EVALUATION OF PARASITOLOGICAL AND SEROLOGICAL DIAGNOSTIC METHODS FOR THE DETECTION OF SLEEPING SICKNESS INFECTION IN ETHIOPIA

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BY
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Comparison of Parasitological and Serological Methods for the diagnosis of African Sleeping Sickness in Ethiopia

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ABSTRACT

Combinations of parasitological and serological methods of diagnosis are becoming standard procedures in epidemiological studies of African trypanosomiasis (sleeping sickness). This is because no single test is specific, sensitive and reproducible enough for an effective diagnosis of this fatal disease. The uncertainity of the relative merits of different diagnostic tests for human trypanosomiasis, particularly for subclinical cases, prompted undertaking the present comparative study in the only known endemic focus of the disease, Gambella, in south western Ethiopia and in two other areas, suspected to be endemic Anger Didessa, in Wollega, and Bodi, in Gemu Gofa. The Parasitological tests included were wet film preparation for clinically suspected individuals, stained blood films (thick and thin) and the Micro-Haematocrit Buffy Coat Technique (MHBCT). Stained films and the MHBCT revealed a positivity of 0.95% where as the wet film preparations were all negative. The serological tests used were IFAT and ELISA, based on crude antigen preparations of T.b. rhodesiense and T.b.brucei. IFAT revealed a 10% positivity in the known endemic area and 6.9% and 7.6% in the suspected foci of Anger Didessa and Bodi, respectively. ELISA values were 14.1% for the endemic area, 9.9% and 8.6% for the two suspected areas, Anger Didessa and Bodi,respectively. Specificities of 92.0% for IFAT and 88.4% for ELISA were demonstrated. It was also shown that both T.b.brucei and T.b.rhodesiense antigens can be used with equal efficiency for the demonstration of anti-trypanosomal antibody in human sera.

INTRODUCTION

According to Bloom (1979), between 500 to 1000 million people in developing countries suffer from tropical diseases and other parasitic infections. Epidemiological studies conducted so far show that it is unlikely that any continent has ever been dominated by a single disease to the extent that Africa is dominated by trypanosomiasis (Murray and Trial, 1987). Trypanosomiasis has profoundly affected the health and socio-economic development of Africa for centuries by causing death of human beings and domestic animals (Ford, 1971; Marray and Trial, 1987).

African trypanosomes are microscopic parasites of mammals belonging to the Kinetoplastid protozoa. They are found in blood tissues and body fluids of human beings and other mammals (Hoare, 1972; ILRAD, 1984) and sometimes cause fatal disease. The disease is manifested as a syndrome of which the final stage is sleeping sickness in man and nagana in cattle (FAO, 1983; ILRAD, 1984; De Raadt, 1984).

Human trypanosomiasis or sleeping sickness, in Africa is caused by two subspecies of Trypanosoma brucei -T.b. gambiense and T.b. rhodesiense occuring in West and Central Africa, and in East Africa, respectively (WHO, 1982,1983).

Records for devastation by sleeping sickness in Africa go back as far as the early 19th century. Epidemics continue to occur even today in the affected countries (WHO, 1986). Human trypanosomiasis occurs in about 200 distinct foci which are

scattered over the tsetse belt of Africa. The disease is endemic in 37 African countries covering 37% of the total land mass of the continent. Its distribution is defined by the range of tsetse infestation (Kolata, 1984; WHO, 1986; Murray and Trail, 1987). Among the affected countries, 23 are endemic for *I.b. gambiense* and the rest for *I.b. rhodesiense* (WHO, 1982, 1983) in which about 50 million people are at risk and about 20,000 new cases appear every year. However, a large proportion of the infected population remains undetected due to socio - economic and political problems. In addition, inadequate methods of diagnosis make the situation even more difficult.

Like in many other African countries trypanosomiasis is endemic in Ethiopia. Animal trypanosomiasis in Ethiopia was noticed as early as in the 18th century, when foreign travellers lost their pack animals in the south western and eastern regions of the country. However, human trypanosomiasis has a short documented record as compared to other African countries. An early record of sleeping sickness in Ethiopia was by Sheppared in 1946 (quoted in Gibson *et al*; 1981) who reported that the disease existed in the south western regions of Ethiopia, near the endemic foci in the Sudan and Uganda. The insect vectors of the *Glossina* species, were also abundant in the region. In addition Kunert in 1956 (quoted in Langridge, 1976) had reported sleeping sickness cases in the low-lying regions of the country to the west and south and that there had been epidemic conditions along the Omo

Valley, where the Mursi and the Bodi people live. These reports were good indication of the possible presence of human trypanosomiasis in Ethiopia, long before the first actual parasitologically positive cases were recorded.

There was no definite record of the disease in Ethiopia until March 1967 when the first two cases were identified from Maji Kaffa (Baker et al., 1970; Hutchinson, 1971). In the same year, two more cases were reported from Gambella - Illubabor, along the Baro River (Hutchinson, 1971). Until the end of 1968, 32 cases were reported from this area. Based on these reports McConnell et al.(1970) conducted a survey in the main suspected foci along the Gilo River in 1969. During that period there was an alarming outbreak of sleeping sickness along the Gilo River. Between 50 to 100 people died in the areabefore the disease was recognized by the villagers, who later started seeking medical aid. The epidemic mostly affected villages along the Gilo river area, where 210 people died. The epidemic subsided at the end of 1970 and by that time 260 cases were recorded, all from Illubabor and Keffa (McConnell and Baker, 1974). Since then, self-reporting cases appeared now and then in the nearby clinics and the hospital in the town of Gambella (Mekasha, 1983).

The trypanosome parasite responsible for the disease outbreak was identified, on clinical grounds as T.b. rhodesiense (Baker et al ., 1970; Hutchinson, 1971). The origin

of its introduction may have been from the nearest endemic focus at Lake Victoria, 1000 km south in Uganda and Nyanza province in Kenya (Gibson, et al ., 1981; Hutchinson, 1971). However Baker et al. (1970) had suggested a recent Sudanese origin for the disease in Ethiopia, in spite of the fact that there was no evidence for the endemicity of T.b. rhodesiense in the Sudan.

According to Hutchinson (1971), sleeping sickness is probably not recently introduced to Ethiopia, even though the epidemic that occurred along the Gilo River was a new phenomenon. The cause for the epidemic may have been that people from Sudan and Gambella were attracted to Dembella to mine gold near the Akobo River. Thus additional cases of the disease may have been imported from the neighbouring endemic foci, creating suitable conditions for the outbreak. Such a view is held by Gibson et al. (1981), who suggested that sleeping sickness in Ethiopia may have existed at low level of endemicity for years, without recognition.

Since the identification of T.b. rhodesiense infections in 1967 in Illubabor and Keffa regions, sporadic cases are appearing and cases have continued to be reported from those regions, as well as from other suspected areas.

Though there is no complete epidemiological study depicting the level of prevalence of the disease in Ethiopia, the reports of WHO (1979, 1986) show that the disease is established at an endemic level. The low level of prevalence of the disease, might

be due to lack of active surveillance using effective diagnostic methods, together with an indadequate procedure of reporting cases. Despite the fact that population movement is frequent between Ethiopia and the neighbouring Sudan, where T.b. gambiense is known to occur,in the Gambella, Gurafereda and Dimma areas, no record of introduction of the parasite into these areas is known (Hutchinson, 1971; Baker et al., 1970). However, one cannot exclude the possibility of future introduction of T.b. gambiense into the region and an increase in the level of prevalence of T.b. rhodesiense. This is predictable from the ever increasing population in the area, as the result of increased agricultural activities and the ongoing resettlement programmes. Thus, the need for adopting effective diagnostic method exists to determine the actual prevalence and the incidence of the disease, and to conduct surveillance activities in endemic and suspected areas of the country.

Immunodiagnosis of sleeping sickness and production of vaccine is made more difficult by the ability of the parasite to survive against the host's immune response by changing its antigenic surface glycoproteins (antigenic variation) (Turner, 1984). The clinical symptoms caused by the disease may be confused with other infections. So far, no single test is adequately specific, sensitive and reproducible enough for clinical diagnosis and field epidemiological studies. Thus the need to improve the diagnostic tests for trypanosomiasis infection has been felt for a very long time (Woo, 1971; WHO, 1976; Magnus et al.,1978).

At present the routine diagnosis of sleeping sickness is based on clinical, parasitological and serological tests (Vanmeirvenne and Le Ray, 1985; ILRAD, 1988). Sleeping sickness due to T.b. gambiense is often a symptomless, chronic disease (WHO, 1982). Infection persists for a long period (more than two years) and the patient produces large quantities of circulating antibodies against several different antigens produced by trypanosomes during antigenic changes (Molyneux et al., 1984; Turner, 1985). Also, the detection of the parasite in the blood is very difficult due to the low parasitaemia. As the disease progresses, clinical symptoms become more elaborate. Surveillance of the disease is primarily based on the palpation of cervical lymph nodes (winterbottom's sign), aspiration of lymphatic glands and of the cerebrospinal fluid (CSF), which can be performed under field conditions.

The disease caused by T.b. rhodesiense is an acute and more virulent type, which kills the patient within a short time. The involvement of central nervous system and enlargement of cervical lymph nodes is less pronounced and not of diagnostic value. Also, fluctuations in parasitaemia may make parasite detection in the peripheral blood very difficult, unless the patient is febrile. This makes it necessary to make several daily blood examinations for diagnosis (Molyneux and Ashford, 1983).

On the other hand, clinical diagnosis in some patients may indicate such signs as mental dullness, apathy and nervous disturbance (De Raadt,1984; Poltera, 1985). Such cases are

misdiagnosed and may be treated for the apparent psychiatric signs. Such misdiagnosis of sleeping sickness cases delays timely and appropriate treatment and management of patients.

Therefore, since the symptoms in sleeping sickness are nonspecific, clinical diagnosis may be confused with other diseases. Differential diagnosis for the disease is most reliably based on parasitological methods.

Among the parasitological techniques, wet film preparation is the simplest, least expensive and sensitive, when the parasitaemia is high. The test is less sensitive for the detection of low parasitaemias and requires examination of more than 20 per high power fields before a slide can be considered negative (Lumsden et al., 1973; WHO, 1983). Examination of slides must be done within 10 to 15 minutes of preparation of the wet film, since the parasites are recognized by their movements and will die if kept any longer. Moreover, the technique may be of particular value especially in T.b. rhodesiense infections during the period of peak parasitaemia.

The other parasitological diagnostic method is stained blood film preparation. The test is less sensitive but more convenient for mass survey especially in areas where T.b. rhodesiense is endemic. The thin blood film reveals individual red blood cells enabling an easy identification of the parasite. In the thick blood film preparations the chances of detecting the parasite is high in a limited microscope field even in a low parasitaemia since the blood is concentrated at one point.

The more elaborate and sensitive parasitological tests developed for field application are the concentration methods. These are the Micro Haematocrit - Centrifugation Buffy Coat Technique (MHBCT) (Woo, 1971; Murray, et al ., 1977; WHO, 1983) and the Miniature Anion Exchange Centrifugation Technique (M-AECT) (Lumsden et al ., 1979; WHO, 1983). They are used to detect low parasitaemias.

The MHBCT also known as the " Woo method"(Woo,1971) was found effective for detection of trypanosomes and microfilariae in the buffy coat fraction of blood following centrifugation in a micro-haematocrit capillary tube. The technique is useful for individual diagnosis as well as for mass surveys. The same test can enable one to correlate the haematocrit value (which indicates possible anaemia) and the level of parasitaemia (FAO,1983; WHO, 1983). The technique is very sensitive because it concentrates parasites from a small volume of blood (50 to 60 ul). Its limitations are that it needs a microhaematocrit centrifuge and electric power which may not be easily available under field conditions. In addition, detection of the parasites in the buffy coat requires experience. Other associated difficulties are breakage of the capillary tubes or spillage from the sealed end during centrifugation, coagulation of the blood and incorrect centrifugation speed.

The other concentration technique is the M-AECT. The test is well adapted for field application especially in areas where T.b. gambiense is endemic (Gashumba, 1981; WHO, 1983; Sachs, 1984).

The principle behind the test is the capacity of the cellulose ion exchange powder, DE-52, that is equilibrated with a buffer and its pH adjusted so that it has a static electrical charge. Thus when infected blood is allowed to pass through a column of the equillibrated DE-52, the negatively charged red blood cells are bound to the cellulose while the positively charged parasites are eluted to the collecting tube. This is due to the fact that the surface charges of the parasites and the red blood cells are different. The test is also used for the detection of blood microfilariae (Gashumba, 1981). This method allows separation of African trypanosomes from large quantities of blood (Lumsden et al., 1979). Quantitative determination of parasites is possible by this method. The limitation of this test is that it needs electrical equipment and experienced technicians to correctly detect parasites in the eluate collected following centrifugation. Under well equiped laboratory condtions, visualization of the centrifugate can effectively be accomplished by using the veiwing chamber developed by Lumsden et al. (1979). The M-AECT is superior to other tests, although it is expensive and requires a sterile column and media in order to avoid misdiagnosis due to contamination with other micro-organisms.

Still other methods used to concentrate trypanosomes, especially from cerebro - spinal fluid (CSF) specimens, are the simple and double centrifugation methods (WHO, 1983). In the simple centrifugation procedure blood or CSF is centrifuged

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and from the pellet a drop is taken and examined microscopically. In the double centrifugation procedure, the pellet from the simple centrifugation step is suspended in a drop of fluid left in the tube transfered in a microhaematocrit tube sealed and centrifuge in a microhaematocrit centrifuge. The tip of the tube is observed microscopically for the presence of trypanosomes.

Among the parasitological diagnostic methods in clinically suspected patients, is the inoculation of susceptible laboratory animals (usually rats or mice) with blood or CSF from patients. Parasitaemia will develop in such animals after some period of time if the specimen contains trypanosomes. However, the method does not provide an immediate diagnosis. Furthermore, the method does not apply for both human parasites equally. For example, the detection and isolation of T.b. rhodesiense parasites from the laboratory mice is more readily accomplished due to the high parasitaemia. On the other hand, the method is almost totally ineffective for T.b. gambiense infections, which characteristically have a very low parasitaemia.

It is a common understanding that the best way of diagnosis is to find and identify the parasite itself. On the other hand, failure to detect parasites does not necessarily indicate that there is no infection. It may simply be an indication of the low level of sensitivity of the direct diagnostic method.

It is therefore often necessary to use additional indirect methods of diagnosis, such as sero-immunological tests to obtain information both on current infection and on previous history of exposure to the infectious agent (Voller, et al ., 1977; Beaver, et al ., 1984).

In recent years sero-immunological diagnostic tests were developed for most of the common parasitic infections in man. Several such tests have been developed for African trypanosomiasis, since most of the parasitological techniques suffer from lack of sensitivity in detecting cases with low parasitaemia and during the remission phase of the parasite cycle.The basis for the serological test is the fact that antibody production in human sleeping sickness starts within 2 to 4 weeks of infection (Vickerman, 1985).

Among the serological tests, the most widely applied are the indirect Fluorescence Antibody Test (IFAT) and the Enzyme Linked Immunosorbent Assay (ELISA), for the determination of anti-trypanosomal IgM levels. Recently, the Card Agglutination Test for Trypanosomiasis (CATT) has also been introduced for T.b. gambiense (Magnus, et al ., 1978; WHO, 1983).

Estimations of the elevation of serum IgM levels are used as an epidemiological tool for screening populations by determining the anti-trypanosomal serum IgM (Cunningham, et al., 1967). High titres of IgM have been shown to indicate current infections. Determination of IgM levels in African sleeping sickness

infection has been evaluated and its application for diagnostic purposes considered favourably in a comparative evaluation of serological tests (McConnell, et al ., 1970). It was also found that an increasing amount of IgM in CSF of patients with sleeping sickness correlates well with the progress of the disease (Houba, 1981). Production of large amounts of IgM both in the serum and the CSF (several times the normal value), in patients with early diseases and in a late stage of infection, was also established.

IFAT has been well evaluated and widely applied for the diagnosis of many parasitic infections, both for individual clinical diagnosis and for mass screening in epidemiological studies. The high sensitivity of IFAT in serum samples is impaired by its lower specificity. Cross reactions with other protozoal infections such as malaria (Houba, 1981) and visceral leishmaniasis (Voller, et al ., 1975; Ruitenberg, et al ., 1977) have been reported. So in areas where other protozoal diseases are co-endemic, and increase in false positivity rates may occur. However, these problems may be overcomed to some extent by testing the sera at high dilutions or grading the amount of fluorescence emitted.

CATT is another useful test for the diagnosis of trypanosomiasis infection due to T.b. gambiense (Magnus, et al., 1978; WHO, 1983; Turner, 1985). The test is easy to run and

appropriate for screening large population. The test can easily be applied in remote areas where no electricity is available, since manual agitation for activation of the antigen with patients blood is adequate. The use of whole blood for the test in the field gives on-the-spot diagnosis of infections. However, CATT positive cases need to be followed up by parasitological methods, especially by the M-AEC technique, for five to six consecutive days for a more definitive diagnosis. The serological tests, ELISA, was also adapted for the diagnosis of trypanosomiasis by Voller, et al. (1975, 1976). High sensitivity was demonstrated by using T.b. brucei antigen for patients infected by both parasites (T.b. gamiense & rhodesiense). A positivity of 94% for T.b. rhodesiense and 100 % for T.b. gambiense was demonstrated in a WHO collaborative study conducted by Voller, et al . (1976). ELISA gave better results, compared with other serological tests such as IFAT, gel diffusion and the Radio Immuno Assay using the same antigen (Voller, et al ., 1977). The test is quantitative and is relatively simple but there is a problem of reproducibility as a result of variation in antigen preparation, fixation of antigen to the solid surface and the choice and preparation of the enzyme conjugated anti-immunoglobulin (Houba, 1981). In addition, there is the problem of cross-reactivity with sera from patients with leishmaniasis. As it gives a quantitative determination, ELISA is preferable to IFAT (Voller and DeSavigny, 1981) and is further advantageous

because the conjugate is more stable and can be stored for a much longer time if refrigerated. Furthermore, the recent developments of monoclonal antibodies specific to particular trypanosome antigens is making sandwich ELISA more applicable (ILRAD, 1984; 1988) for antigen detection in both human and animal trypanosomiasis in Africa.

The main objective of this study was to detrmine the most reliable and practical method for field detection of human trypanosomiasis cases in one known endemic focus and in other two suspected areas in Ethiopia. The study will validate parasitological and sero-immunological diagnostic methods for their sensitivity and specificity in detecting sleeping sickness cases.

MATERIALS AND METHODS

A. Description of study areas

1. Gambella

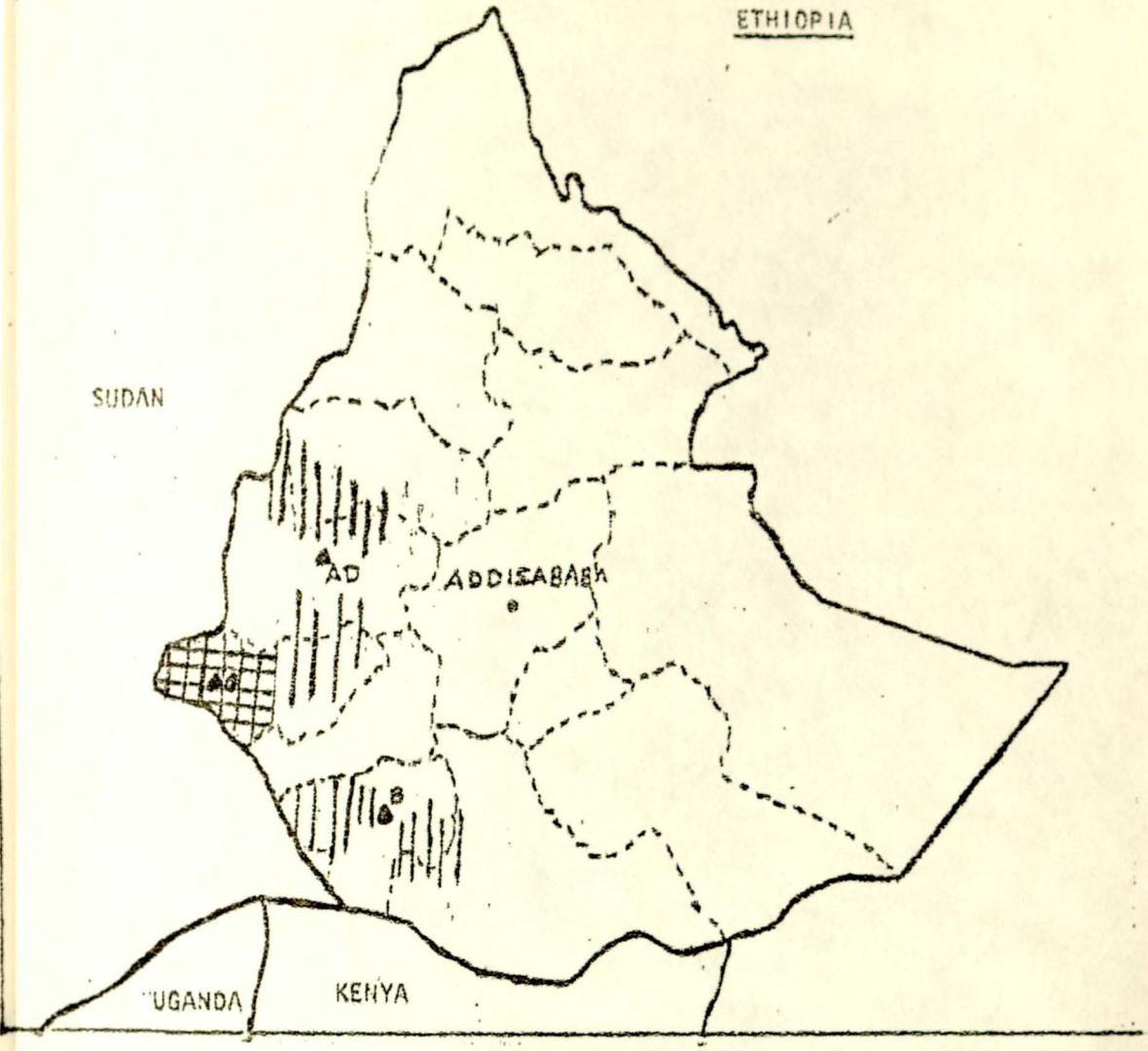
Gambella adminstrative region is the only confirmed endemic area for human sleeping sickness in Ethiopia, (Figures 1 and 2). Gambella is located about 777 km from Addis Ababa on the western escarpment of the central ethiopian massif, latitude 8° N and longitude 35° E. The Baro river, and several other small to moderately sized rivers, crisscross the region. The elevation of the area ranges between 450 and 500 metres above sea level. This study was conducted at Abobo, which is one of the districts about 44 km from the town of Gambella. The study area was selected because it was one of the areas in which many sleeping sickness cases were reported, an intensive resettlement programme is in progress and large state farms are being created. The agricultural developments have drawn a large labour force into the area.

The rainy season begins in late April and continues until the beginning of November with a mean annual rainfall exceeding 1000 mm (Ethiopian Meterological Service, 1979). The rest of the period from late November to the beginning of April is hot. The months of February and March being the hottest. The vegetation of the area ranges from wooded dry savanna grassland to wooded forest in Gok and Jore areas. The dominant trees are *Combretum*, *Acacia* and *Ficus* spp.

A variety of game animals such as warthog, wild pigs, dik-dik, bushbuck, antelopes and different types of monkeys used to be present in large numbers. However, at the moment the number of these animals is decreasing due to extensive hunting and bush clearing.

Due to high tsetse fly challenge and transmission of trypanosomiasis, there were no cattle or other domestic animals in the area until 1986, except dogs and chickens. At present there are some domestic animals, such as cattle, goats and sheep owned by the resettled population. The state farm projects and the resettlement programmes have brought about great ecological change in the area. Due to vegetation clearing, there is a great reduction in the tsetse fly population as compared with the condition before the programmes started. Also, game animals are leaving these areas due to a lack of food and shelter and beacuse of intensive hunting. Glossina species that occur in Gambella are G. morsitans submorsitans, G. tachinoides, G. pallidipes and G. fuscipes fuscipes (Baker et al., 1970;Hutchinson, 1971; Langridge, 1976).G. morsitans is widely distributed and appears to be the important vector in the spread of sporadic cases from natural game reservoirs infection and perhaps G. tachinoides is also involved as well as G. pallidipes and G. fuscipes but to a lesser extent.

The indigenous people of the region, the Anuak, mainly live along the Baro, Gilo and Akobo rivers. They cultivate maize and millet at a small scale subsistence level. The majority are also



LEGEND

AD- Anger Didessa

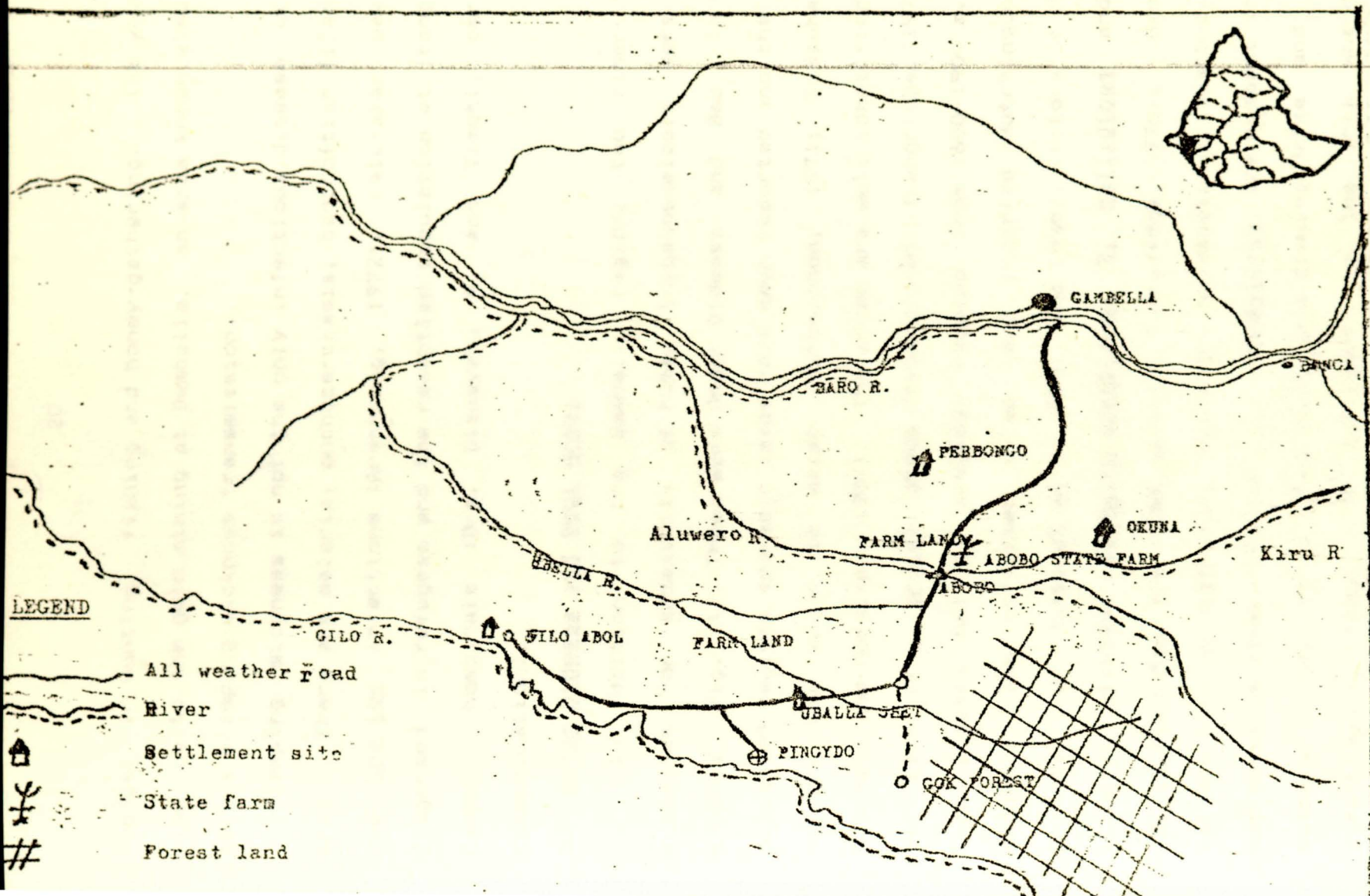
B - Bodi

G - Gambella

T.b. rhodesiense area

T.brucei group area

Fig 2. SKETCH MAP OF THE GAMBELLA SYUDY AREA.



engaged in hunting, fishing and honey-gathering. The young men also go to the gold mining at Dembella, an area known also for active sleeping sickness transmission.

Sleeping sickness is not the only infectious disease of the area. There are malaria, onchocerciasis, bancroftian filariasis and Loa loa infections (Armstrong, 1972). Kala-azar was diagnosed in refugees and the resettled population at Itang and Gambella hospitals (Drs. Bizuayehu A. and Timoti, personal communication).

2. Anger Didessa and Bodi Areas

In addition to the Gambella region, two other regions suspected for endemicity of human trypanosomiasis were also studied (fig. 1). These were Anger Didessa and Bodi. In the two suspected regions sporadic cases have been reported and the potential vectors are known to exist (Hutchinson, 1971; Fisseha 1987; Olfasson and Aorsland, 1981). The areas are well identified to be the belt of T. brucei group Trypanosomes (Langridge, 1976). Anger Didessa has an altitude ranging from 1600-1900 m. Both suspected and confirmed cases were reported (Hutchinson, 1971; Fisseha, 1987; Abebe et al ., 1988). The insect vectors of human sleeping sickness Glossina morsitans, G. pallidipes and G. tachinoides, are known to exist (Langridge, 1976). Game animals such as wild pigs, warthogs, hipoppotamus, bushbuck, that may serve as reservoirs for T.b. rhodesiense, are present in large numbers. There is a large state farm growing Maize and other agro-industry crops (e.g. sunfower), in the area where many

laborours are present. Since 1974, there is also resettlement programme from the drought affected areas of the country.

The second suspected area included in the study is Bodi,in Gamu-Gofa. Here a few sporadic cases were recorded, with some clinically suspected patients who died before diagnosis (Olfasson and Aorsland, 1981). In addition to the presence of a large population of a variety of game and domestic animals, and the presence of potential vectors, G. morsitans and , G. pallidipes (Hutchinson, 1971; Langridge, 1976) were additional reasons for suspecting Bodi as a possible endemic area. Inhabitant of Bodi are nomadic. They feed mainly on milk mixed with blood collected by puncturing the vein of cattle. They grow maize and sourghum on a small scale .

B. Parasitological Methods

Parasitological detection of cases were made on 416 samples by using the Micro-Haematocrit Buffy Coat Technique (MHBCT) (WHO, 1983). Briefly, about 50 ul of blood was taken in heparinaized haematocrit capillary tubes in duplicate from each patient. One end of the capillary tube was sealed with cristoseal and the sample centrifuged in a microhaematocrit centrifuge for 5 minutes. Trypanosomes and blood microfilariae were detected in the buffy fraction of the capillary tube at 10 and 40 magnification, of the microscope.

Preservation of the isolated trypansome parasites was done according to the procedure described by Lumsden, et al . (1973).

In brief, from parasitologically confirmed and clinically suspected cases, about 0.2 ml of blood was inoculated into mice intraperitoneally. Parasitaemia in the mice was checked by taking blood (for wet-film) by tail snip. When the parasitaemia reached 16-32 parasites per high power field, the mice were killed and the blood collected aseptically from the heart, in a 1ml syringe with a 23Gx1 gauge needle containing 0.1ml heparinaized Phosphate Buffered Saline with Glucose, pH 8.0 (PBSG). The collected blood was mixed in an equal proportion with 7.5% glycerol as suggested by Lumsden et al. (1973), and the mixture dispensed into unheprinaized haematocrit capillary tubes. The capillaries were sealed at both ends by a micro-burner. The sealed capillaries were placed inside screw capped test tubes containing methanol. The stabilate was slow cooled, by keeping it in an insulating jacket made of polystyrene, sealed with adhesive tape at the lid and placed into the deep freezer (-80 ^{o}C) for 24 hours. The test tubes were then taken out of the cooling jacket and cryopreserved as a stabilate by directly placing in the deep freezer.

The other parasitological method was thin and thick blood smears prepared following the standard procedures recommended by WHO (1983). The smears were stained with Giemsa and examined for the presence of trypanosomes and other haemo-parasites, at a magnification of X 1000. In addition to the thin and thick film preparations, wet films were prepared and examined on the spot for all clinically suspected cases.

C. Collection of Sera

Using venoject tubes, 210 blood smples from the endemic area (Gambella) were collected. Six people previously known to be parasitologically positive (2 untreated and 4 treated) were included in this collection, these were used as positive controls. The blood was allowed to clot and centrifuged for 10 minutes using an omnifuge centrifuge (Hearaeus Christ, GMBH, Osterode). The serum was separated in the field in labelled plastic vials and was transported to the central laboratory, in liquid Nitrogen . In the laboratory the serum samples were stored in a deep freezer at -80 °C until assayed. Using the same procedure, 206 serum samples were collected and processed from the two regions suspected for sleeping sickness endemicity; 105 from Bodi, and 101 from the Anger Didessa Valley .

Negative control sera were also collected from the same area from inhabitants parasitologically proven negative as well as from Addis Ababa, where tsetse challenge does not occur.

Sera from patients with malaria (*Plasmodium falciparum* and *P. vivax*), from Gambella and leishmaniasis (*L. donovani*), schistosomiasis (*S. mansoni*) from Institute of pathobiology, were also collected for determination of the specificity of the serological tests.

D. Serological Methods

(i) Antigen preparation

(a) Indirect Immunofluorescence Antibody Test Antigen Slides

Local whole parasite antigen was prepared from *T.b. rhodesiense* strains isolated from Gambella and Wollega. Cryopreserved parasite of each species were obtained from the NRIH and inoculated into mice intraperitoneally. The parasitaemia was checked by taking blood by tail snip. When the parasitaemia reached 60-70 trypanosomes per high power field, blood was drawn by cardiac puncture using a 1ml syringe containing 0.1 heparinaized PBSG (10 iu/ml) fitted with a 23Gx1 gauge needle.

Typical thin films, covering the whole surface of the microscope slide were made and allowed to air-dry. The slides were wrapped in blotting paper in batches of five slides and fastened together with scotch tape. The slides were then placed in polyethylene bags with silica gel, sealed and stored in a freezer at -80 °C until used . The change in colour of the silica gel (from blue to white, indicate the presence of moisture in a sealed bags) was checked now and then.

(b) Enzyme Linked Immunosorbent Assay (ELISA) Antigen

Antigens for the ELISA were prepared from *T.b. brucei* isolates from cattle (Gamu Gofa strain) and *T.b. rhodesiense* isolated from humans (Gambella & Wollega strains). Stabilates of the two species were obtained from NRIH, Addis Ababa. The viability of the cryopreserved parasites was checked by wet film

examination and viable stabilates were inoculated intraperitoneally into mice for amplification. When parasitaemia reached antilog 7 (Herbert and Lumsden, 1976), the mice were killed and the blood collected asceptically by cardiac puncture with a 1 ml syringe containing 0.1 ml of heparinaized PBSG, pH 8.0, and fitted with a 23Gx1 gauge needle. The trypanosomes were separated from the blood by filtering the sample through a Preswollen Diethyl aminoethylene cellulose column or DE-52 cellulose column (DEAE, DE-52, Whatman, England).

The eluate was collected by keeping the collecting tube on ice. The eluted trypanosomes were separated by centrifugation in a super speed centrifuge (Sorval, Rc2-B, USA) at 1400g for 15 minutes at 4 °C. The pellet was washed with PBS three times. The supernantnt was discarded and the pellet stored at -80 °C until used. For use in the assay, the stored trypanosome preparations were resuspended in PBS containing protease inhibitor, Phyenyl Methyl Sulfonyl Floride (PBS -PMSF).

Following one cycle of freezing and thawing, the trypanosomes were disrupted by ultra sonication in a ultra sonicator (Ultrasonic B-12, USA) at 60 watts for 20 seconds with intermittent cooling for 30 seconds for a total of 4 minutes in ice. The sonicated preparation was then sedimented by supernatant was separated and its protein content determined spectrophotometerically at 550 nm by the Biuret method. The antigen prepared was stored at -80 °C as a stock antigen, in 0.5 ml aliquots in small plastic vials, until used.

(ii) Indirect Immunofluorescence Antibody Test(IFAT) Procedure

A total of 416 samples were tested by this method using locally prepared antigens. For running the test,the antigen slides were taken out of storage at - 80 °C and incubated for one hour at 4 °C and then at room temperature for 30 minutes. The antigen slides were taken out of the polyethylene bag package and eight small rings (made with a diamond marker on the smear) where the blood film appeared to be most even. The slides were fixed in acetone for five minutes and washed twice with phosphate Buffered Saline (PBS), pH 7.2 for 5 minutes each and then rinsed with distilled water for 30 seconds followed by air drying.

The optimal dilutions of the serum and the conjugate that would give best discrimination between positive and negative sera was determined by running the test on a dilution series. That is, two fold serial dilutions of the positive and negative control sera were tested 1:10 through 1:1280 with a two fold dilution series and of the conjugate 1:25 though 1:800. Based on the findings it was determined that the sample sera should be tested at 1:40 dilution and the conjugate at 1:100 dilution. In each series of tests positive and negative controls, at dilution of 1:80 and 1:20, respectively, were included. This is based on the quantitative test for screening populations for sleeping sickness infection recommended by the standard procedure of the Institute of Tropical Medicine, Antwrep, Belgium.

A drop of the diluted serum was put in one of the circles on the slides coated with the trypanosome antigen (T.b. rhodesiense)

with a pasteur pipette. The slides were then incubated for 30 minutes at room temperature in a humid chamber. After 30 minutes of incubation the slides were washed in two changes of PBS for 5 minutes each and allowed to air dry. A polyvalent conjugate, fluorescein labelled containing anti-human IgM, IgG, IgA, (Diagnostic Pasteur, Paris) was used at a dilution of 1:100 with PBS. A drop of the conjugate was added to each well with a Pasteur pipette and incubated for 30 minutes in a humid chamber at room temperature. Following 30 minutes incubation the slides were washed in two changes of PBS for 5 minutes each, and allowed to air dry.

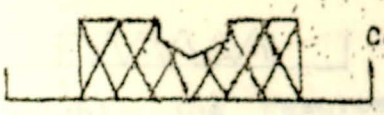
Finally the slides were mounted with glycerol in PBS (1:1) mixture and were examined under fluorescence microscope. The intensity of fluorescence was rated from 0 to +4 where 0 denotes the absence and 1+ to 4+ indicate increasing magnitude of positive reactions. Based on the fluoresence intensity of the positive control, positive and negative values were determed i.e, 1+ = very weak, 2+ = weak, 3+ = strong and bright and 4+ = very strong and bright.

(iii) Enzyme Linked Immunosorbent Assay (ELISA) Procedure

The stock antigen was thawed and diluted in carbonate bicarbonate buffer. The working dilution of the antigen was determined by using checkerboard titration, with positive and negative control sera and the enzyme-labelled anti-human IgM. In this manner, a final protein concentration of 5 ug/ml in a 1:200 dilution was determined to be appropriate. It was also determined that the sera should be tested at 1:500

dilution. A total of 416 samples were assayed by these method from three localities.

In each well of the immunol II ELISA microtitre plate (Dynatech laboratories Ltd., USA), 200 ul of the diluted antigen solution was dispensed. The plates were covered with titre plate sealer and left at 4 °C overnight. The assay was performed follwoing the standard methods of Voller et al., (1975) and Houba (1981) with a few modifications (fig 3). Briefly, the plates coated with antigen were washed three times for three minutes each with 0.05% Tween 20 in PBS (PBS - Tween) pH 7.4 and then shaken dry. 200 ul of test serum diluted to 1:500 in PBS - Tween was added and incubated at 37 °C for 2 hours. The plates were washed as above and 200 ul of peroxidase enzyme labelled rabbit antihuman IgM (Dako, Denmark), diluted 1:500 in PBS Tween 20 was added to each well and incubated at 37 °C for 2 hours. This was followed by washing as above. Finally 200 ul of enzyme substrate, Orthophenylene Diamine (OPD) was added to each well and the plate incubated for 30 minutes. The reaction was stopped by adding 50 ul of 2M H_2SO_4. The result was read spectrophotometrically at 492 nm by using an ELISA reader. The result was interprated based on the mean extinction values (Voller et al., 1975; Rutinberg et al; 1977). i.e, sum of total positives divided by two plus sum of total negative divided by two and the whole divided by two gives the mean extinction value. (In this study the positive and negative controls used were two samples for

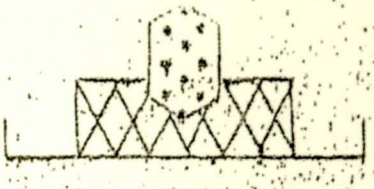


Coat plate with an antigen (at 4°C overnight)

Wash.

Add Test Sample.

Incubation (a 37°C for 2 hours. or at room temp. for 3 hours).

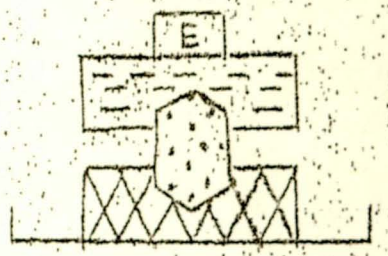


Specific attachment (AgAb complex).

WASH

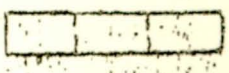
Add Enzyme labelled anti human globulin (CONJUGATE)

Incubation as above.



Specific conjugate attaches to specific antibody.

WASH



Add Enzye Substrate.

HYDROLYSIS (Colour Change)

each). The results above the mean extinction value is considered as positive and below the value as negative. The mean extinction values for three areas were different, because the source of antigen, positive and negative controls varies.

RESULTS

a) Parasitological Methods

(i) Blood films Examination

No trypanosome parasites were detected in all wet film preparation. Microscopic examination of stained thick and thin blood films revealed parasites in 0.95% of the sample from Gambella. In addition malaria (15.7%) and blood microfilariae (0.95%) were also identified in the Gambella samples. No trypanosomes were isolated from the two suspected regions, Anger Didessa and Bodi. On the other hand, malaria parasites were detected in both localities and microfilaria and spirochaetes were seen in the Bodi. These results are summerized in table 1.

(ii) MHCBT

With MHBCT technique 2(0.95%) trypanosome cases were identified in the Gambella samples. In addition blood microfilariae were detected in 3 cases (1.43%) from Gambella and 2 (1.9%) cases from Bodi sample (Table 2).

b) Serological Methods

(i) IFAT.

Of the total of 416 samples from the three regions tested with IFAT, 35(8.0%) were classified as positive, based on the criterion of +3 or more fluorescence intensity. The highest positivity, 21(10%), was obtained from Gambella, whereas the two

other regions had relatively lower ratez of positivety (Table 3). The specificity of IFAT for detection of antibodies against trypanosome parasites was tested by assaying sera positive for malaria, and Leishmania donovani and S. mansoni. Results of this test revealed 3.3% and 20% crossreactivity with malaria and L. donovani, respectively (Table 4).

(ii) ELISA

Based on the established criteria, 31(14.1%) from the endemic area were positive. In the other two suspected regions the mean extinction values were 0.7 and 0.9, with positivity of 10 (9.9%) and 9(8.6%) in Anger Didessa and Bodi, respectively (Table 5). Both T.b. rhodesiense and T.b. bruci antigens can be used equally for determination of anti-trypanosomal antibody in sleeping sickness infection (Table 7 & 8) for epidemiological studies.

Determination of cross reaction with the ELISA method using sera from patients of L.donovani, malaria and S. mansoni showed no cross reactivity with schistosomiasis, 5% and 20% cross reactivity was observed in malaria and leshmania, respectively (Table 6).

The overall positivity of ELISA for the total sample was 12%, the highest being from the endemic region (14.1%), all parasitologically positive cases were also serologically positive, both by IFAT and ELISA (Table 9 and 10). The specificity of ELISA with reference to IFAT was 90.7%. ELISA and IFAT can be equally useful for screening of the sleeping sickness (Table 11).

Table 1. Results of Parasitological Examinations With Stained Blood Films

Locality	Total Samples	Parasite identified	Positive Findings No.	Positive Findings % Total
Gambella	210	Trypanosome (*T.rhodesiense*)	2	0.95
		Malaria (*P. falciparum*) 31 (*P. vivax*) 2	33	15.7
		Microfilaria	2	0.95
Anger Dedessa	101	Trypanosome	-	-
		Malaria (*P.falciparum*) 10	10	9.90%
Bodi	105	Trypanosome	-	-
		Malaria (*P.falciparum*) 7 (*P.vivax*) 1	8	7.62
		Spirochaete (*Borrelia recurrentis*)	1	0.95

Table 2 Micro Haematocrit Buffy Coat Technique (MHBCT) for Trypanosomes and Other Haemoparasites

Locality	Total samples	Parasite Identified	Positive Findings No.	Positive Findings % Total
Gambella	210	Trypanosome (*T.rhodesiense*)	2	0.95
		Microfilaria	3	1.43
Anger Didessa	101	Trypanosome	-	-
Bodi	105	Trypanosome	-	-
		Microfilaria	2	1.90

Table 3 IFAT Determinations With *L. chagasi* Antigen

Locality	Total samples	No. of samples with respective fluorescence intensity +4	+3	+2	+1	% Positive **
Gambella	210	8	13	21	9	10.0
Anger Didessa	101	2	4	8	10	6.9
Bodi *	105	3	5	13	8	7.6

* Positive controls and antigen source for Bodi were from Gambella isolates, since no positive isolate was available from this locality.
** IFAT values > +3 considered to be positive.

Table 4 IFAT Cross Reactivity Test With *L. chagasi* Antigen

Type of Parasite	Total Samples	Fluoresence results* +4	+3	+2	+1	% Cross reactive
Malaria (*P. falciparum*) (*P. vivax*)	30 28 2	-	1	6	12	3.3
Leishmaniasis (*L. donovani*)	5	-	1	3	1	20
Schistosomiasis (*S. mansoni*)	8	-	-	1	2	0

* IFAT values > +3 considered to be positive.

Table 5 ELISA Determinations for 416 Sera with *L.b. chagasi* Antigen

Locality	Total Samples	Ranges of ELISA Readings* 0-.2	.2-.4	.4-.6	.6-.8	.8-1.0	>1.0	No. +ve	% +ve
Gambella	210	74	54	51	13	11	7	31	14.1
Anger Didessa	101	52	19	11	12	6	1	10	9.9
Bodi	105	50	15	12	14	12	2	9	8.6

* Readings > mean extinction values were considered to be +ve
Mean extintion values:
Gambella = 0.6
Anger Dedessa = 0.7
Bodi = 0.9

Table 6 ELISA Cross-reactivity Test with *L.b. chagasi*

Parasites	Total samples	Ranges of ELISA Readings 0-0.2	0.2-0.4	0.4-0.6	0.6-0.8	0.8-1.0	>1.0	Total pos.	% pos.
Malaria	20	9	8	2	-	1	-	1	5
L. donovani	5	-	-	4	1	-	-	1	20
S. mansoni	8	2	6	-	-	-	-	-	-

Table 7 Comparison of Results of ELISA values for 210 Samples by Using *T.b. brucei* and *T.b. rhodesiense* Antigens

Area	Total Samples	Antigen source	Ranges of ELISA reading 0-0.2	0.21 0.41 0.4	0.41 0.6	0.61 0.8	0.81 0.10	>1.0	No. +ve	% +ve
Gambella	110	*T.b. brucei*	39	32	24	12	1	2	10	9
		T.b. rhodesiense	41	29	22	14	2	2	13	11.8
Anger Didessa	50	*T.b. brucei*	18	10	12	9	-	1	4	8
		T.b. rhodesiense	21	12	10	5	1	1	5	10
Bodi	50	*T.b. brucei*	17	6	8	9	7	3	5	10
		T.b. rhodesiense	19	9	6	7	7	2	4	8

Extinction value for positive cases Gambella and Anger Didessa 0.7 and Bodi 0.9.

Table 8 X^2 Test for the Independent use of *T.b. brucei* & *T.b. rhodesiense* Antigens on Human Sera for Diagnosis Sleeping Sickness

	T.b. brucei	*T.b. rhodesiense*	Total
+	19 (20.5)	22 (20.5)	41
-	191 (189.5)	188 (189.5)	379
Total	210	210	420

X^2 Cal (1) 0.05 = 0.26
P < (1) (0.05) = 3.8

So there is no significant difference for crude Antigens for testing human sera by the two trypanosome subspecies.

Table 9 Comparison of IFAT and Parasitological Determinations

	Parasitology		
	+	-	Total
+	2	33	35
-	0	381	381
Total	2	414	416

Specificity of IFAT $= \frac{381}{414} \times \frac{100}{} = 92.0\%$ where

Specificity $= \frac{TN \times 100}{TotN}$

sensitivity of IFAT $= \frac{2}{2} \times \frac{100}{} = 100\%$, where

sensitivity $= \frac{Tp}{Totp} \times 100;$

and TN = True negative
TotN = Total negative
Tp = True positive
Totp = Total positive

True negative = Negative by IFAT as well as parasitologically
Total negative = All parasitologically negative cases.
True positive = All parasitologically positive cases.
Total positive = Parasitologically positive as well as by IFAT.

Table 10 Comparison of ELISA and Parasitological Determinations*

ELISA	Parasitology +	Parasitology −	Total
+	2	48	50
−	0	366	366
Total	2	414	416

Specificity of ELISA = $\frac{366}{414} \times \frac{100}{} = 88.4\%$

Sensitivity of ELISA = $\frac{2}{2} \times \frac{100}{} = 100\%$

* Applying the same formula as in Table 9.

Table 11 X^2 Test of Independence for ELISA and IFAT

	ELISA	IFAT	Total
+	50 (42.5)	35 (42.5)	85
−	366 (373.5)	381 (373.5)	747
Total	416	416	832
% Pos.	12.02%	8.4%	

X^2 Cal = 2.94
P < (1) 0.05 = 3.84

Based on these values it can be Concluded that ELISA is not any superior to IFAT and IFAT is not worse than ELISA. i.e. no significant superiorty of one test over the other.

DISCUSSION

Although Gambella is considered to be the only endemic area for sleeping sickness in Ethiopia, reports of sporadic cases from other regions such as Gamu Gofa (Bodi; Olfasson and Aorsland, 1981) and Wollega (Anger Didessa; Fisseha, 1987) are also documented. The presence of game animals serving as reservior hosts for T.b. rhodesiense and the existence of potential vectors (Hutchinson. 1971; Langridge, 1976; Tikubet and Gemetchu, 1984) in Bodi and Anger Didessa were also well established. These conditions prompted inclusion of Bodi and Anger Didessa regions in the study.

The present study was an attempt to validate different diagnostic methods with the view to determining a method for further field study of sleeping sickness. As expected, microscopic examination of wet film preparations of blood for trypanosome detection in clinically suspected cases did not reveal the presence of the parasite. This indicates that the test is insensitive and cannot be applied as a routine method for trypanosomiasis diagnosis. This is particularly true in late infections when the parasites do not circulate in large numbers in the peripheral circulation. Nevertheless, in the absence of a better alternative, it should be used for on-the-spot preliminary diagnosis of acute infections in clinically suspected cases where T.b. rhodesiense is known to be endemic.

laboratory and field conditions (Woo, 1971; Murray et al ., 1977). The test is also recommended by WHO (1983) as a routine diagnostic test for sleeping sickness surveys and for the detection of filariasis; which usually is co-endemic with African trypanosomiasis. It is also well known that by using the MHBCT, the packed cell volume can be used to determine the anaemic status of the patients. Furthermore, plasma in the capillary can be used for serological test. Thus the method is reliable and can be performed under field condtions in Ethiopia. Based on our observations , MHBCT can be used as confirmatory test to follow-up cases positive by IFAT or ELISA.

In the present study, two serological tests, IFAT and ELISA were evaluated. In ELISA, comparision of the T.b. brucei and T.b. rhodesiense crude soluble antigens for the detection of antibodies showed no statistical difference , ($P < 0.05$). This is in agreement with what was demonstrated by Ruitenberg and Buys (1977), in studies based on experimental animals. This shows that the two species are antigenically very similar and therefore T.b. brucei which is non pathogenic to humans can effectively be used as a substitute antigen in serological tests for African trypanosomiasis. The second test IFAT, showed a 10% positivity in the endemic area (Gambella) and 6.9% and 7.6% for Anger Didessa and Bodi, respectively. On the other hand, screening of the same samples with ELISA yielded a positivity rate of 14.1% for Gambella, 9.9% and 8.6% for Anger Didessa and Bodi,

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respectively. The proportion of positive cases detected by ELISA was greater than that detected by IFAT in both suspected and endemic areas. However, a X^2 test for independence showed that ELISA has no statistical superiority over IFAT ($P < 0.05$). Thus the small discrepency observed may have been only a reflection of ELISA's capability to quantitate and reveal minor differences not identified by IFAT. We therefore conclude that both ELISA and IFAT could be alternatives on a comparable basise for sero-diagnosis of trypanosomiasis as suggested by other workers (WHO, 1976; Ruitenberg and Buys, 1977; Voller, 1977).

The detection of seropositive cases in the suspected areas may be an indication of endemicity of the infection, or it may be attributable to the repeated challenge of antigenic material of nonhuman species of trypanosomes (Molyneux and Ashford, 1983; Vickerman, 1985), or it could be a result of abortive *T.b. rhodesiense* infection (WHO, 1985). Also, cross reactions with antibodies to other infectious agents, such as *L.donovani* and malaria, may result in false seropositivity. In addition the interference of immune complexes cannot be excluded (Vanknapen *et al*; 1977). Since false positive reactions have occured in both tests used in the present study, an attempt must be made to minimize cross reactivities by antigen purifications and by inclusion of appropriate positive and negative controls in every test run.

Tests for crossreactivity of sera with non-trypanosomal infections with IFAT and ELISA have indicated highest cross reactivity with L.donovani. This most probably is because leishmania have related antigens to those of trypanosomes as they both belong to the same order, the Kinetoplastida. Next in crossreactivity was malaria, which showed more cross-reactivity than S. mansoni. Since malaria is caused by protozoan blood parasites and hence the parasites are more phylogenetically related to the trypanosomes than S. mansoni, some common antigenic materials may induce related antibody production. As there were more cases characterized as positive based on ELISA, cross reactivity was also higher in ELISA as compared to IFAT. This may again be attributed to the quantitative value of ELISA absorbance readings. In spite of the apparent cross-reactivity, both tests showed a relatively high specificity for anti-trypanosomal antibody detection.

All the parasitologically proven positive untreated cases of sleeping sickness were positive by both IFAT and ELISA, indicating that positive cases will not be missed by either methods, based on the criterion used for positivity. Although all cases detected by the MHBCT were also positive by IFAT and ELISA, the fact that only a few positive cases were available does not allow a solid conclusion of the relative sensitivity of the two tests. The specificity of IFAT with reference to parasitological results was 92.0% and that of ELISA was 88.4%.

If we condider IFAT as a reference test because of its frequent application for sleeping sickness studies (Wyatt, et al., 1984), ELISA has a specificity of 90.7%. These values are similar to those obtained by WHO (1976) and Voller (1977) while comparing the two tests for the detection of anti-trypanosomal antibodies. The discrepancy in the results of the two methods may in part be a reflection of the differences in the antigens used.

All untreated cases infected with T.b. rhodesiense showed positive results by both tests. Among these, one patient with an absorbance value greater than 1.4 by ELISA, died as he started the treatment. On the other hand, one treated case of 14 weeks duration showed on absorbance value little above the extinction value, and another two of 8-9 months duration of treatment were negative. One individual with more than one year post-treatment showed an ELISA absorbance value of 0.4, which in this study was regarded as negative . This shows the value of ELISA as a follow up method to determine the efficacy of treatment, in addition to its value for routine diagnosis.

The present study has also aimed determining a single serological test that is quantitative, cheap and reproducible for field studies of African trypanosomiasis and ELISA has been demonstrated to be such a test.

With regard to IFAT, the various studies conducted (Kakoma and Rickman, 1981; Wyatt, et al ., 1984) had shown that there is a problem of seroconversion of positives to negatives and weak

negatives to strong positives. Since no such problems have been reported for ELISA , the test can safely be used for epidemiological studies, and the determination of antibody levels in treated paitients. IFAT on the other hand, may be more useful in epidemiological investigation, to determine whether the population has a history of exposure to trypanosome parasites. Furthermore, the use of whole parasite antigens makes the test easily reproducible. But from the experience gained in this study and as indicated by others (Houba, 1981 ; Robert, 1974) the need for a fluorescence microscope and the tedious microscopic observations limit the applicability of IFAT under field conditions.

Thus, ELISA is a good alternative to IFAT for both epidemiological studies in the field as well as for individual diagnosis using an appropriate antigen. In addition, visual estimation of the result in the field, in conjuction with the more specific parasitological methods , such as MHBCT supplemented by inoculation of susceptible laboratory animals, makes it recommendable for field use on clinically suspected individuals.

APPENDEX I

PREPARATION OF REAGENTS

a) Phosphate Buffer Saline Glucose (PBSG) (pH. 7.9-8.0)

This buffer is recommended for ion exchange centrfugation technicic as applied for the diagnosis of African trypansomiasis (WHO, 1983; Lumsden et al., 1979).

Na_2HPO_4 (anhydrous)	-	8.094 gm
$Na\ H_2PO_4\ 2H_2O$	-	0.468 gm
NaCl	-	2.55 gm
Glucose	-	15 gm
Distilled water	-	1 litre

conductivity 1.0×10^{4} mhos. From this heparinaized PBSG was prepared by filtering through 0.22 um pore size filter paper, whatman and 6.2 ml of PBSG is added to 0.1 ml of heparin. It was stored at -20^{o} C and thawed before use for stabilate preparation and to run the column for separation of trypanosomes from blood.

b) DE-52 Equilibration Phosphate Buffered Saline (PBS) (pH 8.00)

Conductivity of the final solution 1.08×10^{4} mhos.

Na_2HPO_4 (anhydrous)	=	8.092 gm
$NaH_2PO_4.2H_2O$	=	0.468 gm
NaCl	=	2.630 gm

The pH is adjusted to 8.00 using diluted 1/20 orthophosphoric acid or 1/20 of NaoH.

c) Preparation of Sterile DE - 52 Column

The column for the separation of trypanosomes was prepared by equilibrating the preswollen DE-52 with PBS. DEAE cellulose (type DE 52 preswollen whatman, England) is equilibrated with batches of PBS, pH, 8.0 in the ratio of 100 gm preswallen adsorbent to 250 ml of buffer. The solution is well mixed and is allowed to settle for about 20 minutes and the supernatant decanted. The procedure was repeated five times until the pH of the supernatant reached approximately 8.00 at the final wash.

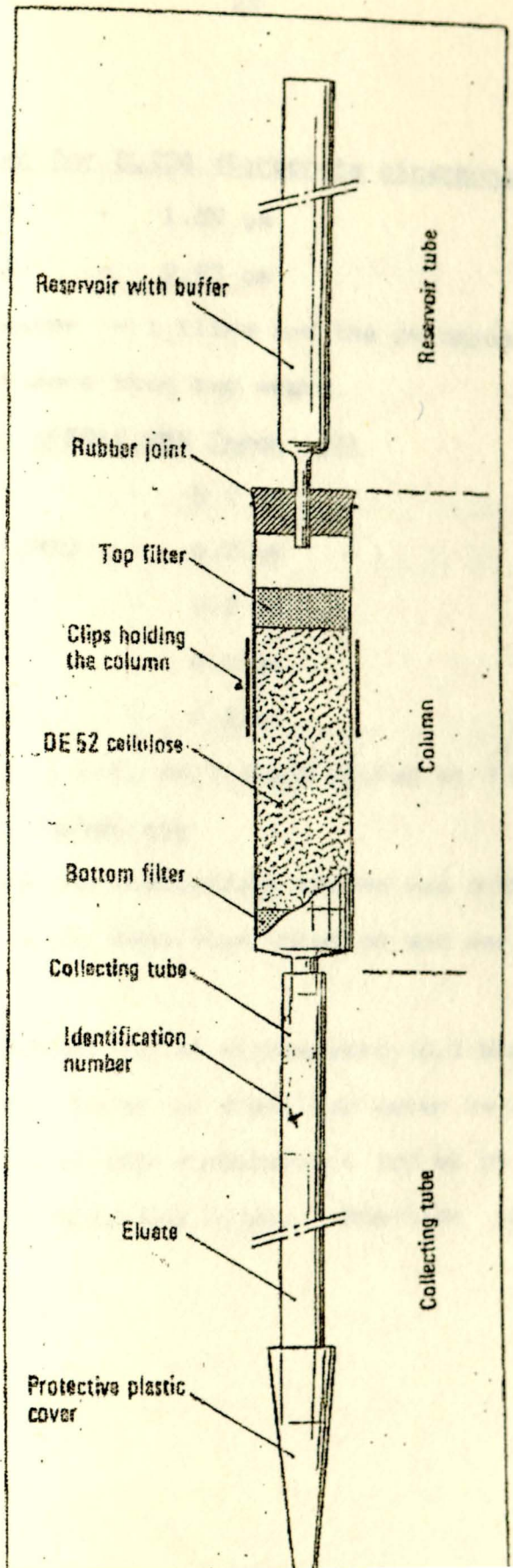
The slurry was poured into small bottles and sterilized in an autoclave 5 lb/sq in for 15 minutes. The sterile slurry was stored at -20 °C.

The column was prepared in a sterile 10 ml syringe. Cellulose sponge was cut out to the diameter of the base of the syringe, sterilized and inserted into it after removing the piston. The DE-52 slurry was thawed and added into the syringe with a sponge to the level of about 7 to 8 ml. Both end of the syringe were covered with Aluminium foil and the prepared column stored at -20 °C until used (fig.4).

d) Preparation of Enzyme Activity Inhibitors

50 mg of Phenyl Methyl Sulfonyl Floride (PMSF) was dissolved in 5 ml of acetone. 2 ml of the solution was mixed with 100 ml of PBS, pH 8.00 for washing and suspension of the pellet for sonication.

Fig 4. Miniature-Anion Exchange Column-for separation of Trypanosomes from blood.



e) Coating Buffer for ELISA (Carbonate bicarbonate) Buffer

Na2 CO3 - 1.59 gm

NaH CO3 - 2.93 gm

distilled water - 1 litre and the pH adjusted to 9.6 and stored at 4 °C not more than two weeks.

f) Washing Buffer (PBS+0.05% Tween -20)

NaCl - 8.0 gm

Na2HPO4.12H2O - 0.2 gm

KH2PO4 - 0.2 gm

KCl - 0.2 gm

Tween -20 - 0.5 ml

Distilled water -1 lit, pH 7.4 and stored at 4 °C

g) Preparation of Substrate

The substrate for peroxidase enzyme was orthophenyl Diamine (OPD). It is a light sensitive solution and was used within a day.

24.3 ml (19.2 gm/lit of citric acid (0.1 M)and 25.7ml (28.4 gm/lit of Na2 HPO4). 5o ml of distilled water is added, and pH, 5.00. Then 40 mg of OPD dissolved in 100 ml of citrate buffer and 0.15 ml of 30% H2O2 was added. Reaction stopping reagent 2M H2SO4 was used.

h) Stock Buffer for IFAT (PBS)

KH2PO4 - 2.15 gm

NaCl - 36.00 gm

Na2HPO4 .2H2O - 9.00 gm

NaN3 - 1.00 gm

distilledwater - 1 litre

The working solution was diluted five times with distilled water. The pH is adjusted to 7.2 and stored at 4 °C for not more than two weeks.

i) Mounting reagents

Glycerol in PBS (1:1) was prepared to mount the slide and to decrease the effect of fluorescence.

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DECLARATION

I under signed, declare that this study is my own work and that all other sources have been dully acknowledged.

Name _____

Signature _____

The thesis is my original work and has not been presented for a degree in any other university.

Tekola Endashaw

Signature _____

This thesis has been submitted for examination with my approval as University advisor.

Dr. Beyene Petros

Signature _____