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Serodiagnosis of African sleeping sickness in vervet monkeys by detection of parasite antigens

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Summary

Sera of vervet monkeys experimentally infected with *T. b. rhodesiense* were examined using a double antibody sandwich ELISA and Procyclic Agglutination Trypanosomiasis Test (PATT) for the presence of circulating trypanosomal antigens and anti-procyclic surface antibodies, respectively. Trypanosomal antigens were detected at 7 days post infection and remained at a detectable level thereafter during the infection. Antigens were not detected in sera prior to experimental infection or at 26 days after trypanocidal drug treatment. Although both the PATT and the sandwich ELISA results correlated with the infection status of the animals, the sandwich ELISA gave a better indication of the disease progression than the PATT, especially during trypanocidal drug therapy. The results illustrate the potential utility of the double antibody sandwich ELISA for diagnosis of African sleeping sickness.

Key words: immunodiagnosis; procyclic culture forms; circulating trypanosomal antigens; *Trypanosoma brucei rhodesiense;* human sleeping sickness.

Introduction

Human African sleeping sickness is an endemic disease to which an estimated 50 million people in sub-Saharan Africa are at risk (WHO, 1986). Depending on the subspecies of the causative trypanosomes, the disease develops as either a chronic form (caused by *T. b. gambiense*) or an acute form (caused by *T. b. rhodesiense*). Current methods for diagnosis, which rely on either parasi-

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tological or serological measurements, have serious drawbacks. Although conclusive results are usually obtained through parasitological methods, they are relatively insensitive, especially in *T. b. gambiense* infections where fluctuations occur in the parasitemia (Van Meirvenne and Le Ray, 1985).

A number of serodiagnostic tests have been developed over the past 20 years (Voller and De Savigny, 1981). The Card Agglutination Trypanosomiasis Test (CATT) (Magnus et al., 1978) utilizes a predominant variable antigenic type of bloodstream *T. b. gambiense* and is the most successfully applied screening technique for the Gambian form of sleeping sickness (WHO, 1986). In order to improve upon the CATT procedure by including the detection of antibodies to *T. b. rhodesiense*, we recently developed the Procyclic Agglutination Trypanosomiasis Test (PATT) (Pearson et al., 1986). Both of these serodiagnostic tests are based on the detection of anti-trypanosome antibodies and thus they do not distinguish between past and currently active infections. An immunodiagnostic test designed to detect circulating trypanosomal antigens may provide a more accurate assessment of the infection status of individuals. Indeed, such a test has been applied to detect trypanosome antigens in goats and rabbits (Rae and Luckins, 1984).

Recently, we reported the use of monoclonal murine antibodies and polyclonal rabbit antibodies to the surface antigens of procyclic trypanosomes for detection of trypanosomal antigens in the sera of infected mice (Liu and Pearson, 1987). Because the monoclonal antibodies employed recognize a single trypanosomal membrane antigen ("procyclin", J. P. Richardson et al., 1988) and the rabbit antiserum was made against procyclic membrane proteins, we felt the assay could be improved by using reagents which bound a larger variety of trypanosomal antigens. In this report, we describe a double antibody sandwich ELISA for detection of circulating trypanosomal antigens in the sera of vervet monkeys infected with *T. b. rhodesiense*. Purified unlabelled rabbit antibodies to whole lysates of *T. b. rhodesiense* procyclic culture forms were used as the antigen capture reagent and biotinylated antibodies as the detection reagent in a sensitive biotin-streptavidin-horseradish peroxidase system for detection of antigens in sera. A comparison of the results with those obtained using the PATT (Pearson et al., 1986) and a discussion of the utility of the sandwich ELISA for diagnosis of sleeping sickness are presented.

Materials and Methods

Parasites. The bloodstream forms of *T. b. rhodesiense* KETRI 2537 or KETRI 2545, derived from EATRO 1989 (Fink and Schmidt, 1979), were used to infect vervet monkeys (see below). *Trypanosoma brucei rhodesiense* ViTat 1.1 procyclic culture forms (PCF) were established from cloned bloodstream populations (Richardson et al., 1986). They were maintained at 26°C in SM medium (Cunningham, 1973) supplemented with 10% Foetal Bovine Serum (FBS). Promastigotes of *Leishmania major* NIH (Seidmann strain) were cloned by micromanipulation and maintained using the same culture conditions. The clone used was designated *L. major* A2.

Monkeys, infection and treatment. Vervet monkeys (*Cercopithecus aethiops*) were infected with *T. b. rhodesiense* and were treated with trypanocidal drugs at 28–42 days post infection as previously described (Pearson et al., 1986). Blood from these monkeys was collected prior to infection, at different time intervals during the infection and after trypanocidal drug treatment. Sera were tested with the PATT (Pearson et al., 1986) and with a double antibody sandwich ELISA (see below).

Preparation of lysates. Procyclic culture form trypanosomes were harvested and washed twice by centrifugation at $800\times g$ for 10 min at room temperature in phosphate buffered saline pH 7.4 containing 1% glucose (PSG). Trypanosomes were then resuspended in phosphate buffered saline (PBS) to give a final concentration of 10^9 trypanosomes per ml. Whole lysates were prepared by 6 cycles of 30 sec bursts of 45 watts (setting 5 in a sonifier cell disruptor, model w185E, Heat Systems-Ultrasonics, Inc., Plainview, New York, USA) sonication on ice. Protein concentrations of the lysate were determined by the Lowry method (Lowry et al., 1951).

Water lysates or membrane fractions of trypanosome PCF and *Leishmania major* A2 promastigotes were prepared as previously described (Liu and Pearson, 1987).

Rabbit antibodies. Whole lysate of *T. b. rhodesiense* ViTat 1.1 procyclic culture forms (1 mg) was emulsified with Freund's complete adjuvant and injected subcutaneously and intramuscularly into a male New Zealand rabbit. Three weeks later a boost of 0.5 mg lysate in Freund's incomplete adjuvant was given. Sera were tested against lysate in indirect ELISA (Liu and Pearson, 1987) 10 days after the boost. The rabbit was then bled out, serum prepared by standard methods and the IgG fractions were purified by ammonium sulphate precipitation followed by protein A chromatography (Wood, 1984). The protein concentration of the isolated IgG fractions was determined by the Lowry method (Lowry et al., 1951). Four milligrams of the purified IgG fractions were labelled with 1 mg of biotin succinimide ester (CAB-NHS; Bethesda Research Laboratories, Burlington, Ontario, Canada) using Focus' (1985) methodology. The activity of the biotin-labelled antibodies was determined by indirect ELISA (Liu and Pearson, 1987).

Sandwich ELISA. Sera from vervet monkeys were examined in an attempt to detect the presence of trypanosomal antigens using sandwich ELISA. Assays were performed as previously described (Liu and Pearson, 1987) with the following modifications: A 1/4000 dilution of unlabelled, ammonium sulphate precipitated rabbit antibodies made against trypanosome PCF whole lysates and a 1/400 dilution of the corresponding biotin-labelled antibodies were used as the capture and the detecting reagents, respectively. Bovine serum albumin (BSA; Sigma, St. Louis. Mo., USA) was used, instead of fish gelatin, in the blocking solution. In addition, 1% BSA was added to the buffer that was used to dilute the test sera and the biotinylated rabbit antibodies. All sera were used at dilutions of 1/4, 1/8, 1/16 and 1/32. Control wells contained PBS, lysates or membrane fractions of trypanosome PCF or sera from uninfected North Americans. Absorbance ratios were calculated by dividing the O.D. readings for test sera with the O.D. readings for negative controls. Samples were considered positive (i.e. presence of antigens) if sera showed absorbance ratios above two.

The sensitivity of the sandwich ELISA was determined by adding known concentrations of trypanosome PCF lysates (ranging from 10^2 to 2×10^7 trypanosomes/ml) and trypanosome PCF membrane proteins (5–0.001 μg/ml). Lysates and membrane proteins of *Leishmania major* A2 were also tested at the same concentrations.

Agglutination test. The titres of antibodies specific for the surface of *T. b. rhodesiense* ViTat 1.1 PCF were measured by the PATT as previously described (Pearson et al., 1986). Both trypanosome PCF and *Leishmania* promastigotes were used as test organisms. Sera from vervet monkeys were tested at doubling dilutions of 1/10–1/320 in PSG. Controls included PSG/10% FBS, sera from humans with *T. b. gambiense* infections (from Daloa, Ivory Coast) and sera from uninfected North Americans.

Results

The double antibody sandwich ELISA using rabbit anti-*T. b. rhodesiense* PCF whole lysate specifically trapped trypanosomal antigens but not *Leish-*

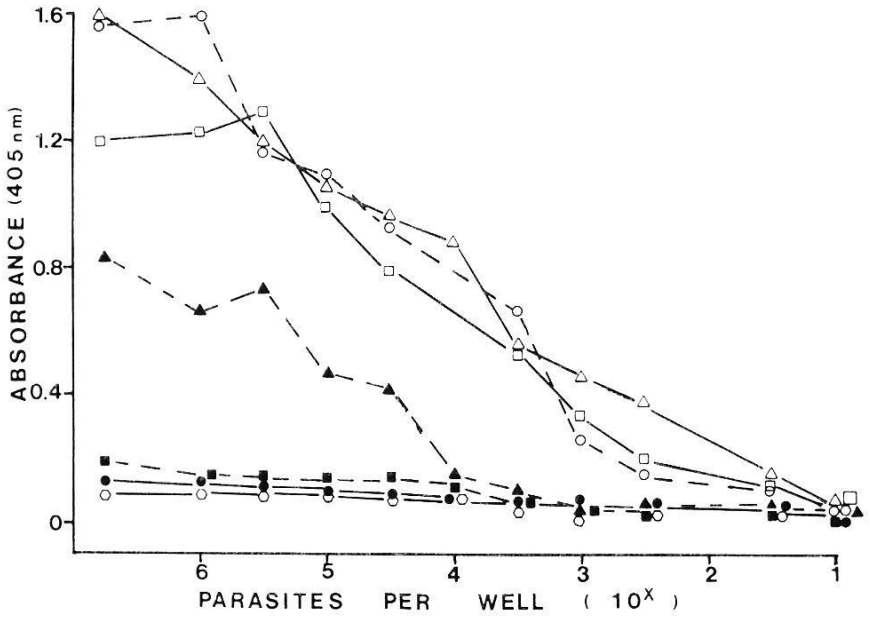

Fig. 1. Detection of trypanosomal antigens in water lysates of parasites by double antibody sandwich ELISA. (△—△) *T. b. rhodesiense* Vitat 1 PCF; (○—○) *T. b. gambiense* U2 PCF; (□—□) *T. b. brucei* 10–26 PCF; (▲—▲) *T. congolense* 44/1 PCF; (●—●) *L. Major* A1; (■—■) *L. donovani* ISD2; (⬡—⬡) PBS.

mania major antigens when parasite lysates or membrane proteins were tested (Fig. 1). This assay was shown to detect antigen from as few as 100 ± 16.25 trypanosomes/well or 5 ± 2.73 ng trypanosomal membrane proteins/well (mean ± S.D.) in four different experiments over a three-month period.

Sera from twelve vervet monkeys infected with *T. b. rhodesiense* were examined using the double antibody sandwich ELISA and the PATT. All sera were numbered consecutively from 1 to 49 as previously described (Pearson et al., 1986). Results for both tests are shown in Table 1. The data are further summarized with respect to the status of infection (Fig. 2).

The sandwich ELISA results correlated with the infection status of vervet monkeys. No detectable antigen was observed in sera collected prior to experimental infection (sera 3, 8, 16, 19, 26, 32, 36) or in sera taken either shortly after drug treatment (12–54 days – sera 14, 18, 23, 30, 31, 40) or long after drug treatment (105–933 days – sera 1, 13, 15, 35, 40 and 41). One serum (no. 2), which was taken long after drug cure, was positive in ELISA but negative in the agglutination assay. During the infection, trypanosomal antigens were detected as early as 7 days post-infection (serum 4). Antigen levels reached a maximum at about 14–16 days infection in all infected monkeys (sera 5, 10, 20, 27, 33 and 37). Thereafter, trypanosomal antigen levels decreased at 28 days and increased slightly prior to drug treatment. Control sera from West African humans infected with *T. b. gambiense* gave positive results in the sandwich ELISA, as did *T. b. rhodesiense* water lysates and membrane proteins. Sera from uninfected North American humans (sera 44 and 45) were negative.

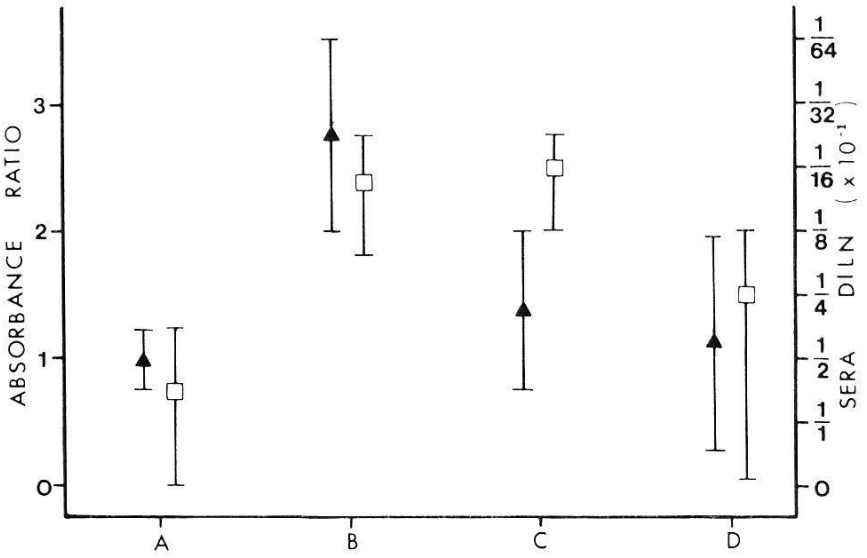

Fig. 2. Summary of antibodies and antigen detection tests with respect to infection status of vervet monkeys infected with *T. b. rhodesiense*. Sera were collected before infection (A), at days 7–56 post-infection (B), at days 12–54 post-drug treatment (C) and at days 105–933 (D). post-drug treatment. Mean ± S.D. values of anti-trypanosomal antibody titres (□) and trypanosomal antigens, as represented by absorbance ratios (▲), in sera are shown respectively.

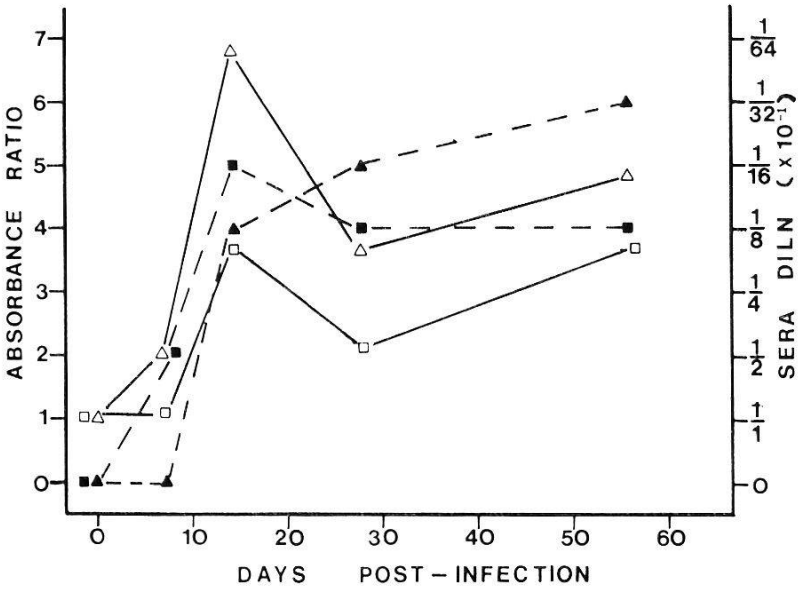

Fig. 3. ELISA measurement of anti-trypanosome antibodies and trypanosomal antigens in sera of vervet monkeys No. 47 and No. 49 during *T. b. rhodesiense* infection. Antibody titres are presented as closed symbols (▲—▲ No. 47; ■—■ No. 49). Antigen levels are indicated by the absorbance ratios and are shown as open symbols (△—△ No. 47; □—□ No. 49).

Parallel testing of the vervet sera for anti-trypanosome antibodies in the agglutination test (PATT) gave similar results to those of Pearson et al. (1986). This is not surprising since the same sera were used in the previous study. Anti-trypanosome PCF antibodies were detected only in sera from vervet monkeys with active, untreated infections or sera taken shortly after drug

Table 1. Measurement of anti-procyclic surface antibodies and circulating trypanosomal antigens in vervet monkeys sera before and during infection with *T. b. rhodesiense* and at various times after treatment with trypanocidal drugs

Serum No.	Monkey No.	No. of days since infection[b]	No. of days since drug treatment (drug in brackets)	Remarks	Agglutination titre vs. *T. b. rhodesiense*[a]	Sandwich ELISA ratio[b]
1	34	940	859 (Mel. B.)		–	1.00
2	35	978	933 (Suramin/M.K.436)		–	3.18
3	47–1	0	0	Preinfection	–	1.00
4	47–2	7	0	Pretreatment	–	2.00
5	47–3	14	0	Pretreatment	1/80	6.80
6	47–4	28	0	Pretreatment	1/160	3.63
7	47–5	56	0	Pretreatment	> 1/320	4.84
8	49–1	0	0	Preinfection	–	1.16
9	49–2	7	0	Pretreatment	1/20	1.16
10	49–3	14	0	Pretreatment	1/160	3.73
11	49–4	28	0	Pretreatment	1/80	2.02
12	49–5	56	0	Pretreatment	1/80	3.72
13	61	875	793 (Berenil)	528 days since last pos. (CSP)	–	0.99
14	85–1	392	130 (Suramin/M.K.436)	29 days since last pos. (blood, CSP) (2nd treatment)	–	0.79
15	85–2	605	343 (Suramin/M.K.436)	213 days since last pos. (blood) (2nd treatment)	–	1.06
16	94–1	0	0	Preinfection	–	1.10
17	94–2	28	0	Pretreatment	> 1/320	3.80
18	94–3	352	158 (Suramin/TS.88)	11 days since last pos. (3rd treatment)	1/320	0.94
19	96–1	0	0	Preinfection	1/20	1.21
20	96–2	14	0	Pretreatment	1/40	2.92
21	96–3	28	0	Pretreatment	> 1/320	0.30
22	96–4	58	14 (Suramin/TS.88)	1st and only treatment	1/80	2.81
23	96–5	71	27 (Suramin/TS.88)	1st and only treatment	1/40	1.31
24	96–6	202	158 (Suramin/TS.88)	1st and only treatment	–	N.D.
26	115–1	0	0	Preinfection	1/80	0.81
27	115–2	16	0	Pretreatment	1/40	2.62
28	115–3	33	0	Pretreatment	1/160	2.51

Table 1 (continued)

Serum No.	Monkey No.	No. of days since infection[b]	No. of days since drug treatment (drug in brackets)	Remarks	Agglutination titre vs. *T. b. rhodesiense*[a]	Sandwich ELISA ratio[b]
29	115–4	56	12 (TS.88)	1st treatment (relapsed later)	1/320	1.83
30	115–5	70	26 (TS.88)	1st treatment (relapsed later)	1/160	1.26
31	115–6	98	54 (TS.88)	1st treatment (relapsed later)	1/320	1.01
32	124–1	0	0	Preinfection	–	0.47
33	124–2	16	0	Pretreatment	1/40	1.97
34	124–3	33	0	Pretreatment	1/320	0.68
35	124–4	352	105 (TS.88)	3rd treatment (Suramin/TS.88, 1st and 2nd)	–	0.84
36	128–1	0	0	Preinfection	1/10	1.07
37	128–2	16	0	Pretreatment	1/320	2.72
38	128–3	33	0	Pretreatment	1/80	0.06
39	128–4	41	0	Pretreatment	1/80	3.61
40	128–5	352	278 Suramin/TS.88)	132 days since last pos. blood (2nd treatment)	1/320	0.75
41	128–6	366	292 (Suramin/TS.88)	146 days since last pos. blood	–	0.94
42	96–CSF	71	27 (Suramin/TS.88)		–	0.56
43	115–CSF	33	0		–	0.49
44	neg.	0	0	Human serum (North American)	–	1.00
45	neg.	0	0	Human serum (North American)	–	1.00
46	pos.	unknown	7 (Arsobal)	Human serum (Ivory Coast)	1/320	4.42
47	pos.	unknown	7 (Arsobal)	Human serum (Ivory Coast)	1/320	2.52
48	pos.	0	0	*T. b. rhodesiense* water lysate (2×10^4 tryps.)	N.D.	3.18
49	pos.	0	0	*T. b. rhodesiense* membrane proteins (0.05 μg)	N.D.	2.4

[a] Agglutination of *T. b. rhodesiense* ViTat 1.1 procyclic culture forms.

[b] Ratio = O.D. for test sera ÷ O.D. for the average of the negative control sera (numbers 44 and 45).

[c] Animals were infected with *T. b. rhodesiense* KETRI 2537 except no. 61 which was infected with *T. b. rhodesiense* KETRI 2545.

N.D. = not done.

treatment (12–54 days post-drug therapy). Prior to drug therapy, infected individuals were usually positive in both sandwich ELISA and PATT assays. The results of ELISA and PATT assays diverged in that the antigen levels as detected by ELISA decreased or disappeared whereas the antibody levels persisted in sera taken soon after drug treatment (Fig. 2).

The results of both antibody detection and antigen detection assays in two monkeys (number 47 and 49) were plotted graphically in order to show their relationship to each other (Fig. 3). Both antibody and antigen levels increased soon after infection. Thereafter, antigen levels had decreased markedly by 28 days and had increased again at 56 days post-infection. Antibody levels remained high throughout the infection period. These trends were also seen with monkeys 96, 115, 124 and 128, that is, an oscillation in antigen levels with a much more stable level of antibodies.

Discussion

It is well established that procyclic culture forms and bloodstream forms of African trypanosomes share antigens (reviewed by Shapiro and Pearson, 1986) which can be recognized by the immune system of infected hosts. The presence of anti-trypanosome PCF antibodies in sera of mice, vervet monkeys and humans (Pearson et al., 1986) illustrates this antigen-antibody recognition. This suggests the possibility of using anti-trypanosome PCF antibodies for detection of circulating trypanosomal antigens in infected individuals. Indeed, our results from the double antibody sandwich ELISA reported here show that anti-procyclic antibodies are useful for the detection of antigen in the sera of experimentally infected vervet monkeys.

Trypanosomal antigens were detected in sera from *T. b. rhodesiense*-infected vervet monkeys as early as 7 days post infection. Thereafter, antigens remained at a detectable level in all sera from monkeys with active, untreated infections with the exception of sera nos. 21, 34 and 38 at days 28, 33 and 56 post-infection, respectively. It is interesting that both sera nos. 21 and 34 showed a high anti-trypanosome antibody titre. It is thus possible that parasite antigens in these sera were in the form of immune complexes which would possibly make antigen detection by the sandwich ELISA more difficult. Immune complexes are often found in the blood and CSF of patients with human African trypanosomiasis (Lambert et al., 1981). After 33 days of trypanosome infection, detectable antigen increased moderately in all sera until drug treatment.

In part through the action of trypanocidal drugs, trypanosomes are quickly eliminated from the bloodstream of the infected host, although trypanosomes may reside in several organs of the host and may initiate bloodstream infections from such sites (Poltera, 1985). A rapid decrease in the amount of circulating antigens is thus expected as the disease begins to regress. A decrease in the

detectable antigen level (as measured by our sandwich ELISA) was in fact observed in sera taken from trypanosome-infected monkeys shortly after trypanocidal drug treatment (14 days post-treatment). Antigens were not detected in sera as early as 27 days post-drug treatment (sera nos. 23, 24, 30 and 31). With the exception of serum no. 2, which was taken 933 days after drug treatment, none of the sera taken long after successful drug treatment showed any detectable trypanosomal antigens. Since this serum was taken from monkey No. 35 which gave no indication of a relapse, the ELISA result must be interpreted as a false positive.

All preinfection or control sera from uninfected individuals were negative. Results from the sandwich ELISA thus correlated with the infection status of vervet monkeys infected with *T. b. rhodesiense*.

Although the antibody level, as measured in the PATT, also indicated the infection status of the trypanosome-infected vervet monkeys, the sandwich ELISA appears to be a more precise method for indication of active infection because there is a lesser tendency for antigen persistence than antibody persistence in sera taken shortly after drug treatment.

Human African trypanosomiasis is characterized by a haematolymphatic and a cerebrospinal stage (Schmidt and Sayer, 1982a, 1982b). A diagnostic test for African trypanosomiasis should ideally detect both stages of the disease. Our present data indicated that circulating trypanosomal antigens were detected in sera of infected vervet monkeys but not in the CSF samples from two trypanosome-infected vervet monkeys. Because the cerebrospinal involvement represents a later stage of the disease (Poltera, 1985), it is possible that these two monkeys (sample nos. 42 and 43) had not progressed to this stage and did not have trypanosomal antigens in their CSF at the time of collection.

The rabbit antibodies used in this sandwich ELISA were specific to *T. brucei* spp. and *T. congolense* and, in this study, they showed no reactivity to leishmania lysates. This implies that the assay is potentially useful in the diagnosis of human sleeping sickness caused by *T. b. gambiense* and *T. b. rhodesiense* and of Nagana in cattle caused by *T. b. brucei* and *T. congolense*. Further testing of well-documented sera from *T. b. rhodesiense* and *T. gambiense*-infected patients and control sera from endemic areas is needed to fully assess the value of this double antibody sandwich ELISA for the diagnosis of African sleeping sickness.

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