

Options for Field Diagnosis of Human African Trypanosomiasis

François Chappuis,^{1,2*} Louis Loutan,² Pere Simarro,³ Veerle Lejon,⁴
and Philippe Büscher⁴

Médecins Sans Frontières, Swiss and French sections,¹ and Travel and Migration Medicine Unit, Geneva University Hospital,² Geneva, Switzerland; World Health Organization, Yaoundé, Cameroon³; and Department of Parasitology, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium⁴

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INTRODUCTION

Human African trypanosomiasis (HAT), or sleeping sickness, is a disease caused by infection with the protozoan *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense*, two morphologically identical subspecies of *Trypanosoma brucei*. The two forms of the disease are transmitted by tsetse flies of the genus *Glossina* (order Diptera) and are restricted to sub-Saharan Africa. Both are fatal if left untreated. HAT is the prototype of a neglected disease, affecting the poorest people of the poorest continent (120). The develop-

ment of new diagnostic tests and drugs has been severely affected by this neglect. Fortunately, new sources of funding and initiatives, such as the Drugs for Neglected Initiative (<http://www.dndi.org>), give some hope for the future (119).

The high toxicity of melarsoprol, the most widely used treatment for second-stage (or neurological) HAT (97, 136), means that both diagnosis and staging of the disease must be highly accurate. The availability of accurate, practical, and cheap screening and confirmatory tests is vital for *T. b. gambiense* HAT control programs, which are usually based on the elimination of the parasite's human reservoir by mass screening of the population and treatment of all infected persons. A correct diagnosis of HAT is thus beneficial for both infected individuals and the community.

This review focuses on the field diagnosis of *T. b. gambiense*

* Corresponding author. Mailing address: Travel and Migration Medicine Unit, Geneva University Hospital, 24 rue Micheli-du-Crest, 1211 Geneva 14, Switzerland. Phone: 41-22-3729620. Fax: 41-22-3729626. E-mail: francois.chappuis@hcuge.ch.

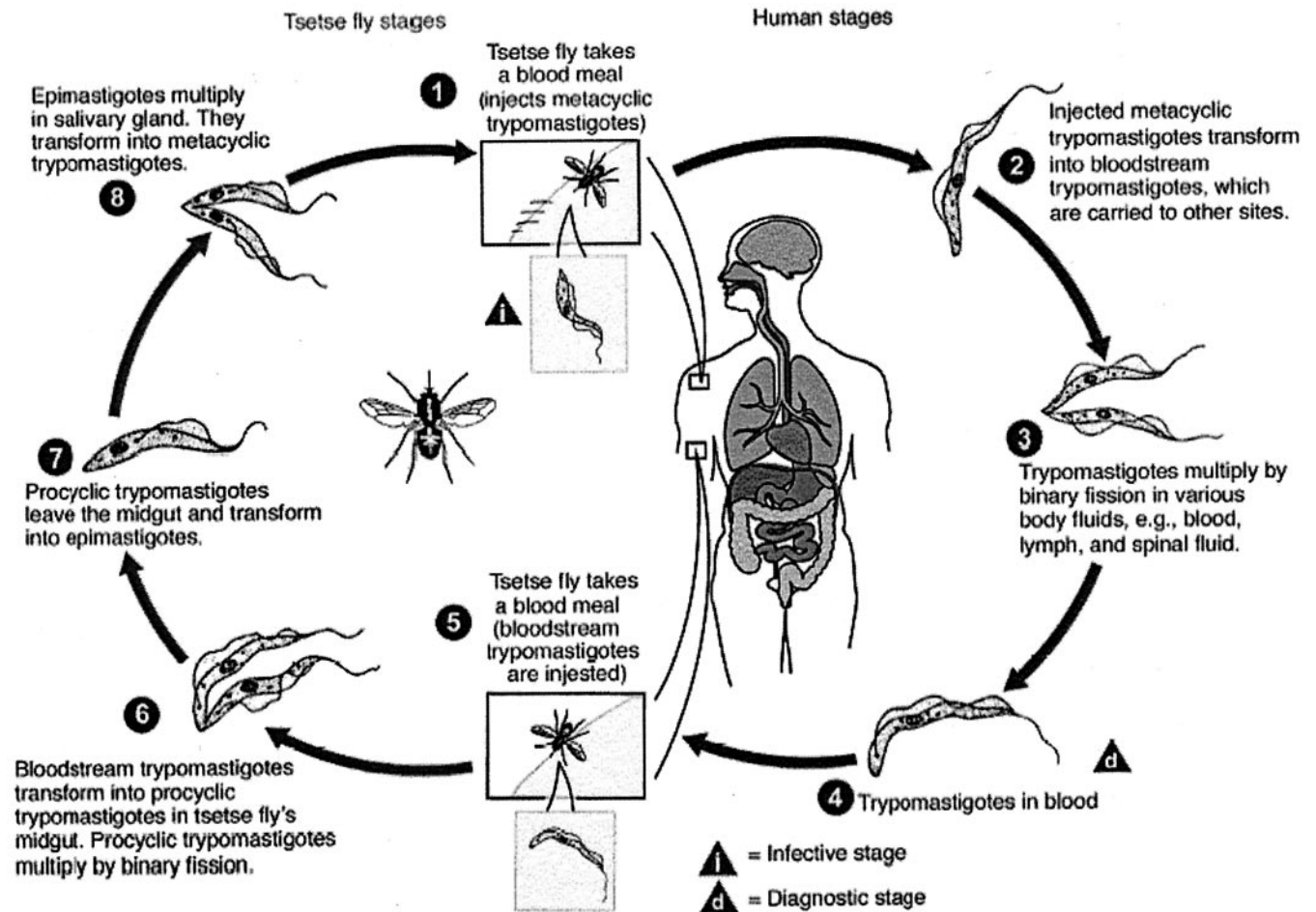


FIG. 1. Diagrammatic representation of the life cycle of *T. b. gambiense* and *T. b. rhodesiense* in humans and the tsetse fly. Copyright Alexander J. da Silva and Melanie Moser, Centers for Disease Control Public Health Image Library. Reprinted with permission from the Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Ga.

HAT, which represents the highest burden of the disease. The diagnosis of *T. b. gambiense* HAT outside Africa has recently been reviewed elsewhere (56).

LIFE CYCLE AND BIOLOGY

T. brucei belongs to the genus *Trypanosoma* within the family of *Trypanosomatidae*, a large group of unicellular protozoan parasitic organisms, and the order of *Kinetoplastida*. It is a spindle-shaped cell (20 to 30 by 1.5 to 3.5 μm) with a single flagellum. This flagellum emerges from the posterior end, runs along the cell membrane, to which it is attached by an undulating membrane, and extends beyond the anterior part of the cell. The base of the flagellum is associated with the kinetoplast, a large particle containing the DNA of the single mitochondrion. The trypomastigote is the only form to be observed in the mammalian host, whereas the epimastigote form occurs during the development phase in the tsetse fly. During the entire life cycle, *T. brucei* cells multiply by binary fission and are considered to be exclusively extracellular.

The life cycle of *T. brucei* is shown in Fig. 1. Infection of the mammalian host starts with the bite of an infected tsetse fly

(*Glossina* spp.), which injects the metacyclic trypomastigote form of the parasite in its saliva before taking its blood meal. The trypanosomes multiply locally at the site of the bite for a few days before entering the lymphatic system and the bloodstream, through which they reach other tissues and organs including the central nervous system (CNS). Two different trypomastigote forms can be observed in the mammalian host: a long, slender proliferative form and a short, stumpy nondividing form. Both forms are taken up by the tsetse fly, but only the latter is able to complete the complex 2 to 3 week life cycle in the fly.

In the mammalian host, the trypomastigote cell is completely covered by a dense monolayer of identical glycoproteins that protect the parasite against direct lysis by complement (17). Only when specific antibodies are present against the surface epitopes is the parasite destroyed. Thanks to a fascinating mechanism of continuous antigenic variation, which has been studied extensively in animal models, a small but sufficient fraction of the parasite population is able to evade the mammalian host humoral immune response and proliferate until the new surface antigen coat is recognized by a new generation of specific antibodies, mainly of the immunoglob-

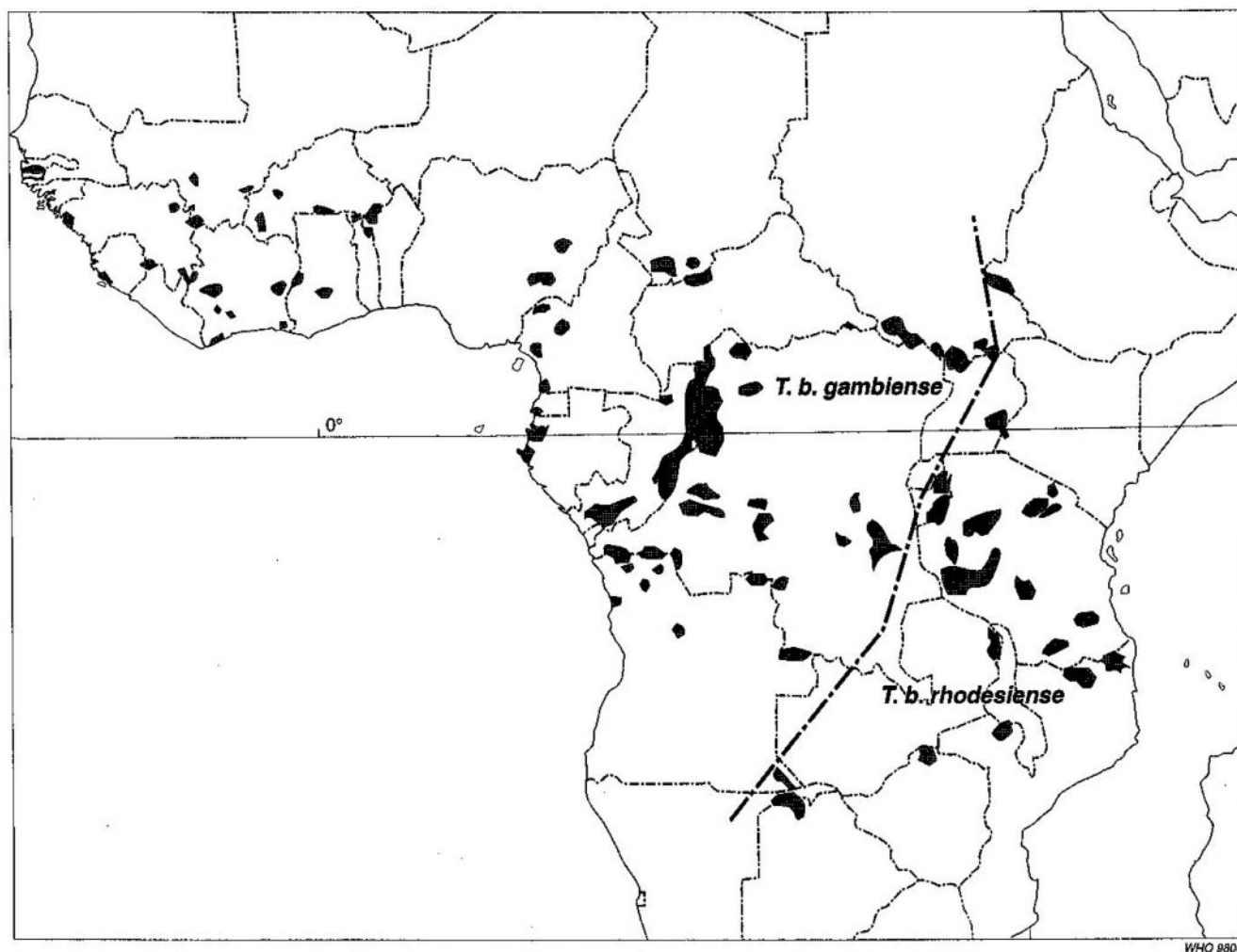


FIG. 2. Geographic distribution of major endemic foci of *T. b. gambiense* and *T. b. rhodesiense* human African trypanosomiasis in Africa (1995). Reprinted from reference 136 with the permission of WHO/CPE.

ulin M (IgM) type. Up to 1,000 different genes encoding the variant surface glycoproteins are present in the *T. brucei* genome (16, 126). This phenomenon explains the fluctuating number of circulating trypanosomes in the patient's blood (105), which contributes to the limited sensitivity of parasite detection methods in clinical practice.

EPIDEMIOLOGY

It is estimated that 60 million people are exposed to HAT in nearly 200 separate active foci from 36 sub-Saharan countries but only 4 million to 5 million are under surveillance (136). The location of endemic foci of HAT follows the patchy distribution of tsetse flies found in a belt that stretches south of the Sahara and north of the Kalahari desert (9), as shown in Fig. 2. Despite the absence of reliable epidemiological figures, the World Health Organization (WHO) reports that 300,000 to 500,000 people might be infected by the *T. b. gambiense* form of the disease in Western and Central Africa (<http://www.who.int/mediacentre/factsheets/fs259/en/>). The most severely affected countries are the Democratic Republic of Congo, the

Republic of Congo, Angola, Central African Republic, and Southern Sudan, where HAT has reemerged during the last decades mainly due to long-standing geopolitical instability and subsequent erosion or collapse of control programs (81, 117, 129). *T. b. gambiense* is transmitted by the bite of tsetse flies of the *Glossina palpalis* or *G. fuscipes* groups. Human-vector contact occurs mostly in forested rivers and shores but is also peridomestic when huts are built in or near plantations (18).

T. b. rhodesiense HAT is a zoonosis present in Eastern and Southern Africa. Wild animals, including game animals, are usually affected, but epidemics occasionally occur in domestic animals and humans. The parasite is transmitted by the bite of tsetse flies of the *Glossina morsitans* or *G. fuscipes* groups, and human-vector contact typically occurs in savanna woodland but can be peridomestic during epidemics (18). The incidence of *T. b. rhodesiense* HAT is currently much lower than that of *T. b. gambiense* HAT, but large epidemics were observed in the past. No more than 50 cases of HAT are diagnosed yearly outside Africa (56). Visitors to some game parks in eastern Africa are at particular risk for *T. b. rhodesiense* HAT (47, 115). Migrants

from countries where *T. b. gambiense* is highly endemic can have HAT that remains unrecognized for years (108).

Studies conducted in Western and Central Africa have failed to find an increased risk of HAT among human immunodeficiency virus (HIV) infected individuals, but no definite conclusion can be drawn from the available data (68, 77, 96). Moreover, there have been no studies describing the clinical presentation and the validity of diagnostic tests in coinfecting patients.

CLINICAL FEATURES

The clinical presentations of *T. b. gambiense* and *T. b. rhodesiense* HAT are remarkably different. While *T. b. gambiense* HAT is generally a chronic illness that lasts for years, *T. b. rhodesiense* HAT usually presents as an acute febrile illness that is fatal within weeks or months if left untreated. Exceptions to this rule occur, since fulminant illness has been described with *T. b. gambiense* infections and chronic disease is caused by *T. b. rhodesiense* in the southern end of its distribution (15, 18, 21). The clinical features can be indicative of HAT but are neither sufficiently sensitive (*T. b. gambiense*) nor specific (both species) to be used to detect all infected individuals, confirm the diagnosis, or stage the disease (12, 14, 26, 29, 46, 61). Diseases such as malaria, enteric fever, tuberculous meningitis and HIV infection can mimic or even coexist with HAT. Therefore, clinical suspicion must be confirmed by laboratory tests.

At the site of the infecting bite, parasites proliferate and, after 5 to 15 days, occasionally lead to a nodule or ulcer called a chancre (or trypanome) that spontaneously resolves within a few weeks. In African patients, chancres are generally absent at the time of diagnosis in both forms of HAT (14, 20). This contrasts with the high prevalence of chancres observed in European patients, in particular those infected by *T. b. rhodesiense* (26, 47). In the largest published review of Europeans diagnosed with HAT, chancres were more frequently observed in patients returning from Eastern Africa (46%) than in patients returning from Western or Central Africa (23%) (26).

T. b. gambiense Disease

After a long but variable initial asymptomatic period, patients with *T. b. gambiense* HAT can present with intermittent nonspecific symptoms such as fever, fatigue, headaches, arthralgia, and pruritus. The skin rash, often observed in patients of European origin (26), is usually not visible on dark skin. Transient limbs or face edema can occur. Enlarged, painless, rubbery cervical lymph nodes in the posterior cervical triangle were recognized as an alert sign for HAT long ago by Sir Thomas Masterman Winterbottom, who noted that slave traders in the late 18th century used neck swelling as an indicator of the sleepiness or abnormal behavior that made particular slaves undesirable (9). However, enlarged cervical lymph nodes may be atypical, absent (in up to 50% of patients), or due to other causes (97). Splenomegaly is another nonspecific sign and is more commonly found than hepatomegaly (14). This highly variable clinical picture grossly corresponds to the first, or hemolymphatic, stage of the disease.

Later in the disease, neuropsychiatric symptoms and signs,

due to the CNS invasion by trypanosomes and the resulting immune response (39), gradually become more prominent. The interval between the start of the infection and the second, or neurological, stage is in the order of months or years (18). The neuropathogenesis of second stage HAT has been recently reviewed (50). The clinical features can be grouped into categories such as psychiatric, motor, sensory, and sleep abnormalities. The mental disturbance may include irritability, lassitude, headache, personality change, and overt psychiatric presentations such as psychosis. Pyramidal (e.g., focal paralysis), extrapyramidal (e.g., rigidity and tremor), and cerebellar (e.g., dysarthria and ataxia) disorders are common. Delayed and increased sensation to pain (Kerandel's sign) can also be noted. Reversal of the normal sleep-wake cycle, with daytime somnolence alternating with nocturnal insomnia, is typical. Weight loss and endocrine abnormalities such as amenorrhea and impotence are also frequent complaints. If left untreated, patients ultimately die from the consequences of severe wasting, dysfunction of the immune system, deep coma, and seizures, often due to bacterial infections such as pneumonia or meningitis.

T. b. rhodesiense Disease

HAT due to *T. b. rhodesiense* infection presents as an acute (sometimes fulminant) febrile illness starting 1 to 3 weeks after the infective bite; it cannot be distinguished clinically from other tropical fevers such as malaria, enteric fever, and bacterial meningitis. Compared to *T. b. gambiense* illness, febrile episodes are more pronounced and frequent and lymphadenopathy is usually generalized. Keratitis and conjunctivitis have been observed. There is less demarcation between first- and second-stage illness, and CNS involvement can be clinically limited to drowsiness and tremor (18, 39). Pancarditis with congestive heart failure, arrhythmia, and pericardial effusion can kill the patient before pronounced CNS involvement becomes apparent (118). Most deaths (>80%) occur within 6 months of onset of illness (88).

LABORATORY DIAGNOSIS OF *T. B. GAMBIENSE* INFECTION

The diagnosis of *T. b. gambiense* HAT follows a three-step pathway: screening, diagnostic confirmation, and staging. The majority of control programs rely on active case detection through mass population screening. Screening tools therefore need to be sensitive, practical, quick, and cheap. For that purpose, the Card Agglutination Test for Trypanosomiasis (CATT/*T. b. gambiense*), currently used in most areas of endemic infection, is a more efficient screening method than the cervical lymph node (CLN) palpation and puncture (103). Diagnostic confirmation then relies on the finding of trypanosomes in the blood, lymph nodes, or cerebrospinal fluid (CSF). Unfortunately, it is estimated that 20 to 30% of patients are missed by the standard parasitological techniques (103). Staging of the disease is a key step that allows classification of the patient into the first (hemolymphatic) or second (meningoencephalitic) stage of the disease. In the absence of reliable blood tests able to detect CNS invasion by the parasite, HAT staging relies on the CSF examination.

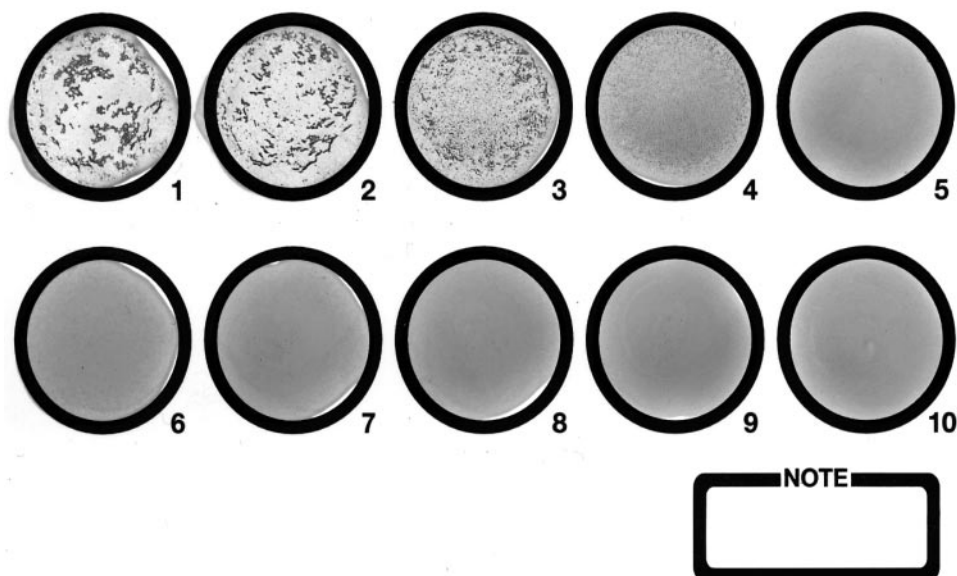


FIG. 3. Example of CATT performed on 10 serum samples diluted 1:4. Samples 1 to 3 are strongly positive, sample 4 is weakly positive, and samples 5 to 10 are negative.

It must be stated that the efficiency associated with implementing accurate diagnostic tools in HAT control programs based on active case finding can be offset by other crucial determinants such as a low attendance rate of the population or an insufficient proportion of patients completing treatment with subsequent cure (103).

Biological Parameters

Biological blood parameters such as increased sedimentation rate and low hematocrit reflect the systemic chronic inflammation present in HAT patients and are therefore non-specific. Thrombocytopenia is generally mild or absent, and features of disseminated intravascular coagulopathy are not found (37). Liver transaminase levels, bilirubin, and renal function tests are usually within normal limits or slightly elevated (12). Protein measurements usually show decreased albumin and increased immunoglobulin concentrations, especially of IgM (12). Low serum C3 levels and split C3 products can be found, reflecting complement activation (38). These findings are of little use in most field settings, where only the erythrocyte sedimentation rate and the hematocrit can be measured.

Antibody Detection

Indirect evidence for trypanosome infection can be obtained by demonstrating specific antibodies in the blood, plasma, or serum of infected hosts. Trypanosomes have a complex antigenic structure and elicit the production of a large spectrum of antibodies. *T. b. gambiense* specific IgG and IgM antibodies are present in high concentrations and are directed mainly against the immunodominant surface glycoprotein antigens of the parasite. The type of antigen(s) used greatly determines the sensitivity and specificity of the test. Current serological tests detect antibodies after 3 to 4 weeks of infection (126). Seropositivity must be interpreted with caution in previously

treated patients since antibodies can persist for up to 3 years after cure (91).

CATT/*T. b. gambiense*. Although not registered by any regulatory agency, the introduction of the CATT/*T. b. gambiense* (CATT) for mass population screening has been a major breakthrough in the diagnosis of *T. b. gambiense* HAT, limiting the number of parasitological examination to patients found with a positive serology. Developed in the late 1970s, the CATT is a fast and simple agglutination assay for detection of *T. b. gambiense*-specific antibodies in the blood, plasma, or serum of HAT patients (74). The antigen consists of lyophilized bloodstream forms of *T. b. gambiense* variable antigen type LiTat 1.3. Antigen production is a fastidious process based on the extraction of trypanosomes from infected rat blood. The trypanosomes are then fixed, stained with Coomassie blue, and freeze-dried. The reagent, which is produced under full quality control, is currently made only at the Institute of Tropical Medicine in Antwerp, Belgium, and field kits containing the reagent, control sera, and a 12/220-V card rotator are available. One drop of reagent is mixed with one drop of blood and shaken for 5 min on the rotator, and the result is visible to the naked eye (Fig. 3). Up to 10 patients can be tested at the same time, and hundreds of individuals can be screened daily. The reported sensitivity of the CATT on undiluted whole blood (CATT-wb) varies from 87 to 98%, and the negative predictive value is excellent during mass population screening (86, 93, 103, 112, 125, 136). Nevertheless, false-negative CATT results can occur (94), as suspected in patients infected with strains of trypanosomes that lack or do not express the LiTat 1.3 gene (27, 31). This could explain the lower sensitivity of the CATT in some areas of endemic infection such as the Ethiopia focus in Nigeria, where an alternative serological test should be used (30). Furthermore, when the CATT is performed on undiluted blood or serum with low dilution (<1:4), the agglutination can be inhibited, a phenomenon called prozone. To

overcome this problem, which is caused by complement factors and affects the sensitivity of the test, addition of EDTA to the dilution buffer has been proposed (89), substantially increasing the sensitivity with only a minor loss in specificity (72, 111). The CATT buffer supplemented with EDTA can remain stable for at least 2 years at 45°C.

Despite a reported specificity of around 95%, the positive predictive value of the CATT-wb remains limited because the test is used for mass screening in populations where the prevalence of HAT is usually below 5% (86, 93, 103, 125, 127, 128, 138). False-positive results can occur in patients with malaria and other parasitic diseases such as transient infection by non-human trypanosomes (74). The specificity of the CATT is further improved when performed on serum diluted to 1:4 (74, 126). This remains insufficient for diagnostic confirmation but allows a significant gain of time and financial resources by decreasing the number of parasitological investigations. The validity of the CATT, when performed with higher serum dilutions, is discussed below.

The CATT can be performed with blood-impregnated filter paper (FP) (85). This method is particularly useful for screening individuals who cannot be reached by full mobile teams during active case finding. The micro-CATT, a protocol using small quantities of both antigen (ca. one-fifth of the standard amount) and FP eluate (sample), showed promising results in Côte d'Ivoire (78, 85). The major constraint for widespread use of the micro-CATT is the rapid decrease in sensitivity when FP are stored for more than 1 day at ambient temperature (125). Moreover, due to the minute volumes of antigen and test sample used, reading and interpretation of the agglutination patterns can be difficult with the micro-CATT. A recently modified method, the macro-CATT, was developed for testing blood-impregnated FP by using a standard amount of antigen and a higher volume of FP eluate. The macro-CATT was evaluated in southern Sudan and showed a sensitivity of 91% and excellent stability when FP were stored for up to 2 weeks at ambient temperature (25 to 34°C) (23).

Other serological tests. The LATEX/*T. b. gambiense* has been developed as a field alternative to the CATT (19). The test is based on the combination of three purified variable surface antigens, LiTat 1.3, 1.5, and 1.6, coupled with suspended latex particles. The test procedure is similar to the CATT, including the use of a similar rotator. Compared to the CATT, the LATEX/*T. b. gambiense* showed a higher specificity (96 to 99%) but a lower or similar sensitivity (71 to 100%) in recent field studies conducted in several Western and Central African countries (45, 93, 125). Further evaluations are needed before it can be recommended for routine field use.

Immunofluorescence assays have been used with success for HAT control in Equatorial Guinea, Gabon, and the Republic of Congo, where they were shown to be highly sensitive and specific (87). The availability of standardized antigen at low cost has greatly improved the reliability of the test (73). It can be used with serum or FP eluates, but the test sensitivity has been reported to be as low as 75% when used with impregnated FP (111). Enzyme-linked immunosorbent assay (ELISA) methods can be performed with serum, FP eluates, and CSF with strict standardization and quantification (57). Interestingly, ELISA could also detect specific antibodies in the saliva from a group of 23 patients with confirmed HAT (59). Anti-

body levels were about 250-fold lower than in the serum and could not be detected by the CATT or the LATEX/*T. b. gambiense* in the vast majority of these patients. The sophisticated equipment required for IFA and ELISA methods limits their use to reference laboratories for remote testing of samples collected in the field during surveys.

Trypanosome Detection

Parasitological diagnosis is made by microscopic examination of lymph node aspirate, blood, or CSF. It provides direct evidence for trypanosome infection and thus allows a definite diagnosis. Unfortunately, parasite numbers in *T. b. gambiense* infection can vary between more than 10,000 trypanosomes/ml, being easily detectable, and less than 100 trypanosomes/ml, being below the detection limit of the most sensitive methods in use. Moreover, parasite detection can be rather labor-intensive. Failure to demonstrate parasites therefore does not necessarily exclude infection. Serial examination of blood on consecutive days can increase the test sensitivity but is rarely performed in practice. When possible, it is recommended to use methods that test a larger quantity of blood and/or that facilitate trypanosome visualization to improve the sensitivity of parasite detection. It is also essential to keep the time between sampling and examination as short as possible to avoid immobilization and subsequent lysis of trypanosomes in the sample. Trypanosomes are rapidly killed by direct sunlight but can survive for a few hours when the sample is kept in a cool and dark place. We review here the main parasite detection methods that are currently available for field use. A detailed description of most of these tests, including figures, can be found in the WHO *Trypanosomiasis Control Manual* (137).

Chancre aspirate. Trypanosomes can be detected in the chancre a few days earlier than in the blood. The chancre is punctured, and the fluid obtained is microscopically examined as a fresh or fixed and Giemsa-stained preparation. This method is very seldom applied in the field because most infections are detected much later, when the chancre has already disappeared.

Lymph node aspirate. CLN palpation should be done systematically in all patients with a positive CATT result. When enlarged CLN are present, they are punctured, the fresh aspirate is expelled onto a slide, and a coverslip is applied to spread the sample and facilitate the reading. The wet preparation is then quickly examined by microscope (magnification, $\times 400$) for the presence of motile trypanosomes. The technique is simple and cheap. The sensitivity varies between 40 and 80% depending on the parasite strain, the stage of the disease (sensitivity is higher during the first stage), and the prevalence of other diseases causing lymphadenopathy (112, 127).

The yield of CLN palpation and puncture in patients with a negative CATT is very low; between 1999 and 2001, trypanosomes were observed in only 316 (0.18%) of 174,295 lymph node aspirates from CATT-negative individuals in Democratic Republic of Congo (112). The authors calculated that a mean of 138 h of work per new case diagnosed would be necessary. Furthermore, all positive lymph node aspirates found in 1,000 individuals from two endemic foci located in Angola and Central African Republic were associated with positive CATT (112). Systematic CLN palpation and puncture is therefore not cost-

effective for use with CATT-negative individuals (L. Lutumba et al., submitted for publication) unless indicative clinical signs are present.

Wet and thick blood films. In wet blood films, 5 to 10 μl of finger prick blood is placed on a slide and examined microscopically (magnification, $\times 400$) under a coverslip. Trypanosomes can be seen moving between the erythrocytes (the movement of the surrounding erythrocytes often attracts attention). Despite its very low sensitivity, with a detection limit as high as 10,000 trypanosomes/ml, corresponding to 1 parasite/200 microscope fields, this method is still used in some centers because of its low cost and simplicity. Giemsa- or Field's-stained thin blood films have a similarly low sensitivity. Examination of 20 μl of stained blood in a thick blood film slightly improves sensitivity, with a detection threshold of around 5,000 trypanosomes/ml. It is the technique of choice for blood examination only when no centrifuge is available (41), although it is quite time-consuming (10 to 20 min per slide) and requires expertise to recognize the parasite, which is frequently deformed in this preparation. Apart from trypanosomes, other parasites such as microfilaria and *Plasmodium* can be detected.

Microhematocrit centrifugation technique. The blood concentration technique of microhematocrit centrifugation (mHCT), sometimes referred to as the capillary tube centrifugation technique or as the Woo test, was developed more than 30 years ago and is still in use in many HAT control programs (133, 134). In brief, capillary tubes containing anticoagulant are filled three-quarters full with finger prick blood. The dry end is sealed with plasticine. By high-speed centrifugation in a hematocrit centrifuge for 6 to 8 min, trypanosomes are concentrated at the level of the white blood cells, between the plasma and the erythrocytes. The capillary tubes, mounted in a special holder, can be directly examined at low magnification ($\times 100$ or $\times 200$) for mobile parasites. The sensitivity of mHCT increases with the number of tubes examined, with an estimated detection threshold of 500 trypanosomes/ml. The optimal number of tubes has not been determined with certainty, but in most programs, six to eight tubes are prepared. This technique is moderately time-consuming, and the concomitant presence of microfilaria in the blood can render the visualization of the much smaller trypanosomes very difficult. Nevertheless, this relatively simple technique can be applied during mass screening by mobile teams.

Quantitative buffy coat. The quantitative buffy coat (QBC; Beckton-Dickinson), initially developed for the rapid assessment of differential cell counts, has been extended to the diagnosis of hemoparasites including trypanosomes (7, 67). It has the advantages of concentrating the parasites by centrifugation and, by staining the nucleus and kinetoplast of trypanosomes with acridine orange, allowing a better discrimination from white blood cells. After high-speed centrifugation of the blood in special capillary tubes containing EDTA, acridine orange, and a small floating cylinder, motile trypanosomes can be identified by their fluorescent kinetoplasts and nuclei in the expanded buffy coat. UV light is generated by a cold light source connected by a glass fiber to a special objective containing the appropriate filter. This objective can be mounted on almost every microscope. A darkroom is needed for the procedure. The relative sophistication and fragility of the material prevents its daily transport during active screening sessions.

The QBC is a very sensitive technique that is very appreciated by most field laboratory workers. It also allows the diagnosis of concomitant malaria, which is very useful for patient care. With a 95% sensitivity for trypanosome concentrations of 450/ml, the QBC can detect more patients with low parasitemia than the mHCT when fewer than eight capillary tubes are used (4). It is as sensitive as the mini-anion-exchange centrifugation technique (mAECT) described below (4, 124). Production of the QBC kit has been abandoned, but the manufacturing of capillaries was recently resumed.

Mini-anion-exchange centrifugation technique. The mAECT was introduced by Lumsden et al. (71), based on a technique developed by Lanham and Godfrey (55). An initial evaluation showed that the mAECT was more sensitive than the thick blood film and the mHCT (70). An updated version has been described by Zillmann et al. (139). The technique consists of separating the trypanosomes, which are less negatively charged than blood cells, from venous blood by anion-exchange chromatography and concentrating them at the bottom of a sealed glass tube by low-speed centrifugation. The tip of the glass tube is then examined in a special holder under the microscope for the presence of trypanosomes. The large blood volume (300 μl) enables the detection of less than 100 trypanosomes/ml, resulting in high sensitivity, but the manipulations are quite tedious and time-consuming. mAECT columns are now produced with the assistance of the Institute of Tropical Medicine—Antwerp at Kinshasa, DRC. Studies validating this newly produced version of mAECT are under way.

Stage Determination: Cerebrospinal Fluid Examination

In the absence of sufficiently specific clinical signs and blood tests indicating the evolution from first- to second-stage HAT, staging of patients still relies on examination of CSF obtained by lumbar puncture. It is a vital step in the diagnosis process. Patients with first-stage disease receive daily intramuscular pentamidine for 7 to 10 days, a treatment associated with less than 1% mortality, whereas patients with second-stage disease are still treated in most centers with melarsoprol, an arsenical derivative associated with a 2 to 10% fatality rate (97). The majority of deaths are due to treatment-related acute encephalopathies (98, 136). Eflornithine (DFMO) is a safer treatment than melarsoprol, but its complicated schedule (four intravenous infusions per day for 14 days) and cost remain an obstacle to a wide field application.

According to WHO recommendations, second-stage HAT is defined by the presence in the CSF of one or more of the following (136): (i) raised white blood cell count (>5 cells/ μl), (ii) trypanosomes, and (iii) increased protein content (>370 mg/liter, as measured by the dye-binding protein assay). As reviewed below, these criteria are not entirely satisfying and might soon be modified by recent studies' findings.

White blood cell count. The CSF white blood cell count is the most widely used technique for stage determination. After collecting the CSF sample, the cell count should be carried out as soon as possible to prevent cell lysis. Due to the small number of cells in normal CSF, cell-counting chambers should have a volume of at least 1 μl , such as the Fuchs-Rosenthal and the Neubauer devices. It is not recommended to dilute the CSF with Türcck solution since this solution can lyse trypanosomes.

If fewer than 20 cells/ μl are counted, it is recommended to repeat the counting procedure and to calculate mean count values. The CSF pleocytosis is of lymphocytic origin, consisting mainly of B cells (40).

The 5-cells/ μl threshold for treatment decision is controversial (12, 63, 80). Some countries use a threshold of 10 cells/ μl (Equatorial Guinea) or even 20 cells/ μl (Angola and Côte d'Ivoire) in their national protocol (25, 63, 117). Patients with 6 to 20 cells/ μl in the CSF are sometimes referred to as being in the "early second stage" or "intermediate stage" of the illness. This group is in fact composed of individuals with or without signs of neuroinflammation, as recently demonstrated (63). This is further illustrated by the effectiveness of pentamidine in HAT patients with 6 to 20 cells/ μl of CSF in Côte d'Ivoire (25), while in a study in Uganda, patients with 11 to 20 cells/ μl or with evidence of intrathecal IgM synthesis (a reliable marker of neuroinflammation) had a lower cure rate, suggesting that these patients should be treated with DFMO or melarsoprol (62). In a smaller study in Angola, the relapse rate after pentamidine treatment was similar in patients with 0 to 5 or 6 to 10 cells/ μl (106). These data support the increase of the CSF cell threshold from 5 to 10 cells/ μl . Furthermore, one should take into account the higher normal cell counts in neonates (109).

There is a general agreement that patients with proven HAT (trypanosomes seen in the lymph node or blood) and with >20 cells/ μl in CSF should be treated as having second-stage HAT. In Médecins Sans Frontières and Malteser programs in Sudan and Uganda, serologically suspected individuals (positive CATT of 1:4) with negative parasitological examination and >20 cells/ μl in the CSF are treated as second-stage HAT patients. This approach aims at partially compensating the insufficient sensitivity of trypanosome detection but exposes some non-HAT patients to unnecessary treatment for second-stage illness. It can be justified in areas with high HAT prevalence, especially where DFMO, a safer drug than melarsoprol, is used as the first-line treatment. Here again, the availability of more sensitive parasite detection methods and more precise staging tools would solve the controversy.

The morular cells of Mott, which are plasma cells with large vacuoles containing IgM, are reported to be highly indicative of HAT when found in the CSF (36, 39, 95). Mott cells are rarely observed in the field and can also be found in other neuroinfectious diseases such as neurosyphilis (52).

Trypanosome detection. The finding of trypanosomes in CSF allows immediate classification of a patient as being in the second stage of illness. It is important to examine the CSF immediately after lumbar puncture, because trypanosomes in CSF start to lyse within 10 min. Direct detection of trypanosomes (e.g., during cell counting) is a simple and cheap technique but suffers from insufficient sensitivity. Increased sensitivity of trypanosome detection is obtained by centrifugation of the CSF sample, especially when a double centrifugation method is used (22). The latter method is relatively time-consuming and requires two different types of centrifuges; therefore, it is not applicable in every field setting. A modified and simplified single centrifugation of CSF using a sealed Pasteur pipette has been proposed as an alternative to double centrifugation (79).

Some authors challenge the value of finding CSF trypanosomes in patients with no sign of CSF inflammation (absence of elevated protein and cell count of $\leq 20/\mu\text{l}$ in the CSF) who were shown to respond to pentamidine treatment (25, 62, 80).

Protein concentration. In normal healthy individuals, proteins in the CSF consist mainly of albumin (70%) and IgG (30%), both originating from the serum. Protein concentrations in the CSF are elevated in HAT patients and range from 100 to 2,000 mg/liter (13, 63). Protein concentrations can also be raised in first-stage illness due to the diffusion in the CSF of IgG, which can be present in very high concentrations in the serum. Recent evidence suggests that the protein concentration threshold set by WHO (370 mg/liter) is too low and should be raised to 750 mg/liter to reflect blood-brain barrier impairment, astrocyte activation, and neurodegeneration (61, 63). Despite its apparent simplicity, accurate determination of the total protein concentration in CSF is rather difficult. CSF protein concentrations obtained by different methods and different standards are not comparable. As a consequence of the sophistication of the methods, the absence of standardization, the instability of reagents, and the limited (if any) added value compared to CSF cell count (80), total protein measurement for staging HAT is no longer recommended and has been virtually abandoned in field laboratories.

Antibody detection. It has been well known for several decades that the CSF of second-stage HAT patients contains high levels of immunoglobulins, especially IgM (12, 36, 54, 132). An increased CSF IgM concentration has thus been considered by some as a strong potential marker of second-stage HAT (39, 75).

The demonstration of intrathecal synthesis of immunoglobulins strongly supports the diagnosis of neuroinflammatory diseases. Immunoglobulins synthesized in the CNS need to be discriminated from blood-derived immunoglobulins by calculation of the intrathecal fraction and antibody index (quantitative approach) or by detection of oligoclonal antibodies (qualitative approach) (101, 114). The origin and composition of the CSF immunoglobulins have been recently studied in experiments with large patients groups. As previously suspected (36), these studies confirmed that the elevated immunoglobulin concentration in the CSF is due to intrathecal synthesis and that the dominant IgM presence was an early marker of CNS invasion whereas blood-CSF barrier dysfunction was found late in the course of CNS involvement (13, 65). These results were further confirmed by studies of 272 HAT patients from different areas of endemic infection, where intrathecal synthesis of IgM was found in 95% of patients with second-stage illness (63). *T. b. gambiense* HAT can thus be classified among neuroinflammatory diseases with a dominant IgM immune response pattern in the CNS, like Lyme neuroborreliosis and, occasionally, neuro-syphilis (101).

Despite its relevance to stage determination, IgM detection in CSF has not been carried out in the field, owing to the lack of simple and robust tests. A latex agglutination test for IgM in CSF (LATEX/IgM) has recently been developed. It is designed for field use and remains stable at 45°C for more than 2 years. Following initial promising results (58), the LATEX/IgM was evaluated with CSF samples from patients from several countries where infection is endemic (60). CSF end titers obtained by the LATEX/IgM paralleled the IgM concentra-

tions determined by nephelometry and ELISA. At a cutoff value of $\geq 1:8$, the sensitivity and specificity of LATEX/IgM for intrathecal IgM synthesis were 89 and 93%, respectively. Future prospective studies with large numbers of patients are needed for LATEX/IgM validation.

Only a small proportion of the very large amount of IgM produced during HAT is specific anti-trypanosome antibody (39). Trypanosome-specific antibodies detected in the CSF by indirect immunofluorescence are specific for second-stage illness, whereas a comparison with serum values by calculation of the antibody index is necessary when measurements are performed by ELISA (54, 57, 69). However, these methods are too sophisticated to be used in remote treatment centers. Unfortunately, the field-designed CATT/*T. b. gambiense* and LATEX/*T. b. gambiense* lack sensitivity when used with CSF for detection of anti-trypanosome immunoglobulins (19, 66, 69).

The CSF of HAT patients also contains antibodies with other affinities. Antibodies against brain-specific components such as neurofilaments and galactocerebrosides (GalC) have been detected and may be promising markers of second-stage illness (6, 61, 64). These autoantibodies, which might result from the CNS damage and immune activation triggered by trypanosome invasion, are associated with markers of neuroinflammation such as the CSF cell count and protein and immunoglobulin concentrations (11, 61). Unfortunately, anti-GalC antibodies detectable in the serum are not correlated with neuroinflammation (11).

Management of Serologically Suspect Individuals

As previously stated, the parasitological methods have a limited sensitivity and therefore do not allow all HAT patients with an initial positive screening (usually a positive CATT on whole blood and on a 1:4 serum dilution) to be confirmed to have the disease and receive treatment (136). These undiagnosed patients will return home and will either be diagnosed at a later stage of the disease or simply die. Moreover, they contribute to the reservoir of parasites and thus to transmission of the disease. One option is to examine these serologically suspect individuals at regular intervals (e.g., every 3 months) for 1 to 2 years (136). However, compliance with follow-up visits is usually low, and the efficiency of this strategy is poor (24).

A much more promising option is to determine a subgroup of serologically suspected individuals at high risk of being infected with *T. b. gambiense* and to treat them. Of 86 serologically suspect individuals (CATT serum, $\geq 1:4$) being monitored in Angola, 52% of individuals with a CATT plasma end titer of $\geq 1:16$ were diagnosed with HAT during follow-up but none of the individuals with lower CATT end titers were diagnosed with disease (113). Similar results were obtained for a group of 749 serologically suspect individuals (CATT, $\geq 1:4$) who were examined at least once during a 12-month follow-up period in Kajo-Keji county, southern Sudan (24). Individuals with a CATT plasma end-dilution titer of $\geq 1:16$ had a 50% person-year risk of being confirmed with HAT during follow-up, compared with a 10 and 14% person-year risk for 1:4 and 1:8 end-dilution titers, respectively. The authors of these studies recommend treatment for all serologically suspect individuals with a CATT end titer of $\geq 1:16$, when the prevalence of

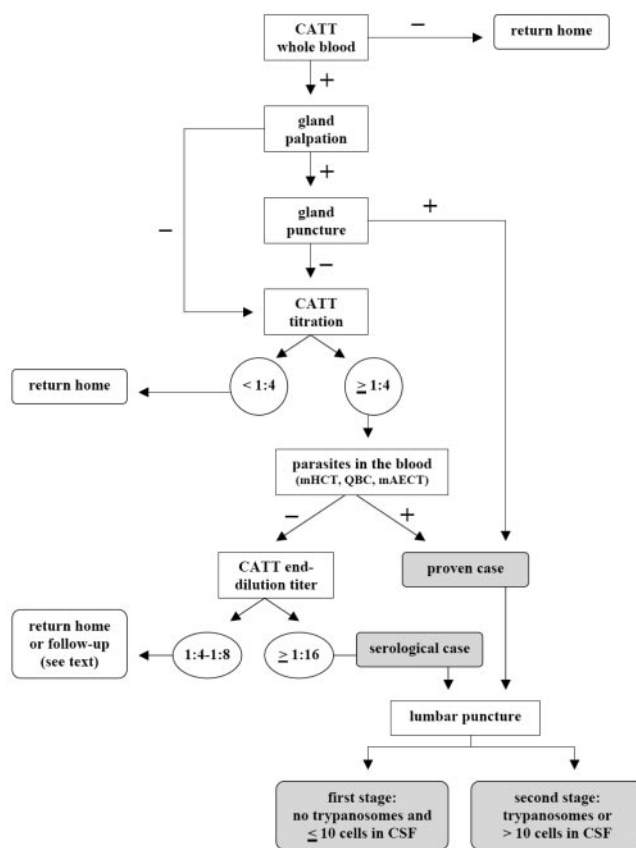


FIG. 4. Example of a field algorithm for the diagnosis of *T. b. gambiense* sleeping sickness according to data from Simarro et al. (112).

HAT in the investigated population is sufficiently high. Which prevalence threshold to choose remains an open question, but it should probably be no less than 1%. In a study conducted in a low-prevalence area in Côte d'Ivoire, only one patient was diagnosed with HAT during a 2-year longitudinal follow-up of 77 serologically suspect patients (33). However, the CATT end-dilution titer was limited to 1:4 in this study. Other factors, such as poor access to care and absence or limited availability of parasitological diagnosis, should positively influence the decision to perform titration of serum and treat individuals with a CATT end titer of $\geq 1:16$.

Example of a Field Diagnostic Algorithm for *T. b. gambiense* HAT

There is no “one-size-fits-all” algorithm for the diagnosis of HAT. The positive predictive value of any diagnostic test varies with the prevalence of HAT among the tested population. Therefore, the diagnostic tree applied in a given area should be adapted to decreasing disease prevalence. Nevertheless, there is a need to decrease interprogram variations in the diagnostic approach. A more standard diagnostic algorithm would improve the external validity of the few studies conducted on the diagnosis and treatment of this neglected disease. An example of a diagnostic algorithm, adapted from a recent publication by WHO experts (112), is shown in Fig. 4. This algorithm is

particularly well suited for populations with a rather high disease prevalence (e.g., above 2%), such as individuals coming on their own to the treatment center, who are more likely to have symptoms and thus the disease, or individuals from a highly active focus of infection. If the prevalence of the disease is lower (e.g., below 1%), it will be more appropriate to set a higher CATT dilution threshold before implementing the time-consuming search for parasites in the blood (i.e., 1:8 instead of 1:4). As stated above, the treatment of serological suspect patients with a high CATT end-dilution titer ($\geq 1:16$) would not be justified for populations with low disease prevalence. In this case, these individuals should return home and be told to come for control visits if symptoms appear or worsen.

The choice of the method for detection of bloodstream parasites will depend on the method's performance (detection threshold), the workload, the number and level of training of laboratory workers, and the financial resources. Blood concentration methods such as the mHCT or the QBC should be preferred to a wet or thick blood smear. The mAECT can be performed sequentially after a negative mHCT, but the efficiency of this controversial strategy is still being studied.

As discussed above, the CSF cell count threshold defining the stage of the disease is still subject to debate. Considering the growing evidence showing a good efficacy of pentamidine in HAT patients with ≤ 10 cells/ μl (25, 106), a threshold of 10 cells is proposed in this algorithm. A 5-cells/ μl threshold remains justified though, particularly in programs using the less toxic eflornithine as first-line therapy for second-stage illness.

Current diagnostic algorithms for HAT tend to neglect investigations for other diseases such as malaria, typhoid fever, tuberculosis, syphilis, and HIV-associated diseases. These conditions can mimic or complicate *T. b. gambiense* HAT. The influence of the varying prevalence of these diseases on the validity of some key steps of the HAT diagnostic algorithm, such as the thresholds for CATT end-dilution titer and the CSF cell count, deserves further investigations.

Other Diagnostic Approaches

Antigen detection tests. Antigen detection is an attractive concept that would allow, unlike methods detecting antibodies, a distinction between active and cured HAT. By detecting antigens released by noncirculating trypanosomes sequestered in the liver, spleen, lymph nodes, or CNS, antigen detection has the potential to improve the sensitivity of current parasitological methods. Following promising results of specific antigen detection by ELISA (83), the card indirect agglutination test for trypanosomiasis (TrypTect CIATT; Brentec Diagnostics, Nairobi, Kenya) was developed for field use. A preliminary evaluation of the TrypTectCIATT showed a high sensitivity compared to other parasitological techniques (82), but the results of subsequent studies raised strong doubts about its specificity (5).

In vitro culture and animal inoculation. When inoculated into a suitable medium, viable trypanosomes multiply but are not detected for days or weeks. A ready-for-use kit for in vitro isolation (KIVI) has been developed (1). After inoculation of 5 to 10 ml of blood, the bottles are kept at ambient temperature. Bloodstream-form trypanosomes transform into proliferating large procyclic forms that can be detected within 3 to 4

weeks. The recorded sensitivity of the KIVI is variable (76, 121, 122). The yield of isolation of *T. b. gambiense* in rodents is low unless neonatal, immunosuppressed, or particular (e.g., *Mastomys natalensis*) rodents are used (136). The high cost and the long delay before obtaining the result are definite obstacles to routine field use of these techniques.

PCR. Different assays now exist; however, none of them have been validated for diagnostic purposes. PCRs targeting repetitive sequences are in theory more sensitive than those targeting low-copy or single-copy sequences like the recently developed tests for distinguishing *T. b. gambiense* and *T. b. rhodesiense* (43, 48, 99, 100, 110, 131).

In principle, PCR can be applied to any patient sample that may contain trypanosome DNA, such as whole blood or buffy coat, lymph node fluid, or CSF. Samples should be stabilized in special buffers or on FP. The FTA FP produced by Whatman is particularly convenient since it is easy to handle and it protects the DNA from degradation, unlike common FP. However, the amount of sample that can be applied on filter paper is small, thus limiting the chance to contain enough DNA for detection. Samples should be protected from sunlight to avoid DNA degradation. PCR results are not always unequivocal. Unexplained false-negative and false-positive results were observed in CATT-seropositive but parasitologically nonconfirmed persons and in CATT-negative controls (33, 116). Also, the significance of a positive PCR on a CSF sample is unclear. True et al. report that PCR is 100% sensitive compared to double centrifugation of CSF, but Jamonneau et al. clearly demonstrate that a number of patients with positive PCR results with CSF were successfully treated with pentamidine, thus showing them to be in the first stage of the disease (44, 123). Efforts to simplify the PCR amplification method itself, such as using an isothermal amplification reaction and visualization of the PCR product by precipitation (53) or by oligochromatography (P. Mertens et al., Abstr. 14th Eur. Congr. Clin. Microbiol. Infect. Dis, abstr. P1842, 2004), may facilitate PCR application in African countries, but PCR is definitely not an option for field diagnosis and for the time being is restricted to research purposes.

Proteomic signature analysis. Proteomic signature analysis is a promising technology that has been recently used with sera from patients with HAT and other diseases (90). The accuracy of this experimental method was high (100% sensitivity and 98.6% specificity) but needs to be confirmed in prospective studies. As the authors stated, this method is impracticable in the field but could identify discriminating biomarkers that could lead to the development of more conventional and simpler tests.

Imaging techniques. Neuroimaging techniques such as computed tomography and magnetic resonance imaging are not available in areas of endemic infection, except in few referral centers. They reveal nonspecific features that are occasionally useful to stage the disease. Brain magnetic resonance imaging may show diffuse white matter abnormalities, ventricular enlargement, hyperintensities in the basal ganglia, and signs of meningitis (35, 107).

LABORATORY DIAGNOSIS OF *T. B. RHODESIENSE* INFECTION

As stated above, *T. b. rhodesiense* HAT usually presents as an acute febrile illness with rather nonspecific symptoms and

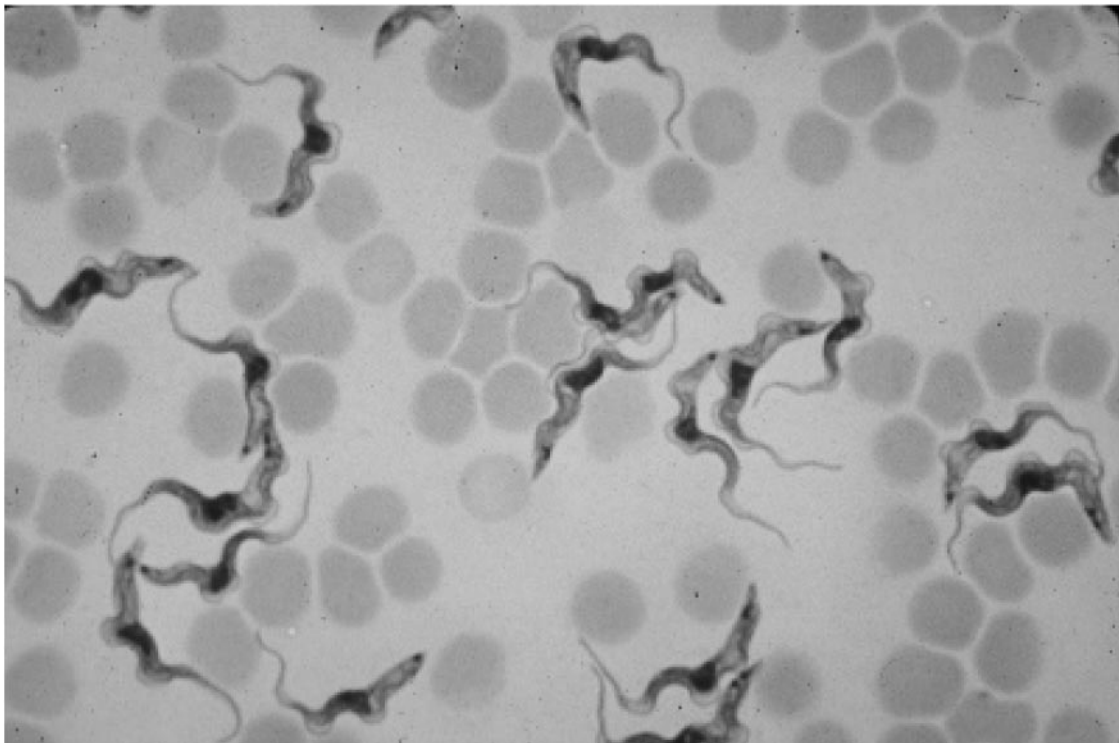


FIG. 5. Bloodstream trypomastigote forms of *T. b. rhodesiense* on a peripheral thin blood smear. Copyright Jean Jannin (WHO/CPE).

signs unless the initial characteristic chancre is present. Biological parameters are generally more abnormal than with *T. b. gambiense* illness. Anemia may be severe, at least partly due to decreased red cell survival (135). Thrombocytopenia may also be severe and can be accompanied by other features of disseminated intravascular coagulation (8, 34, 104).

There is still no equivalent to the CATT widely available for the screening of *T. b. rhodesiense* HAT, despite promising results from initial evaluations of simple agglutination tests such as the trypanosomiasis agglutination card test, the procyclic agglutination test for trypanosomiasis, or a modified and more practical version (3, 84, 92). Immunofluorescence and ELISA-based serological tests exist, but their reported sensitivity is variable and they can be performed only in reference centers with highly trained technicians (2, 49, 51). Field screening thus still relies on clinical symptoms and signs. The diagnostic confirmation and staging of *T. b. rhodesiense* HAT are based on the same methods as described above for *T. b. gambiense* disease. Parasitological confirmation is relatively easy because bloodstream trypanosomes are numerous (Fig. 5). Most patients are therefore diagnosed by examination of a stained thin or thick blood smear (26, 47). Trypanosomes are sometimes detected by chance while searching for *Plasmodium* spp (34, 115). Trypanosomes can also be found in the chancre by microscopic examination of an aspirate, but other methods such as lymph node aspiration or blood concentration techniques are needed less often. Unlike *T. b. gambiense* HAT, the staging of *T. b. rhodesiense* illness has not been recently studied and thus still relies on WHO recommendations (136).

As stated above, *T. b. rhodesiense* infections have a more chronic clinical presentation at the southern end of their dis-

tribution (e.g., in Zambia), with clinical characteristics closer to those of *T. b. gambiense* illness (21, 102). A practical and reliable serological test would be a useful screening tool in these areas to detect infected individuals with few or no symptoms, since simple parasitological methods such as the thick blood smear appears to have a more limited sensitivity in these regions (20, 28).

There are currently no areas of endemicity where *T. b. gambiense* and *T. b. rhodesiense* coexist. Endemic foci of both forms exist in Uganda but are geographically separated. Evidence of a northward spread of cattle carrying *T. b. rhodesiense* has been recently shown (32, 42). A merger of *T. b. gambiense* and *T. b. rhodesiense* foci would create huge problems, since the diagnosis and treatment of these two forms of illness differ considerably (130). Molecular markers such as the serum resistance-associated gene, present only in *T. b. rhodesiense* (131), and a receptor-like flagellar pocket glycoprotein (TgsGP) specific to *T. b. gambiense* (10), may play a role in closely monitoring the distribution of both trypanosome species. The development of a simple assay detecting the circulating serum resistance-associated protein would not only be useful for targeting treatment to cattle infected with *T. b. rhodesiense* (131) but would also be valuable to distinguish the trypanosome species in humans exposed to both forms of HAT.

CONCLUSION

Over the last few decades, only minor improvements have been achieved in field diagnosis of HAT. Parasite detection remains insufficiently sensitive, and a simple serological diagnostic test for *T. b. rhodesiense* infection is still lacking. In

contrast, extensive research has been conducted on stage determination and molecular diagnosis, which eventually may lead to a new generation of field-applicable tests. Since the prevalence of HAT is expected to decrease as a result of ongoing control activities, new diagnostic tests should be highly specific. In the meantime, major diagnostic challenges for the coming years are to improve the access of the existing tools for the population at risk and their rational use in the diagnostic strategy. All this will require determined dedication from scientists from countries with endemic infection and Western countries, WHO, nongovernmental organizations, policy makers, and donors.

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