markers were run along with each sample. Electrophoresis was performed applying 2 volts/cm^{-1} (40 V) of potential difference across the electrodes. On completion of the run, the gel was transferred to a gel documentation system and documented.

Data analysis: The molecular sizes of scorable RAPD bands were estimated by comparing their mobility to that of a standard molecular size marker using a software system of Syngene. To study the degree of polymorphism existing between the *T. evansi* isolates, the analysis of the DNA fingerprinting pattern was carried out (Gilbert *et al.* 1990). The RAPD data were analyzed statistically to identify potentially informative primers based on average percentage difference (APD).

RESULTS AND DISCUSSION

The results of the RAPD reactions are shown in Fig. 1 (a to d). Depending upon the *T. evansi* isolate-primer combination between 3 and 15 reproducible fingerprints of 179 bp to 4039 bp were amplified suggesting minor and major differences in the RAPD profiles. Of the 17 primers analyzed, 14 were able to direct amplification of polymorphic DNA fragments from the genome of *T. evansi*. The primers AP2, AP6 and AP7 produced a monomorphic fingerprinting pattern. The per cent difference between the different isolates of *T. evansi* is presented in Table 2. The presence or absence of a fragment was taken as criterion for the analysis. The APD values of different isolates ranged from 2.94 to 43.30.

Primer AP1 produced the highest difference between *T. evansi* of buffalo and dog origin (47.36%), followed by dog and horse isolates (41.176%), buffalo and camel isolates

Table 2. Random amplified polymorphic DNA patterns generated in *T. evansi* isolates with random decamers

Primer	Percentage difference (%)						Average percentage difference (APD)
	BC	BD	BE	CD	CE	DE	
AP1	35.00	47.36	40.00	33.33	11.11	41.176	34.66
AP3	31.81	26.92	28.57	21.21	5.26	21.73	22.58
AP4	27.16	30.43	34.78	26.08	30.43	9.09	26.66
AP5	0	0	0	0	0	0	0
AP8	38.88	33.33	41.17	50.00	22.44	26.30	41.38
AP9	66.66	66.66	66.66	0	0	0	38.86
AP10	5.88	5.88	5.88	0	0	0	2.94
AP11	0	0	0	0	0	0	0
AP12	7.69	71.42	60.00	64.28	50.00	5.88	43.21
AP13	45.00	30.00	35.00	11.11	37.50	37.50	32.68
AP14	0	7.14	33.33	14.28	33.33	40.00	21.34
AP15	0	33.33	0	33.33	0	33.33	16.66
AP16	52.94	60.00	45.45	41.17	42.10	18.18	43.30
AP17	11.11	11.11	11.11	11.11	11.11	11.11	11.11
Average	23.01	30.25	28.71	21.85	17.38	17.45	23.96

BC: Percentage difference between *T. evansi* genome of buffalo and camel origin; BD: percentage difference between *T. evansi* genome of buffalo and dog origin; BE: Percentage difference between *T. evansi* genome of buffalo and horse origin; CD: Percentage difference between *T. evansi* genome of camel and dog origin; CE: Percentage difference between *T. evansi* genome of camel and horse origin; DE: Percentage difference between *T. evansi* genome of dog and horse origin.

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1a 1b 1c 1d

Fig 1. 1a to1d depict the DNA polymorphism profiles of *T. evansi* DNA with random primers; AP 1, AP 8, AP 12 and AP 16, respectively. Lane M: Molecular weight marker, 100bp ladder plus (MBI fermentas); Lane B: *T. evansi* DNA from buffalo isolate; Lane C: *T. evansi* DNA from camel isolate; Lane D: *T. evansi* DNA from dog isolate; Lane E: *T. evansi* DNA from horse isolate.

(35%) and camel and dog isolates (33.33%) (Fig.1a), whereas AP8 generated greatest polymorphism between camel and dog isolates of *T. evansi* (50%), followed by buffalo and horse isolates (41.17%), buffalo and camel isolates (38.88%) and buffalo and dog isolates (33.33%) of *T. evansi* (Fig.1b).

AP12 generated the highest polymorphism between buffalo and dog isolates (71.42%) followed by camel and dog isolates (64.28%), buffalo and horse isolates (60%) and camel and horse isolates (50%) (Fig.1c). Interestingly, AP16 generated the greatest polymorphism between buffalo and dog isolates (60%), followed by buffalo and camel isolates (52.94%), buffalo and horse isolates (45.45%), camel and horse isolates (42.10%) and camel and dog isolates (41.17%). The least polymorphism was, however, observed between dog and horse isolates (18.18%) (Fig.1d).

Although sporadic, the recent report of *T. evansi* infections in humans from rural India adds a new dimension to the epidemiology of the disease caused by this organism (Joshi *et al*. 2005). From a taxonomical point of view, *T. evansi* is generally considered as monomorphic but polymorphism may also be seen. There is a need for population differentiation of the parasites at molecular level to correlate with the pathogenicity and the changing epidemiology of the disease in the subcontinent. Among the various molecular techniques, DNA fingerprinting by AP-PCR offers a simple and faster analysis of the genome of *T. evansi*, which can be of potential use in intra-species differentiation of epidemiological relevance.

In the present investigation, primers revealing detectable polymorphisms among *T. evansi* isolates were identified and PCR conditions standardized. Detection of polymorphism is primarily limited by the number of primers tested and therefore, it is often possible to identify more polymorphism than ever needed for a particular study. Therefore, the best strategy for RAPD analysis is to screen as many primers as possible and select only those that give highly reproducible bands for scoring rather than trying to optimize every primer-template combination for detection. Under the given PCR conditions between 3 and 15 scorable and reproducible fragments were amplified depending upon the *T. evansi* template primer combination. On the basis of the percentage difference data between each pair of isolates and the APD value for all the isolate pairs (pairs) for a given arbitrary primer, 1 random primer, AP16 with highest APD value of 43.3% was identified as an informative primer on the genome of *T. evansi*. Although the arbitrary primer AP12 generated data on genetic polymorphism with an APD value of 43.21, the percentage difference discerned in the *T. evansi* genome of buffalo and camel as well as between the dog and equine was substantially low with the percentage difference value of 7.69 and 5.88, respectively. On the other hand, despite of the fact that the APD value for the random primer AP1 was comparatively low (34.66), the percentage difference generated between buffalo- camel, buffalo- dog, and dog-equine, of *T. evansi* was 47.36, 41.17 and 40.00, respectively, and moderate 33.33% difference between camel and dog is appreciable. At the same time the percentage difference value between camel and equine isolates with AP1 primer was as low as 11.11. Earlier workers using different techniques reported minor to major heterogeneity in *T. evansi*. The common techniques used were isoenzyme analysis (Boid 1988), kDNA minicircle sequences (Borst *et al*. 1987), VAT repertoires (Bajyana Songa and Hamers 1987), electrophoretic karyotyping (Waitunbi and Young 1994) and RAPD analysis (Waitumbi *et al*. 1994, Watanapokasin *et al*. 1998). Genetic fingerprinting data analysis suggests a greater microheterogeneity between the isolates from buffalo and dog (30.25%) followed by buffalo and horse (28.71%), buffalo and camel (23.01%), camel and dog (21.85%), dog and horse (17.45%) and camel and horse (17.38%) (Table 2).

A pronounced feature of the RAPD profile was the variation in the intensity of bands. The more intense bands

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were probably due to priming within the repeated regions of the trypanosome genome, which could result in more copies being produced during PCR.

The AP-PCR conditions described in this study confirm discernible DNA polymorphism in *T. evansi* stocks derived from cattle, buffalo, horse and dog. The wide geographical distribution and adaptability of the organism to different hosts may have been responsible for the DNA polymorphism seen in this study. Identification of highly polymorphic RAPD fragments followed by cloning and sequencing can be potentially useful for development of species- specific polymorphic markers. This approach is therefore, more interesting for both epidemiologic and population genetic studies.

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