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IDENTIFICATION OF *TRYPANOSOMA EVANSI* BY DNA HYBRIDISATION USING A NON-RADIOACTIVE PROBE GENERATED BY ARBITRARY PRIMER PCR: SHORT COMMUNICATION

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A highly reproducible, dominant, monomorphic fragment of 473 base pair (bp) amplified from the genome of *Trypanosoma evansi* by arbitrary primer - polymerase chain reaction (AP-PCR) was labelled with digoxigenin and investigated for its potential as DNA probe. Dot-blot hybridisation of total genomic DNA with the probe proved useful in detecting bubaline, cameline and equine strains of *T. evansi* down to 10 pg of parasite template DNA. No cross-hybridisation was seen with *Babesia bigemina, Theileria annulata* and the bubaline host DNA. This probe may facilitate laboratory identification of *T. evansi* in developing countries, without the inherent risk associated with radioisotopes.

Key words: AP-PCR, *Trypanosoma evansi*, non-isotopic probe

Molecular techniques such as polymerase chain reaction (PCR), nucleic acid hybridisation and kDNA minicircle analysis are increasingly receiving attention for identification and characterisation of *Trypanosoma evansi* stocks (Masiga and Gibson, 1990; Waitumbi and Young, 1994; Zhang and Baltz, 1994; Wuyts et al., 1994, 1995; Paulo et al., 1998; Omanwar et al., 1999). Gene probes thus far described are based on identification of highly repetitive genomic sequences used as ^{32}P-labelled probes. However, the use of radioactivity makes the methodology impractical for field use. The non-isotopic probes have great potential for use in developing countries as they are stable, not restricted by a short half-life and hazards associated with the radioactive probes.

The arbitrary primer - polymerase chain reaction (AP-PCR) technique has proved to be a powerful and rapid method for detecting polymorphic genetic markers in a number of organisms. Such markers can be used in phylogenetic studies, identification of parasite strains and species, and in comparison of field isolates. It requires little parasite material and is technically easy to perform. A

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large number of anonymous markers can be readily obtained representing independent loci, which are not biased towards particular sequences and so representative of the genome (Carlton et al., 1995). In the present communication, we describe the molecular detection and identification of *T. evansi* using an AP-PCR derived monomorphic DNA marker as a probe.

Isolates of *T. evansi* of bubaline, cameline and equine origin were passaged in laboratory rodents giving short-term fulminating infections. Trypanosomes were then purified from the rodents' blood by diethylaminoethyl (DEAE) -cellulose chromatography using phosphate saline glucose, pH 8.0 as elution buffer (Lanham and Godfrey, 1970). Total genomic DNA was extracted from the trypanosomes, as described by Sambrook et al. (1989).

A 473 bp monomorphic DNA fragment previously identified as strong reaction product by the primer AP_{12} (5'-TGCATCGTAC-3') in AP-PCR (Basagoudanavar et al., 1999) was isolated from the agarose gel using Genei Clean kit (Bangalore Genei, India). The DNA fragment was reamplified using the same amplification conditions described previously for AP-PCR (Basagoudanavar et al., 1999).

DNA labelling of the 473 bp reamplified fragment was carried out by incorporation of digoxigenin-11-dUTP during PCR using DIG-DNA labelling by PCR and detection kit (Boehringer Mannheim, Germany) according to manufacturer's recommendations. The PCR reaction was conducted in 50 µl volume containing 15 ng DNA, 10 mM Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, PCR-DIG probe synthesis mix (200 µM each of dNTPs replacing the dTTP at 30% level with digoxigenin (DIG-dUTP), 15 pmol primer AP_{12} and 1.5 U *Taq* DNA polymerase. The amplification reaction was carried out in a Thermal Cycler (MJ Research, Watertown, MA, USA). Cycling conditions were the same as described previously (Basagoudanavar et al., 1999). The success of the labelling was confirmed by comparing the electrophoretic mobility of 3 µl of labelled product in relation to unlabelled product on 1.4% agarose.

For blotting, the template DNA at various concentrations (1 pg to 25 ng) in 100 µl of 0.1 mM Tris-EDTA (pH 8.0) was boiled for 5 min. To each dilution, 5 µl of 10 N NaOH was added and incubated at room temperature for 10 min. Then an equal volume (105 µl) of 2 M ammonium acetate was added and applied on to the Zeta probe GT (BioRad) membrane using minifold dot-blotting apparatus (BioRad). Similarly, for specificity assay, equal concentration (25 ng each) of genomic DNA of *Trypanosoma evansi* (bubaline strain)*, Babesia bigemina, Theileria annulata* and bubaline host were blotted. The blots were dried and soaked in 2 × SSC, and air dried before subjecting to UV cross-linking (120 $mJcm^{-2}$ energy) for 5 min, using an UV Cross linker (UVP, Upland, CA, USA).

After prehybridisation (68 °C, 3 h), hybridisation was carried out at 68 °C overnight, in a hybridisation oven (Techne, Cambridge, UK) containing hybridi-

sation buffer that included 25 µl of freshly denatured DIG-labelled probe. Afterwards, the membranes were washed with 2 × SSC, 0.5% (w/v) SDS at room temperature for 10 min, followed by two more washing steps at 68 °C for 15 min each in 0.1% SSC, 0.1% (w/v) SDS under constant gentle agitation. The hybridised probe was immunodetected with anti-digoxigenin-AP conjugate (1:1000) in 1% blocking solution and visualised with the colorimetric substrate (NBT/X-phosphate in DMF).

Agarose gel electrophoresis of the reamplified AP-PCR product appeared as a single band of predicted 473 bp size (Fig. 1A). This reamplified DNA fragment when used as DIG-labelled probe in a dot-blot hybridisation, showed a specific signal with *T. evansi* and no detectable signal against *Babesia bigemina, Theileria annulata* and bubaline host DNA (Fig. 1B). When purified DNA from *T. evansi* (buffalo isolate) was blotted in varying concentrations of 1 pg, 10 pg, 100 pg, 1 ng, 10 ng and 25 ng, signals of hybridisation were detected down to 10 pg level. However, no detectable signal was seen with negative control (salmon sperm DNA) (Fig. 1C). The DNA templates of *T. evansi* strains originating from camel and horse also revealed similar sensitivity detection levels (data not shown).

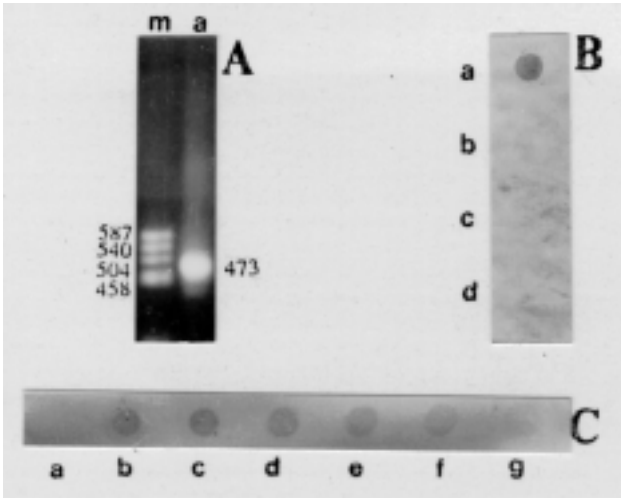

Fig. 1. **A**. Ethidium bromide stained agarose (1.4%) gel showing reamplified AP-PCR product of *T. evansi* using primer AP_{12} (5'-TGCATCGTAC-3'). Lane a – reamplified product (473 bp fragment); m – DNA molecular weight marker pBR322 DNA/*Hae*III digest. **B**. Specificity assay of AP-PCR generated 473 bp DIG-labelled probe of *T. evansi* by dot-blot hybridisation: a to d indicate genomic DNA of *T. evansi* (a), *B. bigemina* (b), *T. annulata* (c), bubaline host DNA (d). **C**. Sensitivity assay: a to g indicate the concentration of DNA: a – negative control; b – 25 ng; c – 10 ng; d – 1 ng; e – 100 pg; f – 10 pg; g – 1 pg

The sensitivity of the nucleic acid based probes thus far reported ranged from 62 to 100 pg of template DNA by Southern or dot-blot hybridisation (Masiga and Gibson, 1990; Viseshakul and Panyim, 1990; Zhang and Baltz, 1994). The development of DNA probes based on identification of repetitive elements, unique genes or spacer DNA sequences and cloning and/or *de novo* synthesis of the oligonucleotide sequences is time consuming. The advent of PCR for generation of RAPD using randomly chosen single oligonucleotide primers has simplified the identification of genetic markers for a number of parasites without the requirement of prior knowledge of their genomic nucleotide information (MacPherson and Gajadhar, 1994; Reddy et al., 1998). In the present study, a highly dominant DNA fragment common to three predominant buffalo, camel and horse isolates of *T. evansi* was used for the preparation of non-isotopic probe. Considering that *T. evansi* is a multi-host parasitising species, identification of a highly dominant DNA fragment common in isolates from various animal hosts is believed to lead to a possibility of application of this technique for sensitive identification of the organism.

Although the PCR assay earlier described (Omanwar et al., 1999) is more sensitive than the probe, the requirement of specialised and expensive equipment may be a limitation to have universal utility of the technique. The convenience of performing the hybridisation assay using the probe in a water bath at 68 °C as a substitute for hybridisation oven, while other steps are carried out at room temperature, makes this probing technique less expensive and relatively simple, allowing the unequivocal detection of *Trypanosoma* DNA. Yet another advantage of this non-isotopic probe is that it can be repeatedly used with proper storage and thus may be ideally suited for use in developing countries.

When used as hybridisation probe, the digoxigenin-labelled 473 bp fragment was found to be of higher sensitivity than that of ^{32}P-labelled probes earlier described using repetitive DNA sequences (Masiga and Gibson, 1990; Viseshakul and Panyim, 1990; Zhang and Baltz, 1994). As it gives no detectable cross-hybridisation signal with *B. bigemina* and *T. annulata,* the two common haemoparasites and bubaline host DNA, this non-isotopic probe may facilitate laboratory identification of *T. evansi* in non-tsetse regions like India, without any of the inherent risks associated with radioisotopes.

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