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DIRECT AND SENSITIVE DETECTION OF *TRYPANOSOMA EVANSI* BY POLYMERASE CHAIN REACTION

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The mechanically transmitted haemoflagellate, *Trypanosoma evansi* causes 'surra', a wasting disease of domestic animals and is highly endemic in distribution in Southeast Asia. The detection of *T. evansi* is important for improving the epizootiological and animal health status of the region. The specificity and sensitivity of polymerase chain reaction (PCR) using oligonucleotide primers constructed from *T. evansi* repetitive DNA sequences were studied in the present investigation. Using the assay, it was possible to amplify template DNA of *T. evansi* derived from buffaloes, camels and horses to a threshold sensitivity level of 0.5 pg and to detect DNA from as few as five organisms in 10 µl crude blood samples. Following experimental infection of calves with 5×10^5 *T. evansi*, positive signals could be observed as early as 12 h post-infection. DNAs from two common haemoflagellates of cattle, *Babesia bigemina* and *Theileria annulata* were not amplified with the primers.

Key words: *Trypanosoma evansi*, polymerase chain reaction, diagnosis

Trypanosomosis caused by *Trypanosoma evansi* is a disease of economic importance in domestic livestock. *T. evansi* has the widest geographical distribution of all the pathogenic trypanosome species outside the tsetse belt in Africa, the Middle East, Asia, Central and South America (Losos, 1980; Mahmoud and Gray, 1980). The importance of *T. evansi* as the aetiological agent of 'surra' (a wasting disease) is often overlooked, presumably because of the enzootic course of the disease, making accurate diagnosis difficult. Serious epidemics of the disease were common in the early years of the century, particularly in India, Indonesia, Mauritius and the Philippines. In Africa and India, trypanosomosis caused by *T. evansi* is the single most important disease of camels. Their most significant impact possibly comes from the chronic form, where abortion, infertility, reduced milk yield and weight gain, and lower work output are the common sequelae of the infection. The clinical signs of trypanosomosis are highly

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variable and the ecological conditions under which the disease occurs are so diverse that in terms of identifying carrier animals, clinical diagnosis is a too imprecise procedure for use in epidemiology. The mouse inoculation method is impracticable for large-scale epidemiological surveillance, and the immunological methods based on antibody and antigen detection do not have absolute predictive value. Furthermore, *T. evansi* consists of a large number of morphologically identical populations that differ significantly in various biological characteristics such as host range, virulence, pathogenicity, and drug sensitivity. A wide range of DNA-based techniques have been described for trypanosome detection, including hybridisation using repetitive DNA sequences, random amplification of polymorphic DNA (RAPD), polymerase chain reaction (PCR) and kinetoplast DNA (kDNA) minicircle analysis, as reviewed by Boid et al. (1996). However, there are differences in the sensitivity claimed for these assays in the literature. Wuyts et al. (1994) described a PCR using a set of oligonucleotide primers constructed from a repetitive sequence probe pMUtec 6.258. The results of laboratory evaluation of the PCR assay against Indian isolates of *T. evansi* are described here.

Materials and methods

Source of parasite DNA

Three *T. evansi* isolates of bubaline, equine and cameline origin isolated from the Indian states of Uttar Pradesh, Haryana and Rajasthan, respectively, were passaged in laboratory rodents (Lumsden et al., 1973). The trypanosome populations tested were derived from single cloned parasites prepared by the method of Rosen et al. (1981). Each clone causes fatal parasitaemia. Trypanosomes were purified from the blood by DEAE-cellulose chromatography (Lanham and Godfrey, 1970).

Isolation of T. evansi *DNA*

Total genomic DNA was extracted from the trypanosomes by proteinase K digestion at 48 °C for 3 h in 5 ml extraction buffer [0.1 M Tris (pH 8.0), 0.1 M NaCl and 5 mM EDTA, 10% SDS and 100 μg ml^{-1} proteinase K]. The resulting viscous solution was extracted three times with equal volumes of phenol, once with chloroform:isoamyl alcohol (24:1), then incubated with 100 μg ml^{-1} of RNase for 1 h. The solution was once more extracted with phenol:chloroform: isoamyl alcohol (24:24:1) and the DNA was precipitated at –20 °C with chilled ethanol and resuspended in an appropriate volume of sterile distilled water. The purity of the DNA was estimated by UV spectroscopy.

Sample collection and experimental infections

Blood samples needed for the PCR were collected in volumes of 10 µl from laboratory-infected mice and calves, following experimental infection with 1×10^4 and 5×10^5 *T. evansi,* respectively. The blood samples collected from mice by venipuncture using a glass capillary tube were directly transferred to a microfuge tube and left to clot. From bovine calves, aged 3 months, blood samples were collected directly from the jugular vein and then transferred to a microfuge tube using either a calibrated capillary tube or an air displacement micropipette. Each sample was then boiled under a mineral oil overlay for 20 min. Blood samples were collected from mice at 12, 24, 48 and 72 h after experimental infection and from calves at 6, 12, 24, 36 and 48 h post-infection to test the course of *T. evansi* infection.

DNA amplification by PCR

A set of *T. evansi* oligonucleotide primers constructed from the repetitive probe pMUtec 6.258 (Wuyts et al., 1994) was used in the present study: a 21-mer sense primer (5'-TGCAGACGACCTGACGCTACT-3') and a 22-mer antisense primer (5'-CTCCTAGAAGCTTCGGTGTCCT-3') synthesised by Bangalore Genei, Bangalore. The conditions were optimised for the PCR assay using 0.125 µM of each primer in a 50 µl reaction mixture containing 200 µM each of dATP, dTTP, dCTP and dGTP, 5 µl reaction mixture buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin), 1.5 units of Taq DNA polymerase and 1 ng of DNA or 10 µl of boiled blood sample. Blood samples were first boiled for 20 min with mineral oil overlay. DNA amplifications were performed using a DNA thermal cycler PTC-200 (MJ Research Inc., USA). The sample was preincubated at 90 °C for 7 min to completely denature the DNA. This was followed by 30 cycles, each with 30 sec at 90 °C (to denature), 30 sec at 60 °C (to anneal), and 30 sec at 72 °C, finally with one extensive polymerisation at 72 °C for 7 min. The tubes were then kept at 4 °C till used for analysis.

For the sensitivity analysis, template DNA concentrations of 10 ng, 5 ng, 1 ng and 0.5 ng per reaction were tested, as described previously (Omanwar, 1998). Sensitivity experiments were further conducted with DEAE-cellulose purified trypanosomes in pool sizes of 25, 10 and 5 parasites directly added to master mix solution in microfuge tubes for DNA amplification. For seeding of the purified parasites, a limiting dilution technique was used.

The specificity of DNA amplification was tested using control PCR assays with blood parasites (*Babesia bigemina* and *Theileria annulata*) and normal bovine blood sample free of these parasites.

For assaying sample stability, unprocessed blood samples collected from experimentally infected mice and calves were stored at room temperature for 15 and 30 days.

A drug sensitivity experiment was designed to assess the use of PCR in monitoring the drug-treated *T. evansi* infections. Swiss albino mice were inoculated with 0.5×10^5 *T. evansi* intraperitoneally. After microscopic confirmation of parasitaemia (48 h post-inoculation), all the mice were administered a potent anti-trypanosomal drug, Triquin® (Wockhardt, Mumbai; quinapyramine prosalt) subcutaneously, 2 mg kg^{-1} body weight. Blood samples collected from mice at 0 h prior to treatment and then at 3, 6, 12 and 24 h post-treatment were similarly processed for PCR assay. Ten µl of the PCR product were analysed by agarose (1.5%) gel electrophoresis. The gels were stained with ethidium bromide and the PCR products were visualised on UV transilluminator.

Results

Sensitivity of DNA amplification

Sensitivity studies were first carried out with the purified *T. evansi* template DNA of three isolates of bovine, equine and cameline origin. A 227 bp fragment could be detected in reaction containing as little as 0.5 ng of parasite template DNA (Fig. 1a). Further studies were then carried out with the bovine isolate of *T. evansi* for evaluating the sensitivity, specificity, stability, and drug sensitivity. When PCR was performed with DEAE-cellulose purified trypanosomes of predetermined pool size, the threshold sensitivity could be increased to the order of 5 parasites per 10 µl sample (Fig. 1b). The presence of phosphate-saline-glucose (PSG) buffer (pH 8.0) in which the trypanosomes were eluted during anion-exchange chromatography seems to inhibit the signal (Fig. 1b). Ten µl of PSG was sufficient in a 50 µl reaction to inhibit PCR, while 5 µl did not completely inhibit PCR, although its presence could influence the intensity of the signal. However, the inhibitory effect of PSG could be eliminated by diluting the PSG or by washing the parasites with sterile distilled water.

Experiments designed for monitoring *T. evansi* infection in blood samples of mice at designated time intervals following experimental infection with 1×10^4 parasites revealed a positive signal as early as 12 h post-infection (Fig. 2a). A progressive increase in the sensitivity of PCR signal was apparent with the maximum intensity at 72 h post-infection.

Following experimental infection of calves with 5×10^5 *T. evansi*, positive PCR signals were gained 12 h post-infection from whole blood processed by the boiling method (Fig. 2b), while microscopically the organisms could be detected only 96 h post-infection.

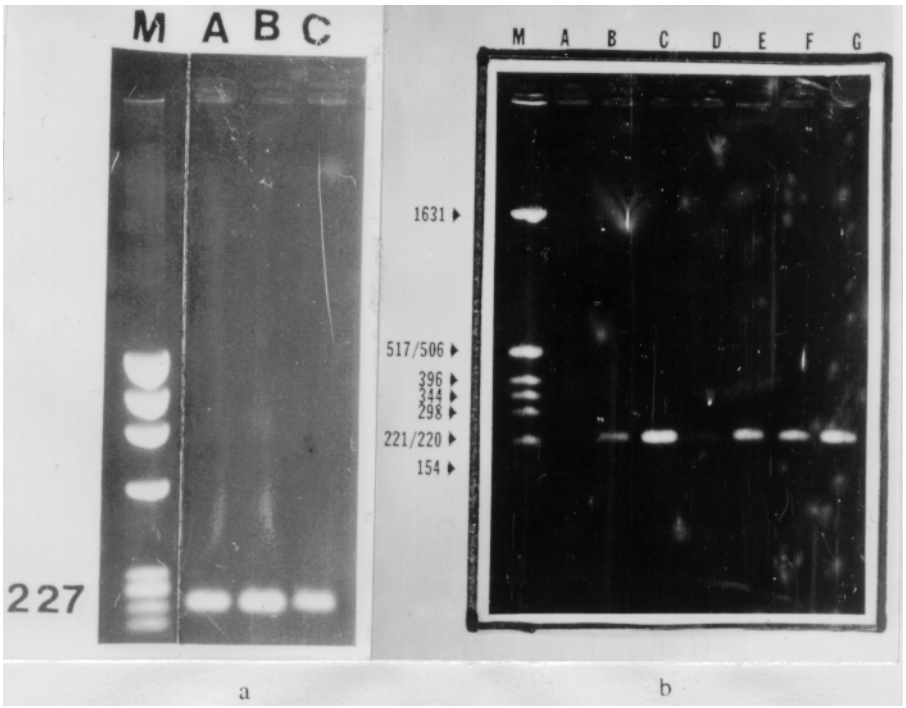

Fig. 1. Ethidium bromide stained agarose gel (1.5%) showing PCR products (227 bp fragment) (a) 0.5 ng purified *T. evansi* DNA. Lanes A, B, and C represent the equine, buffalo, and camel isolates, respectively; lane M shows molecular size marker ΦX174 RF/*Hae*III; (b) Lane A – negative control blood sample processed by boiling; lane B – *T. evansi*, 0.5 ng of purified DNA; lanes C and D – DEAE-cellulose purified *T. evansi*, 5 parasites per 10 µl without PSG and with 10 µl of PSG, respectively; lanes E, F and G – crude blood samples of mice infected with *T. evansi*; sample processed by boiling; lane M – molecular size marker pBR322/*Hinf* I digest

Sample stability

The stability of unprocessed blood samples stored at room temperature (28 °C) was examined at 15 and 30 days after sample collection. Positive PCR signals could be obtained in samples for up to 15 days after collection.

Specificity of the detection method

In order to use confidently the PCR amplification of *T. evansi* DNA from infected bovine blood, specificity tests were carried out with blood samples containing *Babesia bigemina* and *Theileria annulata*, the common haemotropic parasites of bovids, along with normal bovine blood. The amplified product was parasite specific as demonstrated by the lack of DNA amplification in reactions containing *B. bigemina*, *T. annulata* and normal bovine blood.

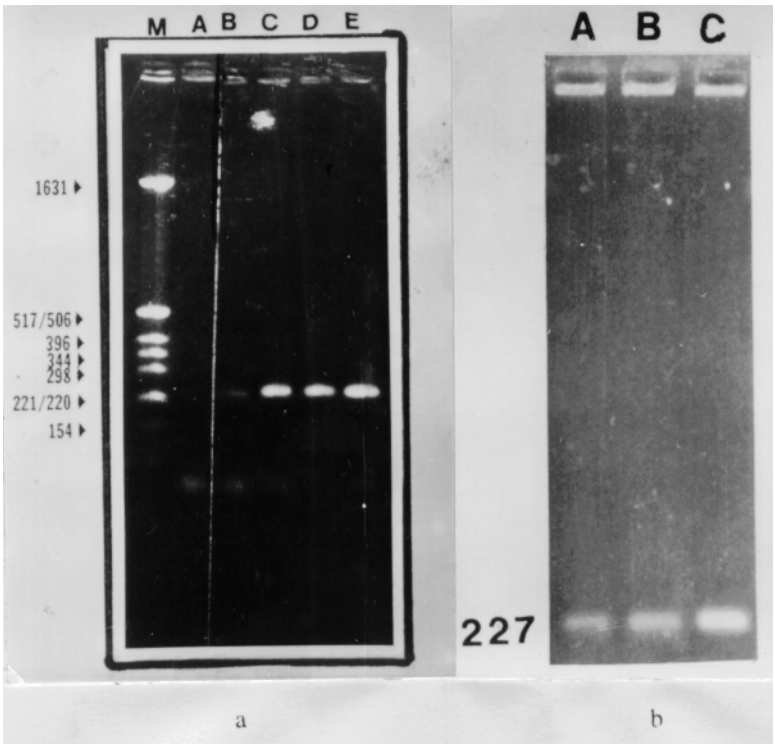

Fig. 2. Agarose gel (1.5%) electrophoresis showing PCR product of whole blood processed by the boiling method. Infection experiment (a) mice: blood taken at 12–24 h interval. Lane A – negative control. Lanes B, C, D and E represent samples assayed at 12, 24, 48 and 72 h post-infection. Lane M – pBR322/*Hinf* I; (b) calves: blood taken at 6 h intervals. Lanes A, B and C represent samples collected at 12, 18 and 24 h post-infection with 5×10^5 *T. evansi*

Drug sensitivity monitoring

Mice experimentally infected with *T. evansi* and treated with Triquin showed parasite clearance from the peripheral blood 6 h after drug administration. No parasite could be detected 3 h after drug administration by microscopy, while PCR revealed the presence of *T. evansi* up to 6 h after drug administration. The PCR signals disappeared totally from 6 to 24 h after drug administration.

Discussion

Diagnosis of cattle with patent infections of *T. evansi* is relatively simple on the basis of microscopic detection of parasites in the peripheral blood, although the clinical manifestations of the disease are not reliable. Accurate diag-

nosis of subclinically infected cattle is, however, more difficult since conventional microscopic and serological techniques lack the reliability and sensitivity in a diagnostic test (Ambrosio and De Waal, 1990).

The use of specific DNA probes and nucleic acid hybridisation to detect *T. evansi* directly in blood from carrier cattle has several advantages over conventional microscopic, serological and animal sub-inoculation techniques. Although radioactively labelled probes derived from cloned segments of *T. evansi* genomic DNA have been reported to be sensitive (Masiga and Gibson, 1990; Visheshakul and Panyim, 1990; Zhang and Baltz, 1994), radioactive probes have the disadvantages of requiring frequent labelling, trained personnel and appropriate facilities. A highly sensitive non-radioactive DNA based hybridisation test would eliminate such shortcomings. However, no such non-isotopic DNA probe with the desired analytical sensitivity is currently available for *T. evansi*. Since the advent of PCR (Saiki et al., 1988), the number of reports on the application of this technique for the diagnosis of infectious diseases has been steadily increasing. Wuyts et al. (1994) reported DNA amplification of *T. evansi* by PCR, and McLaughlin et al. (1996) described a broad-spectrum PCR approach based on small subunit ribosomal RNA gene amplification of trypanosomes from blood. These authors expressed optimism that, in the future, laboratories with ready access to fresh clinical materials and with training in the use of PCR will help to define optimal PCR-based diagnosis and typing assays for parasites. As no single protocol will be appropriate to all situations, each such PCR application requires optimisation and validation, as attempted in the present study.

The PCR-based test reported here has good analytical sensitivity with less cumbersome sample preparation to detect parasite DNA directly in blood. The specificity of PCR in the diagnosis of *T. evansi* infection, however, requires a comment. In the Indian subcontinent, any blood-based PCR assay should be able to differentiate the three common haemoprotozoan parasites of cattle, namely *Theileria annulata, Babesia bigemina* and *Trypanosoma evansi*, while *Trypanosoma theileri*, because of its occult nature, is not of much significance. The PCR system therefore, provides a means to differentiate *T. evansi* from *T. annulata* and *Babesia bigemina* in infected cattle blood and combined with its sensitivity the assay may be of immense use in the field monitoring of surra cases. Sample processing is simple and requires only PCR tubes and glass capillary tubes. The samples can be stored at room temperature for 15 days without loss of parasite DNA stability before processing for the test. The test protocol is a modification of the previously described methods of Tirasophon et al. (1991) and Panyim et al. (1992). Boiling the sample of whole blood was necessary to lyse the parasites and to free the DNA from the blood clot. Porphyrin compounds derived from haem may be the most inhibitory substances to the PCR while working with unprocessed blood. Boiling causes denaturation of haemoglobin, thereby eliminat-

ing inhibitory factors otherwise encountered in unprocessed blood samples. The boiling procedure obviates the need for anticoagulants, which are potential inhibitors of PCR. The thirty thermal cycles protocol yielded reproducible results and the time taken for the test was less than 4 h.

The sensitivity of the test using a sample spiked with parasites (5) was 120 times greater than that of DNA hybridisation (Visheshakul and Panyim, 1990). Given that a parasite contains 0.1 pg DNA (Borst et al., 1982), and 5 *T. evansi* per 10 µl was the smallest number of parasites detectable, this would be equivalent to 0.5 pg of parasite DNA. These observations differ from the findings of Wuyts et al. (1994), who reported a sensitivity of detection to a level of one parasite per 10 µl blood sample (equivalent to 0.1 pg DNA).

The present study confirms the view of Wuyts et al. (1994) on the possible inhibitory effects of PSG buffer of DEAE-cellulose chromatography used for the purification of trypanosomes in the PCR assay. Considering that processing of the test blood sample for PCR precludes the use of PSG or any other buffer, the test procedure is free from such variables.

Most of the trypanocidal drugs currently available have been in use for many years and there are widespread reports of reduced sensitivity of trypanosomes to these drugs in the field. One of the major limitations on studies into trypanosome drug resistance has been the lack of methods for assaying sensitivity to different trypanocidal drugs. The sensitivity of the PCR assay will be valuable for testing the drug sensitivity of *T. evansi* under field conditions when used effectively with other *in vitro* drug assay systems.

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