A SIMPLIFIED AND HIGHLY SENSITIVE DETECTION OF *TRYPANOSOMA EVANSI* BY DNA AMPLIFICATION

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Abstract. In Southeast Asia *Trypanosoma evansi* infection is a disease of economic importance since it affects the health of buffalo, cattle and swine. The acute stage symptoms include abortion, central nervous system disorder and even death, and in the chronic condition working capacity and productivity of the animals are affected. A polymerase chain reaction (PCR)-based detection technique has been developed with a sensitivity of 0.5 pg of parasite DNA or one single parasite in 10 µl of blood samples which were allowed to clot and then boiled before DNA amplification. This permitted storage of blood collection at ambient temperature for at least one month. Phosphate-saline-glucose solution, normally used as trypanosome maintenance buffer, inhibited PCR. Although DNA primers used were derived from *T. evansi* specific sequence, amplification of the genome of *T. brucei* and *T. equiperdum* generated the same 227 bp fragment. This method should now make it possible to detect infections in livestock in the very early stages where microscope examination is equivocal and to monitor groups of animals after trypanocidal treatment.

INTRODUCTION

Trypanosoma evansi has established itself in Southeast Asia for close to a century (Luckins, 1988). In Thailand this hemoflagellated parasite has repeatedly been reported to be a burden of economic importance, affecting productivity and health of buffalo, cattle and swine (Lohr, 1986; Sirivan, 1986). The importance of *T. evansi* as the etiological agent for 'surra' is often overlooked, presumably due to the more enzootic course of the disease, making accurate diagnosis increasingly difficult. Every year however fresh outbreaks are reported from the field. The acute stage symptoms can include abortions, central nervous symptoms and death (Lun *et al,* 1993). Chemotherapy still seems to be the only effective way of dealing with *'surra'* and the choice of drugs is very limited; in practice diminazene aceturate (Berenil®) and suramin are administered to infected animals. With resistance reported to both drugs (Boid *et al,* 1989; Zhang *et al,* 1992), close monitoring of livestock after treatment would provide useful new information on drug-resistant strains and also help to identify trypanotolerant animals. Using DNA-amplification technology we have detected *T. evansi* directly from crude blood. Although Masiga and Gibson, 1992 have used the polymerase chain reaction (PCR) for trypanosome detection, this is the first report on the application of PCR for identification of *T. evansi* in unprocessed blood samples.

MATERIALS AND METHODS

Experimental infections

Male mice were injected with strains of *T. evansi* (Npl, Ps, Ph101, Cm2772) originating from different regions of Thailand. Also the following cloned stains were used: *T. evansi* AnTat 3/1, *T. brucei* AnTat 1/1, *T. equiperdum* BoTat 1/1. Blood was collected either by heart puncture or from the tail vein. Parasitemia was measured as described by Herbert and Lumsden (1976).

Sample collection

Ten µl of whole blood were collected by vein puncture using a plain capillary tube, transferred to a microfuge tube and left to clot. Each sample was then boiled under a mineral oil overlayer for 20 minutes.

Stability experiments

Samples were stored both at 4°C and at 37°C for up to 14 days before boiling and DNA amplification. Samples were also boiled immediately and stored up to one month at room temperature before analysis.

Parasite purification

Trypanosomes were purified from blood cells by DE-52 anion-exchange column chromatogra-

phy using phosphate-saline-glucose (PSG) solution as eluting buffer (Lanham and Godfrey, 1970). *T. evansi* DNA was extracted as described previously by Viseshakul and Panyim (1990). DNA concentrations were calculated from optical density at 260 nm.

Sensitivity experiments

Individual parasites were obtained both by dilution and by picking out parasites using 'hanging micro-drop' microscopy. Dilutions were made in either PSG buffer or in normal rat serum. Individual 'hand-picked' parasites were added to a mixture of normal serum and whole plain blood and subsequently amplified after boiling.

Drug-monitoring experiments

A yearling calf was infected with *T. evansi* strain CM 2772, and blood was taken daily for PCR, microscopy, Card Agglutination (CATT) and mouse inoculation tests. CATT test was performed on serum as described by Bajyana-Songa *et al* (1987). After 15 days, the cow was treated with Berenil® (3.5 mg/kg) and blood samples were taken 3, 6, 12 and 24 hours after treatment. Berenil was kindly provided by the National Institute for Animal Health and Production, Ministry of Agriculture, Bangkok. CATT test-kits were provided by the Antwerp Institute of Tropical Medicine, Belgium.

DNA amplification

Conditions were optimized for a set of *T. evansi* primers constructed from a repetitive sequence probe pMUTec 6.258 (Chokesajjawatee, 1993). A 21 - mer sense primer (5'-TGCAGACG-ACCTGACGCTACT-3') and a 22-mer antisense primer (5'-CTCCTAGAAGCTTCGGTGTCCT -3') were synthesized. Reaction was conducted by adding 50 µl of the master mix solution containing 200 µM of each dATP, dTTP, dCTP and dGTP; PCR buffer (10 mM Tris- HCl pH 9, 50 mM KCl, 2 mM $MgCl_2$ and 0.01% gelatin), 0.25 µM of each primer and 2 units of Taq DNA polymerase onto the boiled blood sample. The first cycle of PCR included a 90°C/7 minutes step to ensure complete DNA denaturation. All the remaining 30 cycles employed 90°/30 sec step for denaturing, 60°C/30 sec for primer-template annealing, and 72°C/30 seconds for primer extension. When purified parasites or DNA samples were used, samples were added directly to the master mix solutions in microfuge tubes. For detection of a single 'handpicked' parasite amplification was extended to 40 cycles and the primer concentration was 0.125 µM.

Detection of PCR product

One fifth of the PCR solution was analysed by agarose gel electrophoresis (Maniatis *et al,* (1982). Gels were stained with ethidium bromide and *T. evansi* DNA fragment (227 bp) was visualized on a UV-light box.

RESULTS

Sensitivity of DNA amplification

PCR amplification was first tested on extracted parasite DNA, followed by experiments on purified parasites and only in the last step was this technique used on crude blood samples. The PCR product of purified DNA samples could be seen using as little as 0.5 pg per reaction (data not shown). This is equivalent to a genome content of 5 parasites, assuming that one trypanosome parasite has a DNA content of 0.1 pg (Borst *et al,* 1982).

In a next step, dilutions of purified trypanosomes (intact parasites without blood cells) gave visible signals down to about 100 parasites (result not shown). Surprisingly, samples which contained undiluted PSG buffer produced lower signals, leading the suspicion that the PSG buffer was inibiting the amplification process and this was confirmed by varying the amount of PSG in the reaction mixture (Fig 1).

Crude blood samples diluted in normal rat serum showed convincing signals down to approximately 300 parasites (Fig 2). The very faint signals from the lowest dilutions (30 and 3 parasites) cannot be readily distinguished in this figure.

Since there is always a certain ambiguity in the accuracy of serial dilutions in relation to exact numbers of parasites, we have 'handpicked' single parasites for testing. With this method individual trypanosomes could be detected but the DNA bands were very faint. Therefore a different set of conditions was used to increase the sensitivity. Amplification reaction was extended to 40 cycles and primer concentrations decreased to 0.125 µM (to avoid the occurrence of non-specific signals). Using this procedure, individually picked

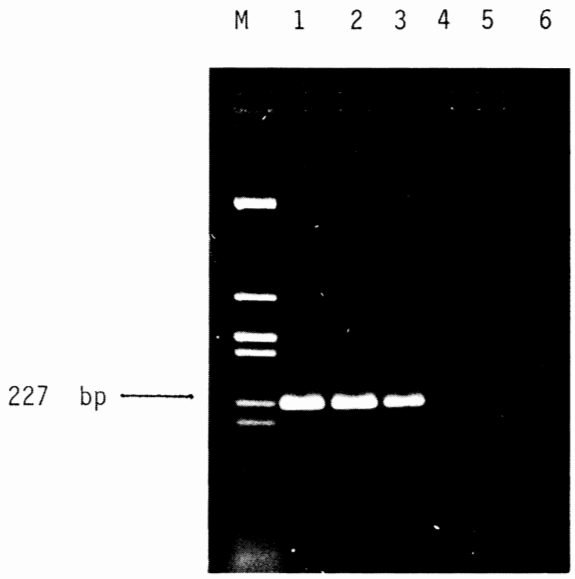

Fig1—Effect of phosphate-saline-glucose buffer (PSG) on PCR reaction. Samples contained 2.5 × 10^4 trypanosomes/reaction. Increasing quantities of PSG were added to the reaction mixture (final volume = 50 µl). 0 µl PSG (lane 1), 5 µl PSG (lane 2), 10 µl PSG (lane 3), 15 µl PSG (lane 4), 20 µl PSG (lane 5), negative control (lane 6). (M) pBR322 Hae II size marker. Electrophoresis on 1.5% agarose gel.

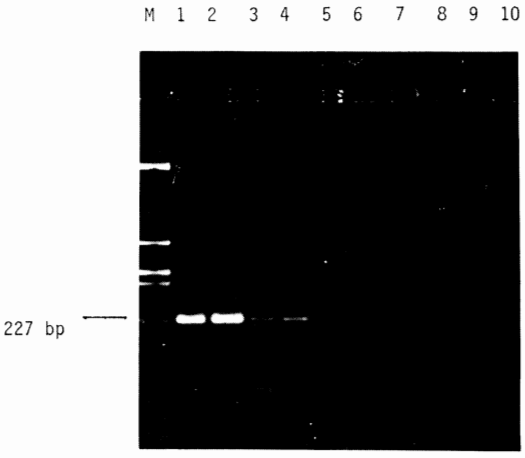

Fig 2—PCR product of *T. evansi* in whole blood. Decreasing concentrations of *T.evansi* in 10 µl whole blood were boiled under mineral oil 100°C/20 minutes. *(duplicate reactions).* 3.10^3 tr/reaction (lane 1 - 2). 3.10^2 tr/reaction (lane 3 - 4). 3.10^1 tr/reaction (lane 5 - 6). 3 tr/reaction (lane 7 - 8). 3.10^{-1} tr/reaction (lane 9 - 10). pBR322 Hae II size marker (M). Electrophoresis on 1.5% agarose gel.

parasites could be detected in 10 µl of whole blood (Fig 3).

Specificity of the detection method

Normal blood from bovine, swine, sheep, goat and equine was tested and no cross reactions of the trypanosome primers with DNA of any of the above mammals were found. Nor were there any cross reactions with the following blood parasites: *Babesia bovis, Babesia bigemina. Anaplasma marginale.* and *Plasmodium falciparum.* On the other hand, *T. brucei* and *T. equiperdum* produced an equally intense 227 bp band when using the *T. evansi* primers' set (results not shown) which, therefore, could be useful in the detection of these related parasites. The specific signal occurs as a distinct 227 bp band on agarose gels. When any specific bands were noticed in our experiments, they never interfered with the 227 bp band. Also, after southern blotting on nylon membranes and subsequent hybridization with the digoxigenin-labeled pMUtec 6.258 DNA probe, these signals never cross-hybridized (Fig 3a, 3b).

Monitoring of infection and of treatment

In an experimentally infected cow, DNA amplification revealed the specific 227 bp band as soon as two days post-infection (Fig 4). Positive detection by microscopy followed two days later. On day six however, the parasitemia plunged below microscopical detection level whereas PCR signals remained positive. The microscopist only saw the parasites reappear at day ten pi, almost simultaneously with the appearance of increased antibody titers on CATT tests. Mouse inoculation tests were started fom day six pi and were always positive until three hours after Berenil® treatment (3.5 mg/kg), which was given fifteen days pi when parasitemia was already very low. About six hours after treatment trypanosomes were cleared from the bloodstream. microscopic detection was negative three hours after treatment and PCR signals disappeared totally between six and twelve hours after treatment, while CATT titers remained high.

Sample stability

From samples stored at 4°C and at 37°C for various periods before boiling it was concluded that they could be kept up to 14 days (Fig 5). Once

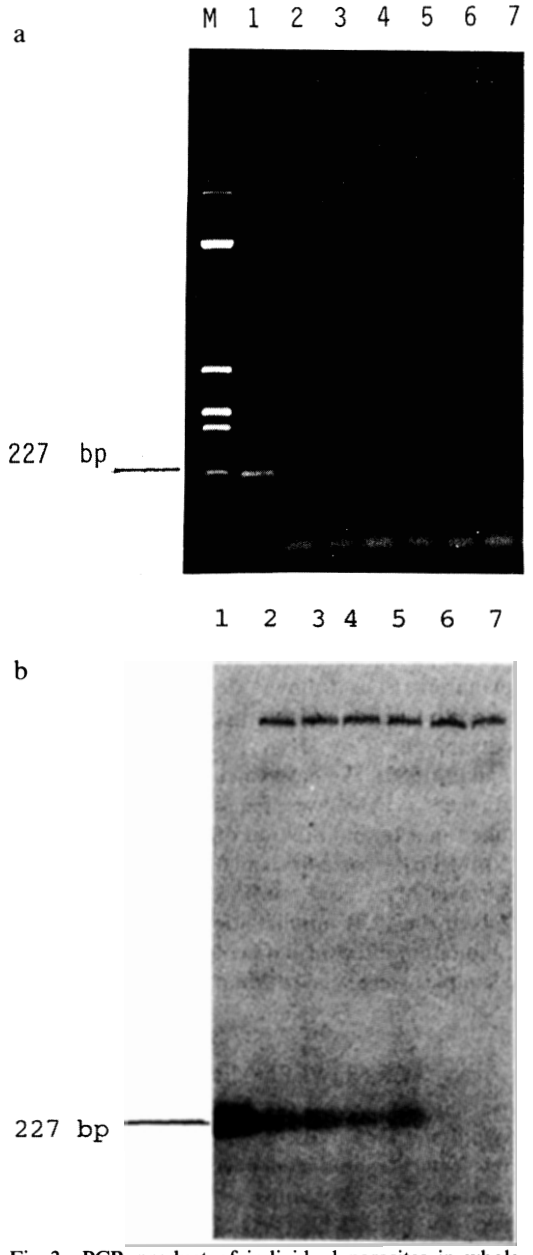

Fig 3—PCR product of individual parasites in whole blood.
3a : 40 cycle-PCR using 0.125 µM of primers. Lane 1 is a positive contol, Lanes 2 to 5 show the signal originating from individual trypanosomes, lanes 6 and 7 are negative controls. (M) pBR322 Hae II size marker. Electrophoresis on 1.5% agarose gel. The very intense bands are primer dimer signals; some non-specific signals are visible immediately below the 227 bp bands. Fig 3b: Digoxigenin hybridization of Fig 3a.Remark the absense of primer dimer and of non specific signals.

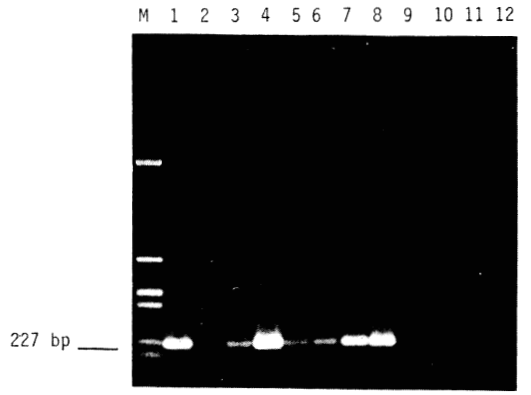

Fig 4—Infection experiment in calf. PCR product from whole blood, processed by boiling method. Blood was taken with 2-day intervals; positive control (lane 1), sample before infection (lane 2), samples 2, 4, 6, 8, 10, 12 and 15 days post infection (lanes 3 to 9). Lanes 10 to 12 represent samples taken 3 - 6 and 12 hours after Berenil treatment. (M) pBR322 Hae II size marker. Electrophoresis on 1.5% agarose gel.

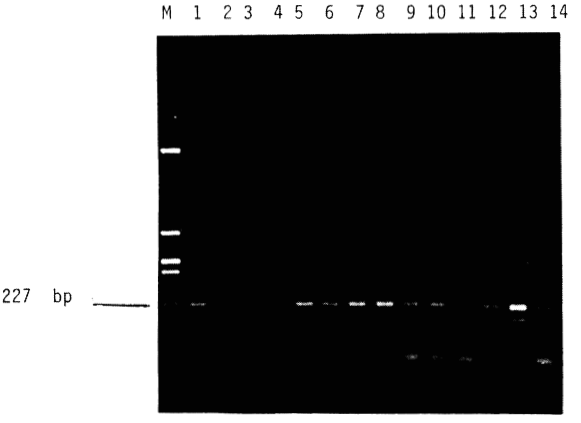

Fig 5—Stability of unboiled samples. PCR product of crude blood samples stored at 4°C (lanes 3 to 8) and at 37°C (lanes 9 to 14), *duplicate reactions,* parasitemia approximately 100 trypanosomes/ reaction. Samples boiled and PCRed immediately (lanes 1 - 2); boiled and PCRed after 3 days (lanes 3 - 4/9 - 10); after 7 days (lanes 5 - 6/11 - 12); after 14 days (lanes 7 - 8/13 - 14); (M) pBR322 Hae II size marker.

boiled, samples could be stored at room temperature at least up to four weeks (Fig 6).

DISCUSSION

The PCR protocol used in this paper is a modification from previously described methods (Tira-

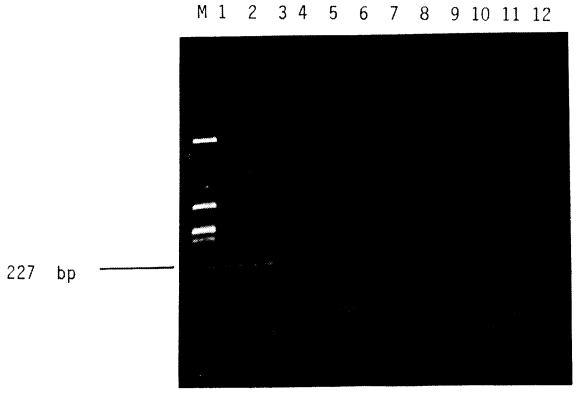

Fig 6—Stability of boiled samples. PCR product of immediately boilled crude blood samples containing approximately 100 trypanosomes and subsequently stored at room temperature. PCRed immediately after boiling (lanes 1 - 2); PCRed 1 week after boiling (lanes 3 - 4); 2 weeks after boiling (lanes 5 - 6); 3 weeks after boiling (lanes 7 - 8); 4 weeks after boiling (lanes 9 - 10). Negative controls (lanes 11 - 12); (M) pBR322 Hae II size marker.

sophon *et al,* 1991; Panyim *et al,* 1992) and from studies on amplification of *Leishmania* tissue samples (Rogers, 1990). Boiling of the blood was necessary to lyse the parasites and to free the DNA from the blood clot. Boiling would also denature proteins like hemoglobin which is believed to inhibit the PCR reaction. The boiling procedure further omits the necessity of anticoagulants, which have also been reported to act as PCR-amplification inhibitors. The 30-cycle protocol should warrant reproducible results, regardless of target-DNA concentrations and should allow a safe margin in variation of reagent-concentrations such as $MgCl_2$, enzyme, and primer concentration. The sensitivity of this method can best be appreciated considering that 1,000 parasites in 10 µl of blood represents 10^5 parasites/ml which requires observation of more than 20 fields under (40 × objective) microscope to detect a single parasite (Herbert and Lumsden, 1976).

The advantages of this PCR based method for the practitioner and the epidemiologist alike are many. Sampling is simple, requiring only PCR tubes and plain capillary tubes. Before boiling, the samples can be kept up to 14 days at ambient temperature without significant loss of detection sensitivity. If samples are collected in very remote locations, they can be preserved a month by boiling under mineral oil layer. Once in the laboratory, many samples can be processed together without need for prior purification or DNA extraction steps, saving considerable time and minimizing the risk of the dreaded PCR contamination.

According to Zhang *et al* (1992), the clearance of trypanosomes from the blood is dosage dependent, some Berenil resistant strains requiring very high blood levels of the drug (up to 89 mg/kg) for elimination. With the current PCR detection method, drug sensitivity tests, field monitoring for incidence and for prophylaxis can be performed with very high accuracy.

Finally, the inhibitory effects of PSG on the polymerase chain reaction should be noted. In our experiments the phosphate concentration in the PSG buffer was 48 mM. Fifteen µl of PSG in 50 µl of PCR solution contains 14.5 mM PO_4^{2-} and this amount is sufficient to completely inhibit the DNA amplification reaction (Fig 1). A probable explanation is as follows: deoxynucleoside triphosphates bind stoichiometrically to Mg^{2+}, necessary for Taq DNA polymerase reaction (Randall, 1989). We have calculated that a sevenfold excess of PO_4^{2-} over free Mg^{2+} concentration resulted in a total inhibition of amplification. Up to 5 mM Po_4^{2-}, or approximate two-fold ratio of PO_4^{2-} over Mg^{2+}, did not have a visible negative effect on the PCR amplification reaction. Hence the inhibitory effect of PSG could be eliminated if the samples were washed, diluting out PSG.

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