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The application of PCR-ELISA to the detection of *Trypanosoma congolense* type savannah (*TCS*) in bovine blood samples

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Abstract. PCR-ELISA was set up to detect strains of *Trypanosoma congolense* type savannah (TCS) in field samples of buffy coats. Results of PCR-ELISA and PCR were compared and the effectiveness of both techniques was also compared with the Murray's method for the detection of TCS in 257 bovine buffy coats. The PCR products were labelled with digoxigenin (DIG-dUTP) during amplification cycles of the repetitive satellite DNA. A biotinylated DNA capture probe was used to detect the PCR products by ELISA in streptavidin coated microplates. Both the PCR-ELISA and PCR were more sensitive and more specific than the Murray's method. Of the 257 buffy coats analysed by the three techniques, PCR-ELISA and PCR detected TCS in 98 and 97 buffy coats respectively, whereas the Murray's method detected only 39 samples. PCR-ELISA and PCR had almost the same sensitivity and specificity. PCR-ELISA and PCR respectively detected TCS in 39.2 % and 38.6 % in all the 334 samples analysed by both techniques in this study.

INTRODUCTION

Trypanosoma congolense is a species of trypanosome of which three sub-species are described in West Africa: *T. congolense* type forest (TCF), *T. congolense* type kilifi (TCK) and *T. congolense* type savannah (TCS). *T. congolense*, like *T. vivax*, *T. brucei*, *T. simiae*, and *T. suis* are responsible for *nagana* in sub Saharan African livestock. *Nagana* causes weight loss, low animal productivity and low reproductive ability, and even death in sick animals. Thus, *nagana* has serious economic impact on livestock production in Africa. Indeed in 2002, FAO (2002) had estimated annual losses due to this disease to be between 1 to 1.2 billion US Dollars.

The field diagnosis of trypanosomosis is based on parasitological techniques. But these techniques are not effective in case of low parasitemia. Even, the Murray method (Murray *et al*, 1977) or the buffy coat technique (BCT), one of the best parasitological techniques, cannot detect trypanosomes below 500 parasites /ml of blood (Paris *et al*., 1982). In order to improve the diagnosis of trypanosomosis, particularly in the event of an outbreak of the disease or in the case of low parasitemia, techniques based on detection of specific antibodies against trypanosomes such as ELISA, or detection of trypanosome DNA, have been developed. In many studies, either ELISA (Bocquentin & Duvallet, 1990; Doku, 1993; Desquesnes *et al*., 1999) or PCR (Solano *et al*., 1995; Mostafa *et al*., 1999; Desquesnes & Dávila, 2002) is applied for the detection of trypanosomes. PCR-ELISA was recently used to detect trypanosomes in blood samples and organs of vector insects

(Chansiri *et al*., 2002; Masake *et al*., 2002). In our study, PCR-ELISA was used to detect TCS DNA in field bovine buffy coat samples. The results obtained by PCR, PCR-ELISA and Murray method is discussed.

MATERIAL AND METHODS

Samples

A total of 334 bovine buffy coats were obtained in our study. Of these samples, 257 were collected in Ouangolodougou area, south-west of Burkina. The remaining 77 samples were from northern Ghana. These two zones have high prevalence of trypanosomosis due to high density of tsetse flies.

Trypanosome strains used as control

Pure DNA of six strains of TCS were used as control in PCR-ELISA. Five were isolated from cattle by the CIRDES1 (Burkina strains Sam.32.1, Kar.83, Sat.91.2) and by the ILRI2 (Kenya strains ILRAD/776, IL 3000). The remaining strain was isolated from a lion in Tanzania (strains IL3575).

Oligonucleotides

The primers TCS1 (5'-CGA-GCG-AGA-ACG-GGC-AC-3') and TCS2 (5'-GGG-ACA-AAC-AAA-TCC-CGC-3') were used to amplify the repetitive satellite DNA sequence of TCS (Masiga *et al*., 1992). A 25 - mer (Proligo Primers & Probes) biotinylated DNA, "*olicongo*" (Biotin-5'-CGG-GCC-TAT-TTG-ACC-GGC-ATA-GTG-A-3'), was used as capture probe.

Murray method

The Murray method or buffy coat technique (Murray *et al*., 1977) represents an improved technique for the diagnosis of animal trypanosomosis. Fresh blood was collected into heparinized capillary tubes and centrifuged at 2000 rpm for 10 min. Subsequently, the capillary tube was cut, with a diamond tipped pencil, around 1 mm below the buffy coat to include the top layer of red blood cells (RBCs). The buffy coat and the top layer RBCs were extruded onto a clean microscope slide and covered with a cover-slip. The preparation was examined for the presence of motile trypanosomes with a dark-ground or a phase-contrast microscope with x40 objective lens. Trypanosome species was identified according to their morphology and mobility in the microscope field. *T. congolense* is a small species of trypanosome (8-25 µm), the undulating membrane is not obvious and the free flagellum is absent. The trypanosome moves very slowly when observed under a microscope.

DNA extraction by Chelex 100®

Extracts of trypanosome DNA by Chelex-100® (SIGMA® chemicals) from buffy coat specimens were processed as described by Walsh *et al*. (1991). The buffy coat was collected into an Eppendorf® tube with 30 µl of sterile distilled water and homogenized by vortexing. Afterwards, 40µl of 5% Chelex 100® was added and mixed by vortexing. The mix was heated at 56°C for 1h then at 95°C for 30 min. The mix was centrifuged at 12 000 rpm for 2 min and 2ml of supernatant was used for DNA detection (Solano *et al*., 1995). The Eppendorf® tube containing the mixture was stored at 4°C for immediate use or at -20°C for long term use.

PCR amplification and digoxigenin labelling

PCR-ELISA DIG-Labeling kit (Roche Diagnostics) was used to label the PCR product during amplification. The PCR mixture for each sample contained 2µl of PCR buffer with magnesium chloride ($MgCl_2$) at 10X; 2µl of DIG-Labeling-Mix [2mM (dATP, dCTP and dGTP), 1.9mM (dTTP) and 1mM (DIG-dUTP)]; 0.25µl of each primer TCS1 and TCS2 at 20µM; 0.1µl of *Taq* polymerase at 5U/µl, 13.4µl sterile water. Then, 2µl of the template DNA was added to make the final volume to 20µl. The mixture was placed in a thermocycler (MJ Research Inc) and pre-heated at 95°C for 5 min and subsequently followed by 30 cycles of the following: denaturation at 95°C for 45s, annealing at 60°C for 1 min

and elongation at 72°C for 2 min. The PCR was ended with a final elongation step at 72°C for 11 min.

ELISA detection of PCR products

For each PCR sample, two ELISA replicate wells were assayed. Duplicates of blank, known negative and positive control samples were included in the assay. The PCR products were analysed by PCR-ELISA DIG-detection, 5 pack kit (Roche Diagnostics). For each sample, 5µl of PCR products were denatured in 20µl of alkaline denaturation solution (NaOH) in a sterile Eppendorf® tube, and left at room temperature for 10 min. A hybridization solution was subsequently added to the mixture to make the final volume to 250µl. The hybridization solution contained biotinylated DNA probe "*olicongo*" at 7.5 pmol/ml. One hundred microliters were overlaid into each well of a streptavidin coated microplate, StreptaWell® (Roche Diagnostics), and incubated with agitation at 37°C for 1.5 h. The microplates were washed four times with washing solution. Then, 100µl of the antibody anti-DIG-peroxidase solution (diluted 100 times) was added into each well and incubated at 37°C for 30 min followed by four times washing with washing solution. Finally, 100µl of ABTS® substrate solution was added into each well and incubated in the dark at 37°C for 10min. The absorbance values were determined by photometry on an ELISA plate reader (Labsystem Multiskan MCC/340) using a 405 nm filter and interfaced to a desk-top computer. For each pair of duplicate samples, the OD value was taken as the mean OD of the two samples. The OD values were converted in the Relative Positivity Percentage (RPP).

$$RPP = \frac{\text{Mean OD (sample)} - \text{Mean OD (negative control)}}{\text{Mean OD (positive control)} - \text{Mean OD (negative control)}} \times 100$$

Detection of PCR products on agarose gel

A mixture of 9µl PCR products and 1µl of loading buffer, was applied onto 1.5% agarose gel stained with ethidium bromide. Electrophoresis was run at 120V for 1hr in an electrophoresis set (Horizontal MGU) containing 1X Tris Borate EDTA. DNA ladder was used to determine the size of the PCR products.

RESULTS

Control PCR-ELISA

PCR-ELISA successfully detected all six positive control strains of TCS (tested in single wells) (Figure 1). The RPP of 23 of the 334 samples tested is shown in Table 1. Samples with RRP > 10% (cut-off) were considered positive.

Results of field samples

PCR-ELISA detected TCS in 33 (38.1%) of the 77 samples from northern Ghana and in 98 (42.9%) of the 257 samples from Ouangolodougou. Simple PCR detected 32 (41.6%) positive cases of the 77 samples from northern Ghana and 97 (37.7%) positive cases of the 257 samples from Ouangolodougou. The Murray method detected TCS in only 39 (15.2%) of the 257 samples from Ouangolodougou. However, samples from northern Ghana were not analysed by the method of Murray. All results obtained by the different techniques are summarised in Table 2

DISCUSSION

The Murray method is regarded as one of the best microscopic examination techniques for the detection of trypanosomes in blood samples (Murray *et al.*, 1977). However, this technique is less sensitive and therefore cannot diagnose the earlier stages of the infection. Furthermore, the Murray method cannot accurately distinguish the different species of trypanosomes. In addition, microscopy is laborious and time consuming as individual slides have to be examined. Thus, to test a whole herd or to monitor livestock in large scale it is more practical to use ELISA (Nantulya and Lindqvist, 1989; Desquesnes *et al.*, 1999). However, ELISA may present cross reactions between different species of

Figure 1. Detection of pure DNA of the 6 strains of *TCS*: A=Blank, B=negative control, C=Sam.32.1, D=Kar.83, E=Sat.91.2, F=ILRAD/776, G=IL3000, H=IL3575.

Table 1. Results of ELISA detection of DIG labelled PCR products from 23 samples

No. Sample	Status	Mean OD	RPP %	SD
1	0	0.015	0.5	0.38
2	1	0.600	29.5	0.15
3	0	0.015	0.5	1.22
4	0	0.018	0.7	0.69
5	0	0.018	0.7	0.69
6	1	2.111	104.5	0.01
7	1	2.091	103.5	0.03
8	0	0.007	0.1	0.61
9	0	0.004	0.0	0.61
10	1	2.087	103.2	0.02
11	0	0.009	0.2	0.47
12	1	2.087	103.3	0.06
13	1	0.144	6.9	0.36
14	0	0.020	0.8	0.04
15	1	0.327	16.0	0.02
16	1	0.905	44.7	0.01
17	0	0.010	0.3	1.41
18	0	0.008	0.2	0.09
19	1	2.203	109.0	0.04
20	0	0.016	0.6	0.96
21	0	0.016	0.6	0.96
22	1	0.664	32.7	0.01
23	1	1.849	91.5	0.01

Note: 1 = positive, 0= negative, sample with RPP> 10% (cut-off) were considered positive,
Mean OD positive control: 2.021, Mean OD negative control: 0.004

trypanosomes (Desquesnes & Bengaly, 2001) and like the other serological assays, it cannot differentiate the sick and cured animals.

PCR is a very good molecular technique for the detection of parasite DNA in blood samples. Specific primers for each trypanosome species have been designed and have been used in many African countries (Majiwa et *al.*, 1994; Masake *et al.*, 1997). However, the requirement of special equipment for the detection of PCR products limits its use in many laboratories. In our study, we used the liquid-phase detection system in order to capitalize on the ELISA technology which has already been established in many laboratories.

The use of specific primers and biotinyled capture probe offers an advantage in that only the PCR products with the authentic TCS DNA will hybridize with the DNA probe. The washing steps remove non-specific reactions which may interfere with the specific ones. Thus, PCR-ELISA is a highly specific and sensitive diagnostic tool. In this study, all the positive cases detected by simple PCR were also positive by the PCR-ELISA. Both techniques were more sensitive than the Murray method, and PCR-ELISA and simple PCR have comparable effectiveness

Table 2. Summary of results obtained

Sample	Numbers	PCR (+)		PCR-ELISA (+)		Murray method (+)	
Ouangolodougou	257	97	37.7 %	98	38.1%	39	15.2%
Northern Ghana	77	32	41.6 %	33	42.9%	–	–
Total	334	129	38.6 %	131	39.2%	–	–

and specificity (P-value < 0.87, OR= 0.98 (0.71, 1.35)). The PCR-ELISA has been described by Landgraf *et al.* (1991) and has been applied successfully for the detection of *Anaplasma marginale* infection (Gale *et al.*, 1996) and mucocutaneous leishmaniasis (Pinero *et al.*, 1999). Masake *et al.* (2002) used PCR-ELISA to detect *T. brucei* and *T. vivax* infections in livestock in East Africa. Chansiri *et al.* (2002) reported the use of PCR-ELISA for the detection of *T. evansi* in animals and vectors in Thailand.

One advantage of PCR-ELISA was that it could detect positive samples which were faintly stained in the agarose gel (Figure 2A, 2B). In the PCR ELISA positive cases could be easily detected through the development of specific coloration in the reaction solution (Figure 2B).

PCR-ELISA can be used as a routine tool for diagnosis as it does not require electrophoresis equipment, dark room plus ultra violet camera facilities, which may be expensive for laboratories in developing countries. PCR-ELISA provides an alternative for laboratories which lack special equipment to benefit from the use of molecular technologies. This is in the view of the fact that PCR-ELISA employs standard equipment in conventional ELISA. The use of microplates, multichannel micropipettes and photometric ELISA reader saves considerable time because many samples can be tested at once. Furthermore PCR-ELISA is safe because it does not involve the use of carcinogenic elements such as ethidium bromide and radioactive probes. Lastly, PCR-ELISA employs standard equipment for processing ELISA and a thermocycler for PCR, thus it can be easily set up in laboratories which already use PCR and ELISA as a routine diagnostic tools.

CONCLUSION

There was no significant difference in the effectiveness of the PCR or PCR–ELISA to detect TCS DNA although ELISA detection of PCR products was greater than the ethidum bromide staining of the amplicons in agarose gel. Furthermore, the capture probe enhances the specificity of the PCR. Lastly, this technique is time saving as multiple PCR reactions can be analysed simultaneously. PCR–ELISA can be used in solid phase hybridization as a means of detecting PCR products, and this option is particularly attractive given that biotinylated PCR products can be detected directly by visual dicernible colour change (Maske *et al.*, 2002). However, cost of PCR-ELISA kits or reagents remains one of the limitations in routine diagnosis.

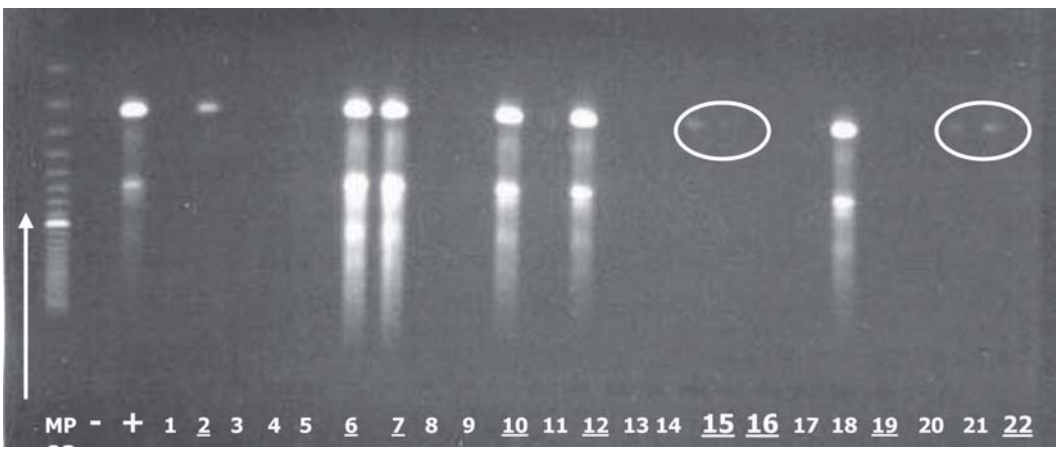

Figure 2A. Photograph showing PCR products in an agarose gel. Samples 15 and 16, 22 and 23 (circled), are weakly positive. However, the positive reaction of these four samples were very obvious in the PCR_ELISA (see Figure 2B).

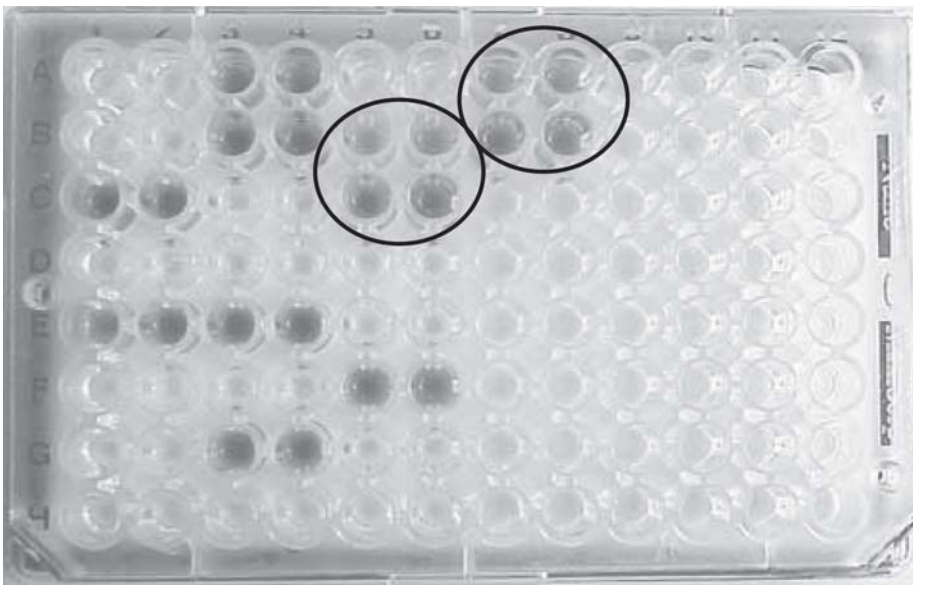

Figure 2B. Positive reaction of samples in PCR_ELISA. Positive samples 15 (B5, B6), 16 (C5, C6) 22 (A7, A8) and 23 (B7, B8) are easily distinguished.

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