The Trypanosomiases

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10 Diagnosis of Human African Trypanosomiasis

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Introduction

Diagnosis of human African trypanosomiasis (HAT) or sleeping sickness can be defined as the demonstration of infection by *Trypanosoma brucei gambiense* (*T. b. gambiense*) or *rhodesiense* (*T. b. rhodesiense*). Evidence of infection may be indirect (clinical and biological features, serological and molecular test results) or direct (demonstration of the parasite). For reasons related to costs and risks associated with treatment, the demonstration of the parasite is mandatory for primary diagnosis. Yet primary diagnosis is not sufficient for deciding on treatment. Since not all drugs pass the blood–brain barrier, it should first be determined whether the parasite has reached the central nervous system. This 'stage determination' may be accomplished by direct (parasite detection) or indirect diagnostic techniques. Finally, after treatment, both direct and indirect diagnostic techniques should repetitively be applied to assess the disappearance of the parasite. It is recommended (but almost impossible) to follow up the patient during 2 years after treatment.

Diagnosis of the infection is important not only for the patient but also for the community. Undiagnosed, patients will remain untreated and will die sooner or later. In the meantime, they act as a reservoir on which teneral tsetse flies will infect themselves to spread the infection. In addition, due to the debilitating character of the disease, patients become a burden to their families. As the disease progresses from first to second stage, treatment becomes more expensive and the risk of severe side effects and sequellae increases.

As long as vaccination or prophylaxis against the infection and effective vector control are lacking, diagnosis will remain one of the cornerstones in the control of the disease, hence the importance of correct application of the different techniques currently available. Since sleeping sickness occurs in rural environments in sub-Saharan Africa, it is clear that only those diagnostic techniques that combine cost effectiveness, rapidity, simplicity and diagnostic performance will be applied within sleeping sickness control activities. Nevertheless, techniques not readily applicable for control activities may be very useful for research purposes, such as studies on epidemiology, on vaccination and alternative treatment, on the animal reservoir and on transmission dynamics.

Clinical Diagnosis

Clinical suspicion might be considered as indirect evidence of trypanosome infection. However, due to the aspecific, variable and

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inconstant character of clinical symptoms and signs, suspects should be confirmed by parasite detection. Only for stage determination and treatment failure cases may clinical signs, indicating central nervous system involvement, be sufficient to decide on the treatment protocol or on retreatment. A comprehensive review of clinical manifestations of sleeping sickness can be found in Dumas and Girard (Dumas and Girard, 1978).

Clinical signs associated with the initial and haemolymphatic stage

The first or haemolymphatic stage corresponds to the invasion of lymph, blood and other tissues by the trypanosomes. The reaction of the reticuloendothelial system to the presence of the parasite is characterized by a great variety of clinical manifestations. The variability and inconstancy of these signs is illustrated by the fact that many patients infected by *T. b. gambiense* are unaware of infection, since they feel in reasonable health for weeks, months or even years. In *T. b. rhodesiense* infections, outward signs of well-advanced disease may occur within 2 months.

Chancre

At the site of inoculation, signs of a local inflammation may appear: itching, erythema, swelling, pain and local heat. The first sign of infection occurs after 5–15 days: the trypanosomal chancre. It expands within a few days from a red skin patch with a little point in the centre (erythematous macula) to a hard and painful round pruritic nodule (erythematous pseudo furuncle) up to 3 cm in diameter. The chancre disappears without leaving a trace within 2–3 weeks. It is more commonly observed in *T. b. rhodesiense* than in *T. b. gambiense* infections and more often in Europeans, but only rarely in Africans.

Fever and general malaise

From 1 to 3 weeks after the infective bite, an initial fever develops in response to the invasion of the bloodstream by the trypanosomes. The fever (often high) lasts for a maximum of 1 week and can be accompanied by headache and general malaise. The first febrile attack is followed by bouts of fever, separated by prolonging remissions as the disease progresses.

Adenopathy

Accompanying the immune stimulation, the enlargement of glands of the posterior cervical and supraclavicular groups is one of the cardinal signs of *T. b. gambiense* infection. Sometimes enlarged neck glands are easily visible (Winterbottom's sign) but usually the neck has to be palpated to discover them. They are the size of a bean or larger, typically feel rubbery and move freely, are painful at the onset and can persist over weeks or months. Later, they tend to shrink. In *T. b. rhodesiense* infections, neck glands are often not enlarged.

Skin rash and pruritus

From 6 to 8 weeks after onset, scattered large erythematous circinate patches 7–10 cm in diameter develop mainly on trunk and shoulders. They often fade and reappear and are invisible on dark skin. They are not tender and do not itch and are therefore not commonly a presenting symptom. The skin rash is too inconstant and aspecific to be of diagnostic value. Pruritus is often associated with skin rash and is observed in almost half of the cases. It may be discrete in the beginning but persists throughout the disease.

Local oedema

Another inconstant characteristic of trypanosome infection is the partly oedematous swelling of lower eyelids and the puffy swollen appearance of the face, which gives some patients an expression of dullness or sadness. The eyes may look alert or stare, contrasting with the stillness of face. Many patients seem unaffected in early phase but this symptom progressively becomes more characteristic. Later, local oedema develops on other body parts, e.g. peripheral oedema on the legs associated with anaemia.

Cardiovascular disturbances

Cardiac involvement is one of the earliest signs suggestive of trypanosomiasis infection and virtually a common feature of *T. b. rhodesiense* and *gambiense* infections. Thorough examination is required, since there is rarely any overt symptomatology. Physical examination can reveal anomalies of rhythm, soft cardiac murmur and hypotension. Thoracic radiography can show cardiomegaly due to pericardial effusion and dilatation of cardiac cavities. Electrocardiogram tracings may have marked abnormalities.

Endocrine dysfunction

Endocrine dysfunction is marked by permanent feeling of cold and by disturbance of vegetative and sexual functions. In women, sterility, amenorrhoea and abortion is observed. Amenorrhoea may appear soon after onset but is common later on. Abortion is often associated with uterine hypoplasia secondary to defective hormonal secretions. Premature birth, stillbirth and perinatal death are frequent. In men, impotence and in later stages excessive development of mammary glands (gynaecomastia) occurs.

Neurological disorders

Neurological disorders may appear very early, indicating the precocity of meningeal and encephalitic involvement. Behavioural disturbances may occur but often remain unrecognized. Headache, debility and weakness are too common in other diseases to be of diagnostic value. Less frequent but almost diagnostic is a peculiar deep hyperaesthesia (Kerandel's sign), a sensation of pain when soft tissues are compressed. Pain is not immediate but occurs after 1–2 s; it rapidly becomes severe and out of proportion to the force causing it, lasts some seconds, fades and disappears within a few minutes.

Clinical signs associated with the meningoencephalitic stage

Signs and symptoms described for the haemolymphatic stage persist or may even worsen but, as the disease progresses, signs of nervous system injury become more obvious. Manifestations depend on which part of the central nervous system is affected. Neurological changes are most extensive in *T. b. gambiense* infection with its duration of years and its characteristic sleep-pattern disturbance. In *T. b. rhodesiense* infections with duration of only 6–9 months, the marked neurological symptoms are not seen, though there may be some drowsiness, tremors and unsteadiness preceding the terminal coma.

Disturbances of consciousness and sleep

Daytime somnolence can be considered as most characteristic of all signs. At any moment, awakening succeeds periods of sleep with variable periodicity, according to the gravity of the disease. Slowing down of mental functions with diminution of attention, total indifference and episodes of somnolence prevail. A state of apathy overcomes the patient and may accompany narcoleptic states, which occur unexpectedly. Together with disorders of awakening, a loss of muscle tone and drooping of the eyelids are seen. Drowsiness gradually becomes more pronounced until sleep is continuous. Finally somnolence deepens to coma.

Disorders of tonus, motility and abnormal movements

These signs vary in distribution, intensity and onset and reveal lesions of the diencephalon and the superior mesencephalon. Tonus disturbances include a hypertonicity (of extrapyramidal origin), which is variable in time and place, or a hypotonicity (of cerebellar origin, due to sensitivity troubles). In association with the disturbed awakening it may sometimes produce the apathic appearance of patients. A great diversity of abnormal movements due to muscle instability is observed. Trembling of hands and fingers and choreiform, athetoid or oscillatory movements of the arms, head, neck or trunk are variable in topography, intensity and rhythm. Exceptionally, real paralysis occurs. Perioral and cheiro-oral reflexes of the cere-

bral trunk are almost a constant. Also in *T. b. rhodesiense* sleeping sickness, tremor of hands and tongue, unsteadiness and walking difficulties may appear from the second or third month on.

Mental changes

In *T. b. gambiense* infections, temporary mental confusion and psychiatric problems may occur. These include emotional lability, indifference, aggression, asocial behaviour, stereotypic behaviour, impulsive actions, fugue states, manic episodes, melancholia, delirium and/or dementia. Even in the early phase subtle changes can be present, whereas psychiatric problems may dominate. Dementia only develops at terminal stages of the disease. In *T. b. rhodesiense* patients, mental slowness, dullness and temporary delirium, mania, confusional or hallucinatory states may be observed.

Parasitological Diagnosis

Definite diagnosis, i.e. direct evidence for trypanosome infection, is obtained by microscopic examination of lymph, blood or cerebrospinal fluid from the putative host. Unfortunately, the demonstration of the parasite still relies on often poorly sensitive yet laborious techniques that have been used for many decades. In practice, therefore, parasitological examination is generally limited to clinical or serological suspects. Failure to demonstrate parasites, however, does not necessarily exclude infection. Due to the sometimes low parasite load, particularly in *T. b. gambiense* infections, trypanosomes can be difficult to detect. Concentration or cultivation techniques, often combined with optimized visualization systems, can improve the sensitivity of parasitological diagnosis. For efficient parasitological examination of samples, it is important to keep the time between sampling and examination as short as possible to avoid lysis of the trypanosomes in the sample. Trypanosomes are rapidly killed by direct sunlight but can survive longer when the sample is kept cool in a dark place. Since parasite detection is mandatory for definite diagnosis, special attention should be paid to the quality of reagents and materials and to proper maintenance of the equipment, in particular the microscope. In general, a 20 × 10 or 40 × 10 magnification is used for screening the sample preparation under the microscope. Few authors have studied the comparative sensitivity of several tests in a more or less standardized way but, in general, the detection limit of a test depends on the amount of sample screened (WHO, 1998).

The following is an exhaustive list of trypanosome detection techniques, of which only some are actually applied for diagnosis of sleeping sickness. More details on some of the techniques can be found in Van Meirvenne (1999).

Chancre aspirate

Trypanosomes can be detected in the chancre 2 days earlier than in the blood. The chancre is punctured and the juice obtained is microscopically examined as a fresh, fixed or Giemsa-stained preparation. This method is seldom applied, since most of the patients are detected much later after infection when the chancre has already disappeared.

Lymph node aspirate

If enlarged lymph nodes are present, they are punctured and the fresh aspirate is microscopically examined. Due to its simplicity and low cost, this technique remains widely applied, particularly in *T. b. gambiense* infections. Sensitivity varies from 40 to 80% and seems to depend on the parasite strain, on the occurrence of other diseases causing lymphadenopathy and on the disease stage (more common in the early stage).

Blood

Wet blood film

About 5–10 µl of finger-prick blood are applied on a slide and examined microscopically under a coverslip. If present, try-

panosomes are mainly revealed by their moving between the red blood cells. Although the detection limit is only 10,000 tryps/ml and the sensitivity is therefore low, this technique is still in use most probably because of its low cost, its simplicity and its immediate result.

Thick blood film

A small drop (20 µl) of finger-prick blood is spread over an area of 1 cm on a microscope slide and defibrinated with a toothpick or the corner of another slide. The slide is dried in a horizontal position, protected from direct sunlight, and is stained without fixation by Giemsa or Field stain prior to microscopic examination at 100 × 10 magnification. Proper preparation of the sample is important to avoid artefacts: correct thickness and defibrination, purity and quality of the staining reagents and cleanness of the slides. The microscopist should pay attention to the morphology of the trypanosomes (long-slender, short-stumpy with often serious deformation). The detection limit is about 5000 tryps/ml. In those situations where a centrifuge is not available, thick blood film is the technique of choice for blood examination although it is time consuming (10–15 min per slide). Apart from trypanosomes, other parasites such as microfilaria and *Plasmodium* can be detected.

Microhaematocrit centrifugation technique

Capillary tubes containing anticoagulant are three-quarters filled with finger-prick blood. The dry end is sealed with plasticine. By high-speed centrifugation in a haematocrit centrifuge, 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 screened at low magnification for mobile parasites (Woo, 1971). By preparing more than one capillary tube, fewer than 500 tryps/ml can be detected. Unfortunately, the presence of moving microfilariae in the blood often makes it impossible to observe the much smaller trypanosomes.

Quantitative buffy coat (QBC)

Acridine orange is a fluorescent dye that binds to the nucleus and kinetoplast DNA of mobile trypanosomes in fresh blood, thus allowing discrimination from white blood cells in which only the nucleus becomes fluorescent. The quantitative buffy coat (QBC, Becton-Dickinson), originally developed for *Plasmodium* detection, combines both this fluorescent technique and the concentration of parasites by centrifugation. QBC has been used with success in sleeping sickness diagnosis (Bailey and Smith, 1992). After high-speed centrifugation of the blood in special capillary tubes, coated with ethylenediaminetetraacetic acid (EDTA) and acridine orange and provided with a floating cylinder, mobile trypanosomes can be identified by their fluorescent kinetoplast and nucleus between the white blood cells in the expanded buffy coat. Ultraviolet light is generated by a 'cold light source' connected by a glass fibre to a special objective containing the appropriate filter. This objective can be mounted on almost every microscope. The technique is highly sensitive and robust. Unfortunately, Becton-Dickinson recently decided to stop its production.

Mini anion exchange centrifugation technique (mAECT)

The mAECT has been introduced by Lumsden *et al.* (1979). The technique consists of separating the trypanosomes from venous blood by anion chromatography and concentrating them at the bottom of a sealed glass tube by low-speed centrifugation (3000 rpm). The large blood volume (300 µl) enables detection of fewer than 100 tryps/ml, resulting in high sensitivity, but the manipulations are quite tedious. In the case of low parasitaemia and failure of all other techniques to detect the parasite, mAECT can make the difference. The price may be prohibitive for large-scale use. mAECT columns and accessory materials are available from Institut National de Recherche Biomédicale (Kinshasa, Democratic Republic of Congo).

In vitro *culture*

Detection of trypanosomes through inoculation of culture medium with blood is possible but, due to low success rates, *in vitro* culture is seldom applied for diagnosis. The kit for *in vitro* isolation (KIVI) was originally developed for isolation of trypanosomes under field conditions (Aerts *et al.*, 1992) but can be used for diagnosis when other techniques fail. The technique consists of inoculating aseptically a large volume of venous blood (10 ml) into culture medium whereafter bloodstream trypanosomes transform into procyclics and start to multiply. The culture flask is kept in the dark at ambient temperature (ideally 27°C) and should be examined for the presence of trypanosomes during several weeks. The KIVI can be ordered at the Institute of Tropical Medicine, Antwerp.

Xenodiagnosis

Xenodiagnosis can be performed by inoculating rodents with the blood of the putative host or by feeding teneral tsetse flies on the host blood. Xenodiagnosis is seldom applied for obvious reasons: need for animals, low success rates, long incubation time.

Cerebrospinal fluid (CSF)

Examination of the CSF is usually undertaken for stage determination and not for primary diagnosis, except in cases of strong clinical or serological suspicion in which trypanosome detection in blood or lymph has failed. Parasitological examination of CSF will be discussed below under stage determination.

Serological Diagnosis

Serological indirect evidence for trypanosome infection can be obtained by demonstrating inflammatory responses, specific antibodies or parasite antigens in the putative host. The introduction of simple antibody detection tests for screening the population at risk, thus limiting tedious parasitological examination on the seropositive suspects only, has been a major breakthrough in diagnosis of human African trypanosomiasis. Reliable antigen detection tests remain to be developed. Seropositivity in antibody detection tests must be interpreted with caution, since antibodies can persist for up to 3 years after cure (Paquet *et al.*, 1992). Also cross-reactivity with other parasitoses can occur, particularly at low serum or blood dilutions and when immunoglobulin M (IgM) is participating in the reaction.

Non-specific biological alterations related to inflammation

Numerous non-specific alterations of the blood and the CSF composition have been observed in sleeping sickness patients of which the tremendous increase in total serum IgM, 8–16 times the normal concentration, is still the most suggestive for African trypanosomiasis. Anaemia is also observed frequently in sleeping sickness patients but can have other causes.

Detection of trypanosome-specific antibodies

Several techniques for detection of trypanosome-specific antibodies in blood, serum and CSF exist. The type of antigen(s) employed greatly determines the sensitivity and specificity of the test. For *T. b. gambiense*, the better tests make use of selected variable surface glycoproteins, while for *T. b. rhodesiense* non-variable antigens are preferred for reasons of much higher antigenic variability of this subspecies.

Only rapid agglutination tests are readily applicable in the field while immunofluorescence assays, ELISA, immune trypanolysis and plate agglutination tests are appropriate for remote laboratory testing of samples collected in the field during surveys.

Immunofluorescence assay (IFA)

IFAs have been used with success for *T. b. gambiense* sleeping sickness control in Equatorial Guinea and in Gabon. Immune

fluorescence can be applied on serum and on filter-paper eluates (Wéry *et al.*, 1970). The availability of standardized and stabilized antigen for *T. b. gambiense* at low cost has greatly improved the reliability of the test (Magnus *et al.*, 1978a). For testing of serum, it is important to use strictly IgG-specific fluorescent conjugates, thus avoiding cross-reactive IgM. Research is still needed to identify better antigens for *T. b. rhodesiense* sleeping sickness. The investment costs for a fluorescence microscope can be reduced by recurring to an external light source (e.g. QBC system or similar, or fluorescent light-emitting diodes).

ELISA

Numerous ELISA tests for sleeping sickness have been described. There is a tendency to use purified antigens instead of crude trypanosome lysates but investigations should continue into the use of recombinant or synthetic peptides. ELISA is interesting for those who wish to perform large-scale surveys on serum, filter-paper eluates and CSF with strict standardization and quantification. Furthermore, it is possible to study different immunoglobulin classes and isotypes separately in serum and CSF (Lejon *et al.*, 1998). However, the need for sophisticated equipment and large volumes of pure water remains a serious drawback for widespread application of the test. Alternative formats such as lateral flow tests for blood and serum and testing of saliva are currently being considered in our laboratory.

Immune trypanolysis

This test for antibody detection makes use of live bloodstream trypanosomes and is restricted to laboratories that have facilities to maintain cloned populations. The test is based on recognition of the variable epitopes on the surface of the trypanosomes by the corresponding antibodies resulting in complement-mediated lysis. The test is highly specific and is used in our laboratory as the reference test for evaluation of other antibody detection systems (Van Meirvenne *et al.*, 1995). Unfortunately, the test sensitivity is limited in the case of *T. b. rhodesiense* sleeping sickness, due to the higher antigenic variability of this subspecies compared with *T. b. gambiense*.

*CATT/*T. b. gambiense

The card agglutination test for trypanosomiasis (CATT) is a fast and simple agglutination assay developed for detection of *T. b. gambiense*-specific antibodies in the blood of sleeping sickness patients (Magnus *et al.*, 1978b). Although its diagnostic performance is not perfect, the test is now widely used in different countries where *T. b. gambiense* sleeping sickness is endemic. The CATT is currently produced in the Institute of Tropical Medicine, Antwerp. Evidence exists for limited sensitivity of CATT in the Ethiope East focus in Nigeria for which solutions have been found (see LATEX/*T. b. gambiense*, below). To overcome complement-mediated prozone effects, the addition of EDTA in the dilution buffer has been proposed thus increasing the sensitivity considerably combined with only minor loss of specificity (Magnus *et al.*, 2002). To reduce costs of reagent in seroepidemiological studies and in passive case detection, a miniature CATT version using dried blood samples on filter paper has been described (Miézan *et al.*, 1991). As with IFA, special attention should be paid to correct sampling and to dry storage of the filter papers. The occurrence of parasitologically non-confirmed CATT seropositives remains a matter of concern (WHO, 1998).

Procyclic agglutination test for trypanosomiasis (PATT)

The PATT has been introduced for detection of *T. b. rhodesiense* or *T. b. gambiense* antibodies in blood (Pearson *et al.*, 1986; Liu *et al.*, 1989). The original test, using live procyclic trypanosomes, has obvious limitations for field application but the modified test with fixed trypanosomes (MOPATT) is being used with success at Kenyan Trypanosomiasis Research Institute (KETRI), Kenya, and deserves thorough evaluation. According to our experience, the sensitivity of the test for *T. b. gambiense* sleeping sickness is low.

Haemagglutination

As early as 1975, a capillary haemagglutination test had been described for field application (Boné and Charlier, 1975), but it silently disappeared due to the complexity of its execution. A plate agglutination format, particularly useful for *T. b. gambiense* sleeping sickness, is still available from Behring (Cellognost-Trypanosomiasis). Its use may be considered for large-scale surveys on serum samples or filter-paper eluates, especially in those cases where sophisticated equipment for ELISA or indirect fluorescent antibody test (IFAT) is not available. The use of other particles instead of red blood cells coated with more defined antigens (*T. b. gambiense* or *T. b. rhodesiense*) might improve the performance of the assay.

*LATEX/*T. b. gambiense

The latex agglutination test for *T. b. gambiense* (Büscher *et al.*, 1999) has been developed as an alternative to CATT/*T. b. gambiense*. The test is based on the combination of three purified variable surface antigens resulting in high sensitivity, e.g. in the Nigerian samples where CATT/*T. b. gambiense* fails. The test is currently under evaluation and research continues to replace the purified native antigens with recombinant antigens or synthetic peptides.

Detection of trypanosome antigens

ELISA

ELISA tests for trypanosome antigen detection have been developed by Liu and Pearson (1987), Olaho-Mukani *et al.* (1994) and Nantulya (1988), making use of polyclonal and monoclonal antibodies. The tests can be performed on serum and CSF samples from *T. b. gambiense* as well as from *T. b. rhodesiense* patients. Apart from the need for sophisticated equipment, the contradictory results obtained with similar test systems for animal trypanosomiases have diminished the interest in implementation of these tests in sleeping sickness control programmes.

Card indirect agglutination test for trypanosomiasis (CIATT)

The CIATT is the result of the combination of latex agglutination technology with monoclonal ELISA experience (Nantulya, 1997). CIATT should not be confused with CATT, the latter being an antibody detection test for *T. b. gambiense* infections widely applied in the field (see above). In contrast to ELISA, the CIATT is intended to be applied as a pen-side assay generating immediate results. The test is still under evaluation (WHO, 1998) but preliminary results indicate specificities tested in non-endemic areas ranging from only 61% in Côte d'Ivoire to 98% in Tanzania (Meda *et al.*, 1999). It should be noted that a positive result in tests such as the CIATT may result from the presence not only of trypanosome antigens but also of agglutinating anti-mouse-Ig antibodies and of anti-idiotypic antibodies. Furthermore, circulating antigen may be incorporated in immune complexes and not available for the reaction, thus giving false negative results.

Molecular Diagnosis

Molecular techniques can be applied on various sample types to provide evidence of infection. However, and often overlooked, evidence is still indirect and molecular tests can also generate false negative and false positive reactions. The different assays published so far remain to be evaluated on a larger scale. Molecular diagnostic techniques are far from simple and their present application is limited to research purposes. Although molecular techniques are very sensitive, the test sample should be large enough and well stabilized to contain the required minimum amount of template DNA or RNA.

DNA detection

Polymerase chain reaction (PCR) is a molecular technique which theoretically is very sensitive and specific. Several groups have already published research on its diagnostic

potential and use in sleeping sickness (Schares and Mehlitz, 1996; Kabiri *et al.*, 1999). A wealth of variations on the sample preparation and detected sequences exists from which the most convenient can be chosen. However, some practical considerations should be kept in mind. For example, some authors propose the collection of blood on filter paper for PCR testing of the eluted DNA, thus limiting the sensitivity of the PCR to one trypanosome per volume of blood retained in the filter paper. Furthermore, filter papers should be protected from direct sunlight to prevent DNA degradation, unless special filter paper (e.g. Whatman FTA) is used. In our laboratory, excellent recovery of DNA from 180 µl blood samples is obtained with the commercially available QIAamp DNA blood kit.

PCR results should be interpreted with caution. For example, in our laboratory, positive PCR results have been observed in animals long after treatment without evidence of cryptic infection. Furthermore, Garcia *et al.* (2000) observed unexplained false negative and false positive results in CATT seropositive but parasitologically non-confirmed persons and in the CATT negative control group. It is probable that much work still has to be done before PCR can replace parasite detection in sleeping sickness – if it ever does. Nevertheless, some improvements can be expected in the near future, including: (i) simplification of sample preparation; (ii) visualization and quantification of the PCR result by ELISA or fluorescence; and (iii) a reduced need for expensive equipment. PCR tests that are able to distinguish *T. b. gambiense* and *T. b. rhodesiense* from the other Trypanozoon taxa have now been developed (Welburn *et al.*, 2001; Radwanska *et al.*, 2002a).

RNA detection

RT-PCR

Reverse transcriptase PCR is a technique to detect mRNA, which is far less stable than DNA and which is indicative for active transcription of DNA sequences. In this way, the technique is less prone to contamination during sampling and may allow differentiation between an active infection and persisting DNA derived from dead trypanomes. RT-PCR might be of importance in clinical trials on treatment as they are in progress, in drug resistance studies and in vaccination trials. Again, this technique probably will be used exclusively for research purposes, with only indirect impact on control activities in the field.

Hybridization

Fluorescent molecular probes that hybridize with ribosomal RNA have recently been developed for facilitated detection of the parasite in thick blood films or blood smears (Radwanska *et al.*, 2002b).

Stage Determination and Follow-up

Accurate stage determination is essential to select an adapted treatment with minimal risk for the patient. Since there are no exclusive clinical signs, nor any clear changes at blood level, indicating the evolution from the haemolymphatic to the meningoencephalitic stage, stage determination is performed by examination of the CSF obtained by lumbar puncture, assuming that the CSF composition reflects the events going on in the central nervous system.

For follow-up after treatment, the blood and the CSF of the patient should be re-examined on several occasions (ideally at 1, 3, 6, 12, 18 and 24 months). In theory, a patient is considered cured when during this 2-year follow-up period no trypanosomes are detected in the blood, lymph or CSF and when the CSF stays or returns to normal (WHO, 1998). In practice, this strict follow-up is seldom achieved since patients tend to stay away from control lumbar punctures as soon as they feel healthy. Relapsing patients, therefore, are too often in a very advanced stage when recognized. Diagnosing a relapse is not always easy and only a few guidelines for follow-up can be found. Persistence or reappearance of clinical symptoms gives a good indication but is no absolute proof. Serological tests on blood are useless, since

antibodies persist after cure. Trypanosome detection relies on poorly sensitive techniques and often the parasite can only be detected in the CSF. Other CSF parameters sometimes never return to normal values. Finally, differentiation between a relapse and a reinfection is almost impossible.

Following the WHO recommendations for stage determination and follow-up (WHO, 1998), the CSF has to be examined for the presence of trypanosomes for direct diagnosis and white blood cell number and total protein concentration for indirect diagnosis. If at least one of these parameters is beyond the normal value, a patient is considered to be in the meningoencephalitic stage and should be treated accordingly.

Alternative CSF parameters for indirect stage determination and follow-up have been proposed, such as trypanosome-specific antibodies, anti-galactocerebrosides, the total IgM concentration and detection of trypanosomal DNA.

Detection of trypanosomes in CSF

In poorly equipped or non-specialized laboratories, trypanosomes are generally detected by the relatively insensitive but simple direct examination of CSF. Increased sensitivity of trypanosome detection can be obtained by centrifugation of the CSF sample or by *in vitro* culture (Kronenberger and Miezan, 1988). For obvious reasons, the latter technique is restricted to research purposes. Not only for detection of trypanosomes but also for cell count, it is important to examine the CSF immediately after lumbar puncture, because trypanosomes and cells in CSF seem to be more fragile than those in blood and they lyse quickly. The finding of trypanosomes in CSF allows immediate classification of a patient in the meningoencephalitic stage, though in some cases patients with trypanosomes in otherwise normal CSF have been cured with first-stage drugs (Doua *et al.*, 1996).

Detection of trypanosomes in CSF during the follow-up period is the absolute proof of treatment failure. Therefore, the use of the most sensitive techniques is recommended during follow-up, particularly when other CSF parameters remain abnormal or when clinical signs for nervous system involvement are present.

Detection of trypanosomes in the cell counting chamber

When the number of trypanosomes in CSF is very high (at least one trypanosome/µl, depending on the volume in the counting chamber), trypanosomes can be seen during enumeration of white blood cells. This is often associated with pleocytosis.

Single centrifugation of CSF

Several millilitres of CSF are centrifuged at low speed (2000–4000 rpm) and supernatant is removed without touching the bottom of the tube. Part of the remaining drop or sediment (invisible at low cell counts) is transferred to a slide for microscopic examination. The sensitivity of this technique is limited.

Double centrifugation of CSF

The sensitivity of trypanosome detection in CSF can be improved further by double centrifugation (DC) (Cattand *et al.*, 1988). After a first low-speed centrifugation of up to 5 ml of CSF, the sediment is taken up in one or two microhaematocrit tubes which are flame-sealed and centrifuged at high speed, whereafter the bottom of the tube is examined under the microsope. In spite of its high sensitivity and the availability of low-budget centrifuges connected to a car battery through a 12–220 V adapter, DC is not widely applied in the field. This is probably due to the number of manipulations and the need for both an ordinary and a microhaematocrit centrifuge.

Modified single centrifugation

The modified single centrifugation technique is a simple, sensitive, rapid and cheap alternative to double centrifugation of CSF. Up to 2 ml of CSF are centrifuged at low speed in a flame-sealed Pasteur pipette, such as used in the mAECT, and the pipette is mounted in a

viewing chamber (Miézan *et al.*, 2000). The test can be performed within 10 min. Although it remains to be evaluated on a larger scale, sensitivity seems equal to or even better than that of double centrifugation.

White blood cell count in CSF

CSF white blood cell count is without any doubt the most widely used technique for stage determination and follow-up. Miézan *et al.* (1998) stated that 'in poorly equipped laboratories, the diagnosis of CNS involvement in patients with confirmed systemic infection should be based only on the white cell count'. However, the authors disregarded the fact that concurrent infections inducing pleocytosis in CSF may interfere with the interpretation of cell count results.

The upper limit for normal and the cut-off value for the haemolymphatic stage are set at five cells/μl in CSF (WHO, 1998) but it has been suggested that the cut-off should be raised to ten or 20 cells/μl. It is true that *T. b. gambiense* patients with up to 20 cells/μl in their CSF have been successfully treated with pentamidine (Doua *et al.*, 1996) but a considerable amount of relapses has been observed during a clinical study carried out in Uganda on such patients. It should also be noted that, in children, normal leucocyte counts in CSF range from 30 cells/μl when younger than 1 year down to 10 cells/μl in puberty.

Counting chambers with a volume of at least 1 μl should be used, such as the Fuchs-Rosenthal, Nageotte or Neubauer counting chambers. The currently used cut-off value of 5 cells/μl is near the detection limit of most counting chambers, resulting in large variations when counting on samples with low cell numbers is repeated. In addition, due to time constraints or lack of money, rules for cell counting are often not followed, resulting in unreliable cell counts. For example, the rule of taking enough CSF (at least 5 ml), examining it immediately and mixing it before taking the sample for cell counting is often not followed. Other general mistakes are the use of worn-out cell counting chambers, or using ordinary microscope coverslips to replace broken original coverslips, negligent mounting of the coverslip (without visible Newton rings), wrong application of CSF and immediate counting without allowing the cells to settle down in the counting chamber. The use of disposable cell counting chambers such as KOVA slides (ICL), retaining a fixed volume of liquid and eliminating manipulation errors, should be encouraged.

Only a few guidelines can be found for follow-up, although cell count is often the only parameter checked. Pépin *et al.* (1994) considered cell counts higher than the previous determination and above 50 cells/μl to be a relapse, even if the patient is asymptomatic. Cell counts higher than the previous one and between 20 and 49 cells/μl are only considered as relapse when symptoms recur. When cell count is high (> 50 cells/μl) but lower than the previous one, they advised against treatment as it can take months before the cell count returns to normal. It is also known that there can be a significant increase in CSF cell number, also termed 'fluid storm', immediately after Melarsoprol treatment, which is not at all predictive of relapse. According to the guidelines of the Bureau Central de la Trypanosomiase in the Democratic Republic of Congo, a significant increase in cell numbers compared with previous controls, even in the absence of trypanosomes, is indicative of treatment failure.

Determination of CSF total protein concentration

Protein quantification on CSF is not a current practice in sleeping sickness control centres, probably due to the need for more or less sophisticated material (spectrophotometer), the instability of the reagents and the belief that CSF protein concentration is highly correlated with CSF cell number and adds no additional information.

A variety of protein quantification methods for CSF is available: precipitation methods such as sulphosalicylic acid precipitation, the Sicard and Cantaloube method and the trichloroacetic acid precipitation and colorimetric methods such as the Coomassie bril-

liant blue method and the BCA method. Depending on the method applied, largely differing cut-off values are prescribed for sleeping sickness: 250 mg/l (Sicard and Cantaloube method), 370 mg/l (colorimetric methods) or 450 mg/l (sulphosalicylic acid precipitation). Moreover, different results may be obtained depending on the protein standard, IgG or albumin, which is only rarely used in current practice. This leads to contradictory results when the same sample is measured with different methods and when the associated 'normal' cut-off is applied, if a cut-off is available at all. On top of the practical drawbacks, determination of the proteinorachia for stage determination suffers from one major theoretical shortcoming. The baseline protein concentration in CSF is determined by serum Ig and albumin concentrations. Pathological increases are the result of intrathecal Ig synthesis and/or blood–CSF barrier dysfunction. In trypanosomiasis patients without CNS pathology, CSF protein concentrations are relatively high due to the very high Ig concentrations in serum. On the other hand, since blood–CSF barrier dysfunction occurs only in a very advanced stage of sleeping sickness and usually remains moderate, the resulting increase in proteinorachia will also be a late and moderate event. Instead of total protein, the quantification of intrathecal IgM seems more appropriate for stage determination (Lejon *et al.*, 2003; see below).

Detection of trypanosome-specific antibody in CSF

The detection of trypanosome-specific antibodies in CSF of second-stage patients by IFA or ELISA has been described extensively. Furthermore, by ELISA it is possible to study semi-quantitatively the different immunoglobulins in serum and CSF (Lejon *et al.*, 1998), allowing the calculation of intrathecal synthesis of specific antibodies, which indicates an inflammatory response in the central nervous system. However, the need for sophisticated equipment precludes the widespread application of these techniques.

The detection of trypanosome-specific antibodies in CSF by techniques applicable in the field, such as CATT and indirect haemagglutination, has been explored. The sensitivity of CATT appears too low whereas indirect haemagglutination presents the highest sensitivity and specificity, combined with a clear correlation with the CSF cell number. LATEX/*T. b. gambiense* can also be performed on CSF samples for specific antibody detection (Büscher *et al.*, 1999). The reagent seems to be not 100% sensitive, but is highly specific for second-stage trypanosomiasis.

It has been described that CSF specific antibody concentrations drop down quickly after successful treatment (Smith *et al.*, 1989). The decrease of trypanosome-specific antibody concentrations in CSF might therefore be a good parameter for definite cure.

Anti-galactocerebrosides in CSF

Several antibodies directed against brain-specific proteins have been detected in serum and CSF of sleeping sickness patients. The presence of anti-galactocerebroside antibodies in CSF, assessed by ELISA, seems a promising marker for central nervous system involvement (Bisser *et al.*, 2000). Further studies on the applicability of this parameter for stage determination and the development of a simpler assay are necessary before implementation in the field. No data are yet available on the disappearance of these antibodies after cure.

IgM detection in CSF

The CSF of sleeping sickness patients can contain high levels of IgM, which are explained by the high serum levels but also and mainly by the exceptionally strong intrathecal IgM synthesis in patients with central nervous system involvement, and/or by blood–CSF barrier dysfunction (Lejon *et al.*, 2003). The latter two are both indicators of inflammation in the central nervous system due to parasite invasion. However, it should be borne in mind that IgM in CSF is only pathognomic when the CSF has not been contaminated with blood during lum-

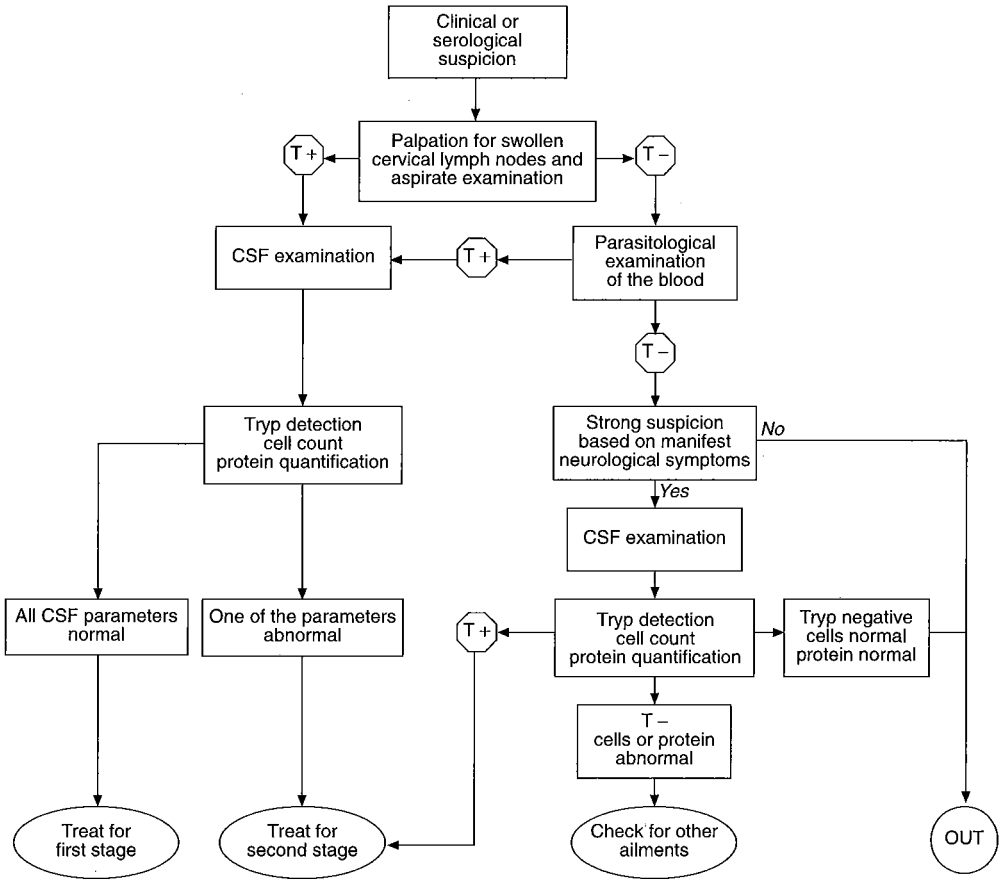

Fig. 10.1. Simplified algorithm for sleeping sickness diagnosis starting from clinical or serological suspicion of infection up to stage determination. T + trypanosomes detected in the sample, infection confirmed; T – no trypanosomes detected in the sample, still suspicion of infection.

bar puncture, since even small volumes of blood will cause abnormally high IgM concentrations in CSF.

Due to the lack of appropriate reagents to quantify IgM in CSF under field conditions, IgM determination is seldom applied for stage determination. A rapid latex agglutination test (LATEX/IgM), which combines stability, sensitivity and simplicity, has been developed (Lejon *et al.*, 2002). Twofold serial dilutions of the CSF are tested and the highest dilution causing an agglutination of the reagent (the end titre) is determined. End titres ≥ 8 have been shown to be 89% sensitive and 93% specific for presence of intrathecal IgM synthesis. Moreover, such high end titres were prognostic for a relapse in sleeping sickness patients with fewer than 20 cells/μl who were treated with the first-stage drug, pentamidine. The test is currently under evaluation in different control centres for sleeping sickness.

Detection of trypanosome DNA in CSF

Theoretically, the detection of trypanosomal DNA in CSF could provide a more sensitive tool for stage determination and follow-up than trypanosome detection but its implementation in the field is not straightforward. PCR with *T. brucei* specific primers on CSF of *T. b. gambiense* patients and suspected sleeping sickness patients has been described to

be 100% sensitive compared with double centrifugation (Truc *et al.*, 1999). However, the fact that the number of CSF samples positive in PCR systematically exceeds the number of trypanosome positives, casts some doubts on the relevance of the results. As for trypanosome detection in CSF, one might question the meaning of PCR positivity when other CSF parameters are normal. The detected DNA could originate from the blood or from trypanosomes that have invaded the CSF without actually causing injury or inflammation to the central nervous system. Finally, as for other techniques, it cannot be excluded that PCR also suffers from aspecific reactions.

Although very limited data are available from follow-up studies, it looks as if PCR becomes quickly negative after treatment (Truc *et al.*, 1999). No data on relapsing patients have yet been published.

Organization of Diagnostic Activities

The practical set-up of a diagnostic procedure depends on several variables, including the infecting agent (*T. b. gambiense* or *T. b. rhodesiense*), number of expected patients, size of population at risk, access to laboratory facilities, technical skill of health personnel, active or passive case detection etc. However, some general rules apply to all situations where the intention is to treat the patients correctly (WHO, 1998). An example of a simplified diagnostic flow chart is given in Fig. 10.1. First of all, one should know whether *T. b. gambiense* or *T .b. rhodesiense* is involved, depending on the distribution of the subspecies. Next, a brief anamnesis is obtained paying attention to subjective signs and to signs observed by the patient's family, followed by a clinical examination of the patient (neurological signs, palpation of the cervical lymph nodes). In cases of possible *T. b. gambiense* infection, a serological test such as CATT can confirm suspected sleeping sickness.

Clinical or serological suspects undergo parasitological examination following the general diagram in Fig. 10.1. The procedure can be adapted to particular situations, such as mass screening of the population at risk where, in the first instance, a serological test can be performed on the whole population to focus on only the seropositive cases.

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