Restriction site detection in repetitive nuclear DNA sequences of *Trypanosoma evansi* for strain differentiation among different isolates

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Abstract The differences or similarities among different isolates of *Trypanosoma evansi* through endonuclease profile was identified in the present study. The repetitive nuclear DNA of *T. evansi* isolated from infected cattle, buffalo and equine blood was initially amplified by PCR using specific primers. A panel of restriction enzymes, *EcoRI*, *Eco91I*, *HindIII* and *PstI* were for complete digestion of PCR products. Agarose gel electrophoresis of digested product did not show cleavage fragments and only single DNA band of the original size was visible in the ethidium bromide stained agarose gel. This indicated that the 227 bp PCR product from repetitive sequence had no site-specific cleavage sites for the REs used in this study. No heterogeneity in the repetitive nuclear DNA restriction endonuclease profile among the different isolates was recorded.

Keywords Buffalo · Cattle · Equine · PCR amplification · Trypanosomes · Restriction digestion

Introduction

Trypanosoma evansi, a blood protozoan parasite, causes a serious disease known as ‘surra’ in domestic and wild animals. In India, *T. evansi* infection is widely prevalent in different parts and is of significant economic importance in livestock production (Juyal et al. 2007). Surra may occur in acute, sub-acute, chronic and inapparent forms. It is of primary importance for the research scientists, epidemiologists and clinicians to characterize the morphologically identical parasites responsible for different diseases. (Godfrey 1978). The objective of this study was to find out whether trypanosome associated diseases in animals are

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caused by single form of *T. evansi* or by subpopulation of various isolates.

A new method of characterization, restriction endonuclease digestion of mitochondrial DNA has differentiated species and strains of trypanosome (Borst et al. 1981; Frascch et al. 1981) and leishmania (Lopes et al. 1984). The present study was undertaken to determine the similarities or differences among isolates of *T. evansi* from different mammalian species of various regions of Haryana by restriction endonuclease profile.

Materials and methods

Collection of blood and serum samples from domestic animals

T. evansi was isolated from infected blood samples from cattle (n = 58), buffaloes (n = 35), and equines (n = 31) from different places of Haryana state. Infected samples were confirmed by wet blood film. The positive sample for *T. evansi* parasites was inoculated (@0.2 ml) into rats intraperitoneally for bulk harvests of parasites for extraction of DNA.

Extraction of genomic DNA and its amplification

The genomic DNA was extracted from *T. evansi* positive rat blood samples according to the method modified from Sambrook and Russell (2001). Briefly, 5 ml of *T. evansi*-positive rat blood was centrifuged at 1,500 rpm for 10 min to separate plasma from the blood cells. To 3.0 ml of pelleted erythrocytes, 30 ml of lysis buffer was added, mixed well and incubated for 1 h at 37 °C. Proteinase K was then added to a final conc. of 100 µg/ml and mixed by inverting the tube. The tube was incubated at 50 °C for 3 h with intermittent shaking. Equal volume of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1) was added and mixed by gentle rocking for 3 min. The tube was centrifuged at 3,000×g for 10 min at room temperature (RT) and upper aqueous phase collected in a clean centrifuge tube. The aqueous phase was once more treated with equal volume of PCI and then once with equal volume of chloroform: isoamyl alcohol (24:1). Upper aqueous phase was finally collected after centrifugation at 3,000×g for 10 min at RT. One-tenth volume of 3 M sodium acetate, pH 5.2 was added to the aqueous phase and DNA was precipitated by adding equal volume of ice-cold isopropanol and incubating the mixture at 4 °C overnight. The DNA pellet was obtained by centrifugation at 4,000×g for 15 min at RT and the pellet was washed once in 70 % ethanol, and air-dried. The dry pellet was suspended in 250 µl 10 mM tris-HCl pH 8.5, kept at RT for 30 min for dissolving DNA

Fig. 1 *T. evansi*-specific PCR using DNA extracted from infected blood. [Lane M 100 bp DNA ladder, Lane 1 5 µl *T. evansi* DNA from infected blood, Lane 2 10 µl *T. evansi* DNA from infected blood]

and then stored at −70 °C until further use. PCR (TE-PCR) was standardized for detection of *T. evansi* in infected blood samples. Primers (5'TGCAGACGACCTGACGCTA CT3' Forward and 5'CTCCTAGAAGCTTCGGTGTC CT3'-Reverse) specific to *T. evansi* repetitive DNA sequence probe pMUTec 6.258 as described by Wuyts et al. (1994). TE-PCR was carried out in 50 µl reaction mixtures containing 10× PCR buffer with KCl (Fermentas), 1.5 mM MgCl₂, 200 µM each dNTP (Fermentas), primers each at 20 pM, 2 U of thermostable Taq DNA polymerase (Fermentas) and 10 µl template DNA. The cycles included an initial step at 95 °C for 4 min followed by 29 cycles of denaturing at 95 °C for 1 min, primer annealing at 60 °C for 1 min and primer extension at 72 °C for 1 min. This is followed by last cycle of denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 10 min and hold at 4 °C for indefinite time. Amplification products were resolved in 2 % agarose (Biogene) stained with ethidium bromide (5 mg/ml).

Restriction endonuclease analysis of TE-PCR products

The purified TE-PCR products were subjected to restriction endonuclease (RE) analysis using a panel of four restriction enzymes, viz. *EcoRI*, *Eco9II*, *HindIII* and *PstI*, for complete digestion with the recommended RE buffers in separate PCR tubes in the following reaction volume: 7 µl nuclease-free water, 10 µl DNA, 2 µl 10× RE buffer, 1 µl (10 U) *EcoRI* (New England Biolabs)/1 µl (10 U) *Eco9II*

Fig. 2 RE digestion of TE-PCR product with *EcoRI*, *PstI*, *Eco91I* and *HindIII*. [Lane M 100 bp DNA ladder; Lane 1 TE-PCR product with out RE; Lane 2 TE-PCR product with *EcoRI*; Lane 3 TE-PCR product with *PstI*; Lane 5 TE-PCR product with out RE; Lane 6 TE-PCR product with *Eco91I*; Lane 7 TE-PCR product with *HindIII*]

(Fermentas)/1 μ l (10 U) *HindIII* (Fermentas)/1 μ l (10 U) *PstI* (Fermentas). The components were mixed properly and incubated at 37 °C for 3 h. RE was inactivated at 65 °C for 20 min, cooled to 4 °C and analysed by agarose gel electrophoresis.

Results and discussion

TE-PCR was first standardized using parasite template DNA isolated from infected blood (Fig. 1). Distinct PCR product of approximately 227 bp size was obtained. Digestion of TE-PCR products with *EcoRI*, *Eco91I*, *HindIII*, *PstI* and resolution in agarose gel, did not show cleavage fragments and only single DNA band of the original size was visible in the ethidium bromide stained agarose gel. This indicated that the 227 bp PCR product from repetitive sequence had no site-specific cleavage sites for the REs used in this study (Fig. 2). The different isolates of *T. evansi* from different regions and different species of animal were found to belong to single form with enzymes used in the study.

Trypanosoma spp. contain both genomic or nuclear DNA and kinetoplastic (kDNA) or mitochondrial DNA. The kDNA is a network of thousands of interlocked DNA minicircles and about 50 maxicircles. However, Borst et al. (1987) reported the absence of detectable maxicircle and homogeneity in the sequence of minicircles in different

strains or isolates of *T. evansi*. The homogeneity in the sequence of minicircles DNA of kinetoplasts in 6 different isolates of *T. evansi* originating from bovine, buffalo, mule, horse and camel was reported by Ou et al. (1991). However, Gibson et al. (1985) detected minor difference in the minicircle kDNA in 5 isolates of *T. evansi* originating from Africa and South America. Songa et al. (1990) studied the nuclear DNA restriction site polymorphism of 9 stocks of *T. evansi* and opinioned the DNA sequences were homogenous and conserved. The restriction endonucleases banding patterns of the repetitive nuclear DNA from the different isolates of *T. evansi* were studied with a view to identify an isolate specific restriction site. Similarly, Tungapradabkul et al. (1983) found no characteristic banding pattern of repetitive sequence of DNA fragment in the digested samples in any of the isolates of *T. evansi* excepting the samples digested with either *DraI* or *AluI*. Highly abundant repetitive DNA sequences of nuclear DNA have been described for several *Trypanosoma* species. Gonzales et al. (1984) identified repetitive sequence of 195 bp size in *T. cruzi*. A repeat element of 550 bp of unknown chromosomal location was found in *T. simiae* DNA (Majiwa and Webster 1987). Moser et al. (1989) reported the presence of a DNA fragment of 369 bp in genomic DNA of *T. congolense* digested with *DraI*. However, no heterogeneity in the nuclear repetitive DNA extracted from any of the isolates of *T. evansi* could be detected upon restriction enzyme digestion. In fact, isolates with identical banding pattern may not be identical but may differ in a manner not detected in the present study.

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