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Evaluation of ELISA using crude *Trypanosoma brucei brucei* antigen and CATT in the detection of human African trypanosomiasis

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ABSTRACT: An antibody Enzyme Linked Immunosorbent Assay (ELISA) for the detection of parasite induced IgG and IgM using excretory-secretory products of *Trypanosoma brucei brucei* (*T. b. brucei*) as antigen was carried out on human sera and cerebrospinal fluid samples collected from a field survey. Card Agglutination Technique for Trypanosomiasis (CATT) was also performed on dried blood samples from the same subjects. Anti-trypanosome antibody was detected in 85.7% and 74.5% of the 98 samples using ELISA and CATT respectively, 36 of these subjects were confirmed parasite positive, 57 were negative and 5 were non-endemic controls. The sensitivity of the assays using sera from parasite-positive cases for the detection of antibodies by ELISA (IgG + IgM) was 100% and 94.4% for CATT. The sensitivity of the antibody detection assay was 77.8% for IgG and 88.9% for IgM respectively and the specificity was 60% for CATT, 40% IgG and 100% IgM. A statistically significant relationship between IgM titres and parasite presence in subjects ($P < 0.05$) was observed. Thirteen CSF samples from 6 parasite positive and 7 aparasitaemic subjects were analyzed. IgG antibodies were detected in 7 (53.8%) and IgM in 6 (42.2%) of these. The specificity of the ELISA assays was 46.2% (IgG), 53.8% (IgM) and sensitivity was 66.7%. The findings of this study showed that the crude extract of *T. b. brucei* was highly immunogenic and the antibody ELISA using this antigen was more specific than CATT in the diagnosis of human trypanosomiasis. The field sensitivity and application of CATT in field survey was also highlighted.

Key Words: African Trypanosomiasis; *Trypanosoma brucei brucei*; ELISA; CATT; Detection of trypanosomes.

Introduction

Trypanosomiasis is still one of the major tropical diseases with approximately 2,500 new cases diagnosed annually and 50 million people living in 200 endemic foci in affected countries of tropical Africa (WHO, 1990). *Trypanosoma brucei gambiense* causes a chronic infection which remains asymptomatic for several months before clinical manifestations develop and even when they do there are no pathognomic signs. Hence, clinical diagnosis needs to be supported by the identification of trypanosomes in a patient's tissue fluid. Parasitological diagnosis of trypanosomiasis is generally more accurate but less sensitive than the serological methods. This is because the fluctuating character of *T. b. gambiense* makes diagnosis difficult (WHO, 1984) resulting in several cases going undetected.

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Several serological techniques have therefore been developed and extensively evaluated in the field as alternatives to the direct detection of trypanosomes even though they would enable only a presumptive diagnosis to be made (Aiyedun et al., 1976; Magnus et al., 1978). Also, some of the serological techniques employed have low sensitivity while those that have adequate sensitivity are not readily applicable in the field (Nantulya, 1989, 1990) resulting in the need for the development of alternative diagnostic methods (WHO, 1986). CATT has been recommended for use because of its simplicity and ease of performance under field conditions (WHO, 1984; Noireau et al., 1991), while ELISA was introduced for African trypanosomes because of its suitability for screening large numbers of samples (Voller et al., 1976). This paper reports the findings of tests using *T. b. brucei* extract as antigen in ELISA while comparing with CATT in the detection of *T. b. gambiense* infection in human subjects in parts of Nigeria.

Materials and Methods

Procurement of trypanosomes

Trypanosomes (1 x 10^6/ml) inoculated into adult male Wistar rats were harvested at peak parasitaemia and separated from blood by DEAE cellulose (Bio-Rad) chromatography (Lanham and Godfrey, 1970).

Preparation of antigen

Purified trypanosomes were suspended in phosphate saline glucose buffer (PSG) pH 8.0 and counted using a haemocytometer. An aliquot containing 1 x 10^8 trypanosomes/ml PSG was incubated for 30 minutes at 4^{o}C and centrifuged at 600 x g for 45 minutes in Bio-Rad Centricon 10 tubes. The protein content of the antigen prepared was determined by the method of Bradford (1976).

Test Subjects

A total of 98 people were involved in the study. They consisted of 36 parasite confirmed individuals from a previous survey of parts of Delta State of Nigeria, 57 parasite-negative subjects from the same location and 5 non-endemic negative controls from Kaduna, Nigeria. Parasitological diagnosis involved the identification of trypanosomes in the blood or cerebrospinal fluid of subjects (Woo, 1970; WHO, 1983).

Card Agglutination Technique for Trypanosomiasis (*CATT* ®)

Whole blood from finger pricks of the subjects were blotted onto filter paper in the field, air dried and stored in jars containing silica gel. Dried blood samples were examined in the laboratory for haemagglutination as described by Noireau et al. (1991) using CATT test kit. Briefly, four holes were punched into blood blotted paper and each of the cut pieces was put into a cavity of round bottomed titre plates. Dulbeccos Phosphate Saline Buffer (PBS) pH 7.6 was added (50 µl/well) to the paper and incubated for 18 hours at room temperature. Equal volume of eluent (50 µl) from incubated paper and CATT test reagent were placed on the test card and stirred thoroughly for 5 mins. The card was then examined in clear light and the agglutination pattern was recorded as follows: +++ (very strong reaction), ++ (strong reaction), + (weak reaction) and – (negative reaction).

Enzyme Linked Immunosorbent Assay (ELISA)

Titration experiments were carried out to determine the optimal concentration of the antigen, antibody, conjugate, and the blocking buffer. Optimal concentrations were dilutions in which antibody-antigen reactions have a high titre with low or no background activity. Also, at such values there were no non-specific binding.

The trypanosome antigen was diluted 1:40 (5 µg/ml) with coating buffer (0.1M carbonate buffer, pH 9.6). Marxisorp immunoplates (Nunc) were coated (100 µl/well) and incubated overnight at 4^{o}C. Unbound components were poured off and the plates were washed five times in 0.3% Analar grade sodium

chloride/Tween-20 (washing buffer). Non-reactive sites in wells were blocked with 0.15M Dulbeccos phosphate buffered saline pH 7.4 containing 0.5% Tween-20 (PBS-Tween) and 2% bovine serum albumin (BSA, Sigma Chemical Company) for 1 hour at room temperature (200 µl/well). Plates were washed several times and flipped dry afterwards. Each serum sample was diluted in PBS-Tween (1:250) and added to triplicate wells at 100 µl/well. The plates were incubated for 2 hours at room temperature. After careful washing, rabbit anti-human IgG/IgM Dako horseradish peroxidase conjugate (1:500) were added to the appropriate wells (100 µl/well) and incubated for 2 hours at room temperature. Adding (100 µl/well) of freshly prepared 1% orthopenyl diamine (OPD, Sigma Chemical Company) for 15 minutes at room temperature developed the enzyme-substrate reaction. The reaction was terminated with 0.2M sulphuric acid (30 µl/well) and the optical density of each well was measured at 492 nm on a Dynatech MR4000 ELISA reader. One known positive and one negative sample were used as control on each plate.

Assay on the thirteen CSF samples were also carried out in triplicate. Wells were coated with antigen (1:10) and left overnight at 4°C. Unreactive sites were blocked after washing with 2% BSA in 0.05% Tween-20 for 1 hour at room temperature. The wells were thereafter washed thoroughly and antibody (CSF) was added at 1:10 and 1:2 dilutions for IgG and IgM conjugates respectively at room temperature for 2 hours. Rabbit anti-human IgG and IgM peroxidase conjugate (1:500) was added to wells after washing for 1½ hours at room temperature. Colour development was carried out using OPD substrate and terminated using 0.2M H_2SO_4 (30 µl/well). The optical density value of each well was read at 492 nm.

Results

The sensitivity of the ELISA from the study data was compared with the results of the CATT in the diagnosis of trypanosomiasis by the use of sera from human subjects. Table 1 shows results of both the sensitivity and specificity of CATT obtained from 98 sera which were from 36 *T. b. gambiense* positive subjects, 57 parasite-negative subjects and 5 non-endemic controls. The agglutination reaction varied from negative to very strong, the ability of the test to detect 94.4% of parasite positive subjects indicated a significant relationship between agglutination and parasite presence in subjects. The test gave a sensitivity of 94.4% sensitivity and 60% specificity.

Table 1: Performance of CATT in trypanosomiasis detection.

Source of serum	No. CATT positive (%)	No. CATT negative (%)	Total
Parasite positive subjects	34 (94.4)	2 (5.6)	36
Parasite negative subjects	39 (68.4)	18 (31.6)	57
Control	2 (40.0)	3 (60.0)	5
Total	73 (74.5)	25 (25.5)	98

Sensitivity = proportion of positive correctly identified.

Specificity = proportion of true negative correctly identified.

The positive and negative control sera used in the ELISA titration experiments for IgG gave absorbance values ranging from 0.01 – 1.35 and 0.02 – 0.23 respectively at the dilutions employed. The readings for IgM assays ranged from 0.016 – 0.759 and 0.007 – 0.386 for the known positive and negative sera respectively, establishing a gradient.

Test samples with absorbance values ≥ 0.25 and 0.19 were considered positive for IgM and IgG respective. These values were equivalent to the mean absorbance + 2SD + 0.05 estimated error of ELISA reader. Seventy-two (73.5%) of the 98 samples were IgG positive and 65 (66.3%) IgM positive while 44 (44.9%) were positive for the presence of both IgG and IgM antibodies. Table 2 shows the sensitivity and specificity values for IgG and IgM assays. The assays showed no significant difference between the IgG

and IgM antibody levels. ANOVA showed a statistically significant difference between IgG and IgM antibody levels ($P < 0.05$). Figs. 1 and 2 show the absorbance profiles of the samples for both IgG and IgM respectively.

Table 2: Sensitivity and specificity of ELISA in trypanosomiasis detection.

Source of serum	No. positive (%)	No. negative (%)	Total
IgG Assay			
Infected subjects	28 (77.8)	8 (22.2)	36
Negative subjects	39 (68.4)	18 (31.6)	57
Control subjects	5 (100)	0 (0)	5
Total	72 (73.5)	26 (26.5)	98
IgM Assay			
Infected subjects	32 (88.9)	4 (11.1)	36
Negative subjects	33 (57.9)	24 (42.1)	57
Control subjects	0 (0)	5 (100)	5
Total	65 (66.3)	33 (33.7)	98
IgG + IgM			
Infected subjects	24 (66.7)	12 (33.3)	36
Negative subjects	20 (35.1)	37 (64.9)	57
Control subjects	0 (0)	5 (100)	5
Total	44 (44.9)	54 (55.1)	98

Thirteen cerebrospinal fluid samples from 6 parasite positive and 7 aparasitaemic subjects were analyzed using ELISA. Seven (53.8%) were IgG positive, 6 (46.2%) IgM positive and 4 (30.8%) both IgG and IgM positive. the specificity recorded for the assays were 46.2% and 53.8% for IgG and IgM respectively (Table 3).

The positivity rate of detecting either IgG or IgM using ELISA for the 98 (86.2%) subjects was higher than for CATT (74.5%) though the Wilcoxon test indicated there was no statistically significant difference ($P > 0.05$) in the sensitivity of the two assay methods. Figs. 1 and 2 also show absorbance values for IgM and IgG; IgM had a higher absorbance value at 492 nm though there was no significant difference in the values ($P > 0.05$).

Discussion

The findings of the tests indicate that the *T. b. brucei* extract used as antigen for the ELISA gave a higher specificity that CATT. The ability of ELISA employed to identify all the parasite-confirmed subjects in the study made it superior to CATT which showed 8.4% false negative diagnosis. This higher detection ability of ELISA over CATT corroborates the findings of Aiyedun et al. (1978). These workers compared ELISA with other serological tests and reported a higher sensitivity in ELISA. However, the results for the different immunoglobulins showed a 22.2% and 11.1% false negative diagnosis for IgG and IgM

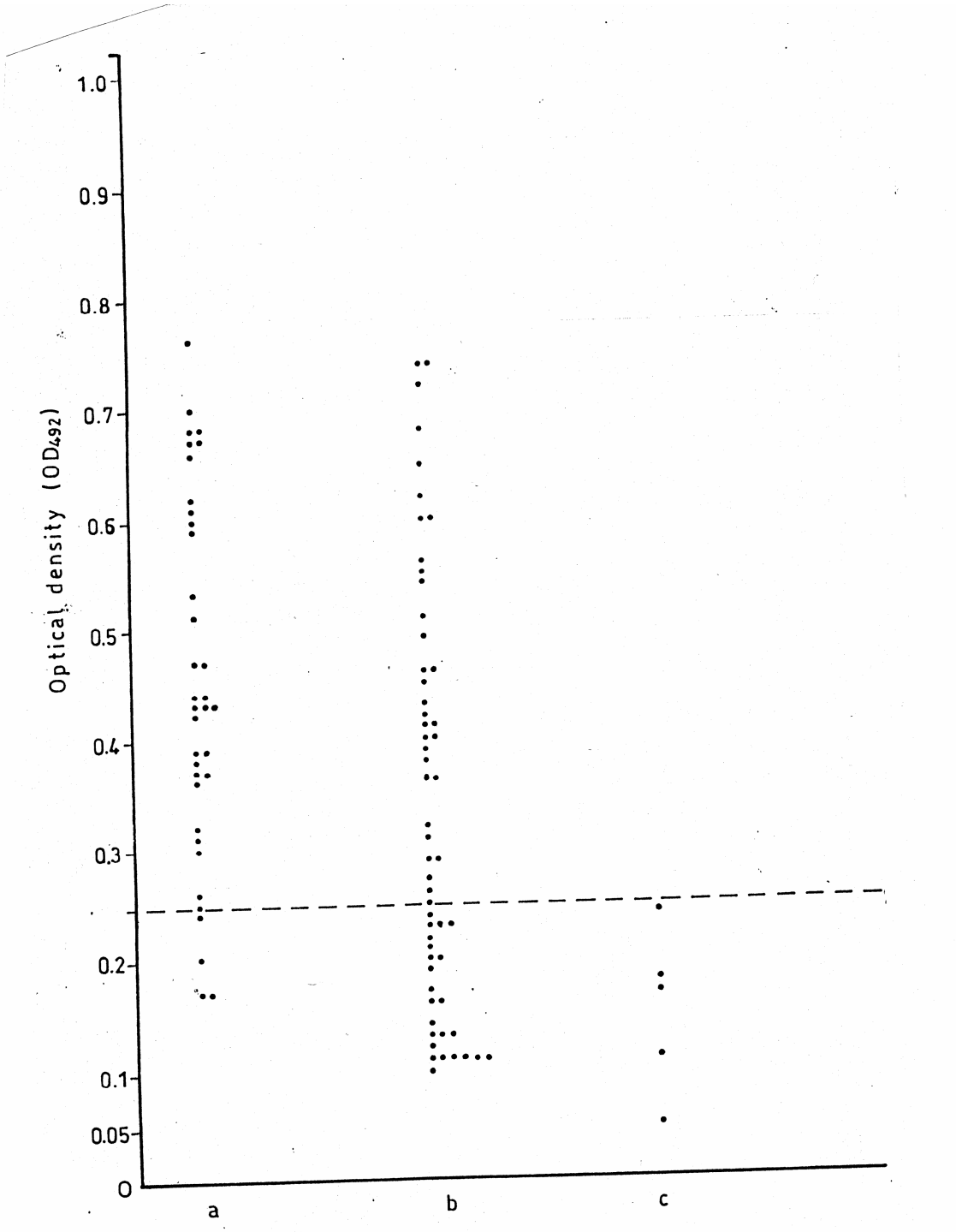

Fig. 1. Optical density (O.D) readings showing detection of parasite induced IgM in a) Field sera from T. b gambiense positive subjects b) Parasite negative subjects c) controls from trypanosome-free areas. O.D values of 0.25 (horizontal broken line) and above were regarded as positive.

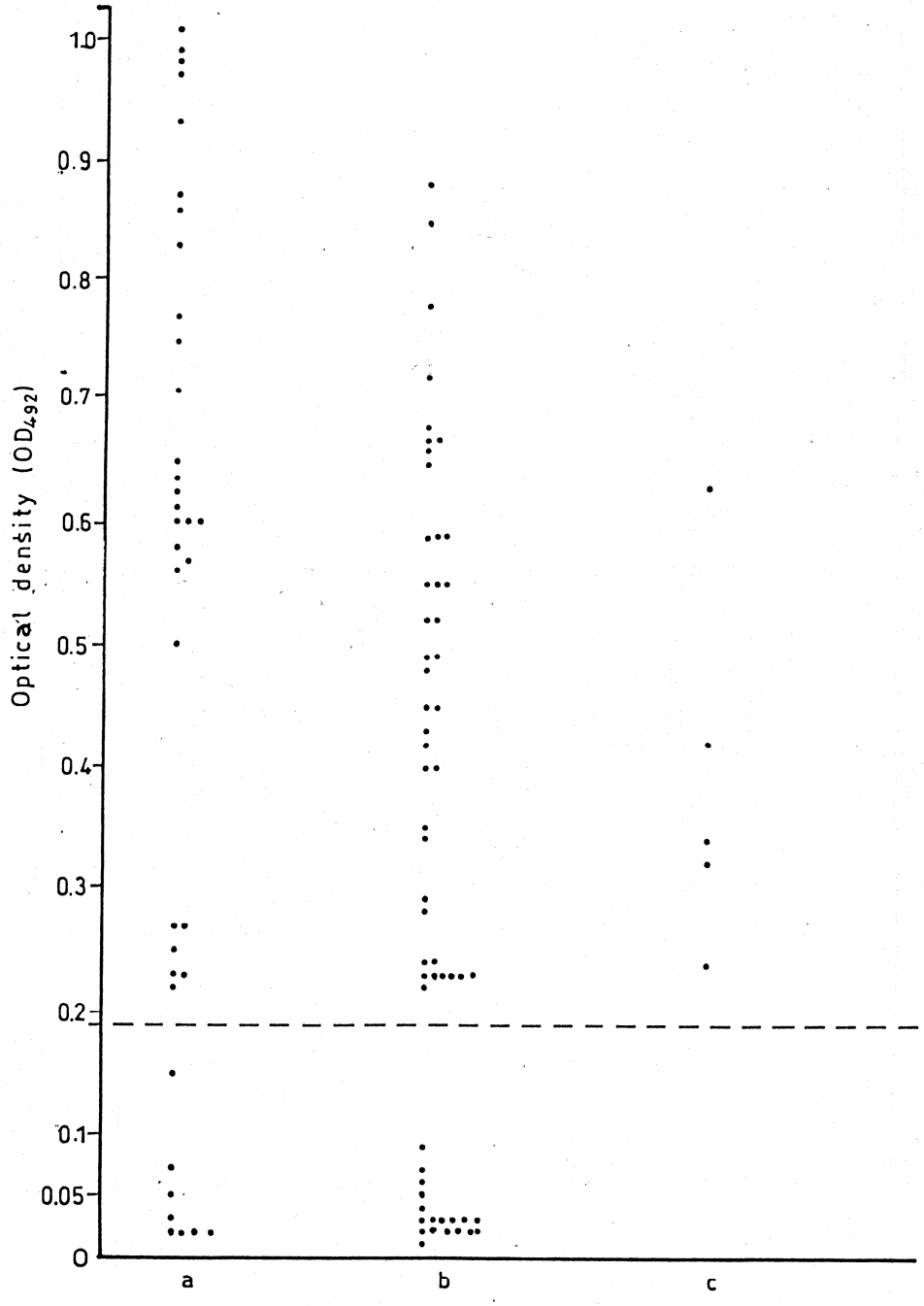

Fig. 2. Optical density (O.D) for parasite induced IgG level in human sera from a) T. b gambiense positive subjects b) T. b gambiense negative subjects c) Control subjects. Horizontal lines indicate cut-off point (0.19 and above is positive).

respectively in the identification of parasitologically confirmed subjects. This could probably have been lower if *T. b. gambiense* extracts were used as antigen in place of the *T. b. brucei* extract. A small proportion of the subjects that were sero-positive from the results of the assays were parasite negative. They could be infected subjects that were not identified in parasitological screening due to the fluctuating nature of the parasites which renders parasitological methods less effective in detecting infected subjects.

Table 3: Detection of trypanosome infection in human CSF using ELISA.

Source of serum	No. positive (%)	No. negative (%)	Total
IgG Assay			
Parasite positive subjects	4 (66.7)	2 (33.3)	6
Parasite negative subjects	3 (42.9)	4 (57.1)	7
Total	7 (53.8)	6 (46.2)	13
IgM Assay			
Parasite positive subjects	4 (66.7)	2 (33.3)	6
Parasite negative subjects	2 (28.6)	5 (71.4)	7
Total	6 (46.2)	7 (53.8)	13

The higher level of IgM absorbance values could be explained by the observed 8 to 16 times rise in serum IgM in Gambian trypanosomiasis reported by White et al. (1977). He further suggested that in surveys over 95% of cases of trypanosomiasis could be detected by IgM measurement. Raised serum IgM consists of antibodies against surface antigens of trypanosomes. The comparatively high IgG titres in the study could also be attributed to the fact that IgG antibodies are easier to detect and are therefore of higher value in diagnosis (Whittle et al., 1977).

The IgG seropositivity recorded in some control subjects outside the endemic area could indicate an overlap between normal values and those of patients since the study showed no relationship between IgG values and parasitaemia. This is similar to that in the report of Cunningham et al. (1967) in which such an overlap was observed in Rhodesian sleeping sickness. However, attention would be paid to these subjects in subsequent surveys to establish their infection or otherwise. Furthermore, based on the high level of specificity and sensitivity recorded in this study, it has been shown that secretory extracts of the parasite have antigenic characteristics and it would be recommended that extracts of *T. b. brucei* could be used as antigen for serodiagnosis of human trypanosomiasis. This would reduce the risk of infection involved in using *T. b. gambiense*. Although CATT has been recommended as a technique for field screening (Noireau et al., 1991), the result of this study suggests that ELISA should further confirm such field results in the laboratory. This would be necessary particularly if interest were not only on the disease profile in a locality but also in individual cases.

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