THE AFRICAN TRYPANOSOMES

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DIAGNOSIS OF HUMAN AND ANIMAL AFRICAN TRYPANOSOMIASIS

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ABSTRACT

Diagnosis of African trypanosomiasis has been the subject of intensive research for decades, which is reflected by the bulk of publications related to this topic. From this literature, it appears that the current parasite detection techniques can hardly be improved and that reliable antigen detection tests will remain wishful thinking. Much more success has been obtained in the development of antibody detection tests of which some have even reached the end-user, *i.e.* health personnel and veterinarians in rural regions of Africa, Latin America and Asia. Unfortunately, the production of diagnostics for African trypanosomiasis has no economical value. As a consequence, potentially excellent diagnostic tests never reach the validation phase, not to mention production, and sooner or later will belong to history unless they have some scientific value. In this context, international human and animal health organizations have largely missed the opportunities to provide validated diagnostics to those who need them. Recent literature clearly reflects the general shift from serological diagnostics to molecular diagnostics. Molecular diagnostics have unequalled potentials for sensitive and specific detection of human and animal African trypanosomiasis. However, the risk that molecular diagnostics will remain the property of the scientific world with only minor impact on real disease control should not be underestimated.

Key words: Diagnosis, African trypanosomiasis, surra, nagana, dourine, sleeping sickness, *Trypanosoma brucei*, *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma evansi*, *Trypanosoma equiperdum*.

INTRODUCTION

African trypanosomiasis is a general term for infections in many different hosts (man, bovine, buffalo, goat, sheep, camel, horse, pig and wild animals) caused by various trypanosome species (*Trypanosoma brucei*, *T. congolense*, *T. vivax*, *T. evansi*, *T.*

equiperdum) and subspecies. As a consequence, a chapter like this must remain superficial. Nevertheless, some interesting general features on the development of African trypanosomiasis diagnostics can be observed.

Despite decades of research into the development of reliable diagnostic tests for African trypanosomiasis, little has changed in current practice, particularly at the level of farmers, veterinarians and health personnel in developing countries where African trypanosomiasis prevails. In fact, diagnosis of African trypanosomiasis in animals is too often based on clinical suspicion only, whereafter individual or herd treatment measures are undertaken. Only in human African trypanosomiasis (sleeping sickness), is parasitological confirmation of clinical or serological suspicion compulsory, because treatment is expensive and not without risk for the patient.

Most probably, the reason why simple and reliable diagnostic field tests are still rare must be found in the combination of limited economical interest and the inconstancy of research priorities and related financial resources. It is amazing to see how many publications describe new or improved diagnostic techniques, mostly evaluated on a restricted, well-controlled set of samples and presenting promising 'preliminary' results. Some of them are indeed promising and based on sound scientific research. Unfortunately, competition for the same restricted funding resources and the constant pressure to come up with something 'new', whether or not it will ever be applicable, results in a lack of encouragement for researchers to undertake multicenter validation of diagnostic tests that could then become applied on a large scale. Indeed, the researcher may encounter actual barriers against undertaking multicenter validation of diagnostic tests. It might be interesting to evaluate some day the role and activities of international organizations like WHO, FAO, ISCTRC, EU etc. in the light of the overt failure to really improve basic diagnosis of African trypanosomiasis.

Only for epidemiology and surveillance purposes, where perfect performance, simplicity and low cost are not too important, have some major improvements in African trypanosomiasis diagnosis been achieved with recent techniques such as ELISA and PCR. However, also in this field, standardization is definitely lacking. With few exceptions, most research groups prefer to develop their own system which is then applied in their action region with minor attention to multicenter validation of the tests. Faced with increasing drug resistance, expansion of the distribution area of some trypanosome species and their re-appearance in 'controlled' regions,

one might expect researchers to focus onto the validation and standardized quality production of diagnostic tests.

Students who have no experience with African trypanosomiasis diagnosis may not be aware of the intrinsic difficulties to be overcome. Generally, clinical symptoms are rather unspecific or may be even obscure, particularly in trypanotolerant hosts. In many instances, the parasite load is extremely low which makes the detection of trypanosomes rather cumbersome and poorly sensitive. For example, with the most sensitive techniques, *T.b. gambiense*, the causative agent of sleeping sickness in West and Central Africa is probably detected in only 80% of the patients. The same applies to *T. evansi* in buffalo and camel or *T. congolense* and *T. vivax*, often occurring as mixed infections, in trypanotolerant cattle. The situation is even worse for *T. equiperdum* (dourine) in horses where parasite detection sensitivity is almost zero. Indirect tests based on antibody detection may be very sensitive but specificity may be poor due to cross-reaction with other infections, depending on the purity of the antigen applied. In addition, antibodies tend to remain detectable for months after cure, which makes antibody detection tests useless for follow-up studies. Finally, except for agglutination tests, for every host species, the appropriate conjugates should be incorporated in the test. This conjugate should preferentially be an anti-IgG to avoid cross-reactions due to aspecific IgM. In many instances, related to the low parasite load, the amount of circulating antigens is largely insufficient to detect by simple techniques. The same may apply for DNA detection tests such as PCR although these tests are theoretically very sensitive. The occurrence of mixed infections and of different taxa within each trypanosome species further complicates the picture. Special attention should be paid to choosing primers able to discriminate the described taxa, knowing that poorly studied taxa (e.g. within the *T. vivax* or *T.b. gambiense* group) may not be detected.

In the following paragraphs, an overview of some more or less recent achievements in the diagnosis of African trypanosomiasis is given.

PARASITE DETECTION

As stated above, parasite detection is cumbersome in the many cases where only low numbers of trypanosomes circulate in the host body fluids. Techniques for concentration of the trypanosomes by centrifugation of a blood sample are still the most widely applied in animal trypanosomiasis (*T. congolense*, *T. vivax*, *T. brucei*, *T. evansi*). After centrifugation of some blood in a capillary tube, the

trypanosomes can be detected directly under the microscope at the level of the white blood cell layer, the buffy coat (Woo 1969). Where differential diagnosis is needed, the capillary tube is broken and the buffy coat is spread on the microscope slide for examination according to Murray et al. (1977). It is clear that the latter technique is not without risk of infection when human samples are manipulated. Our experience has also shown us that the much bigger and very motile microfilaria, often present in the blood of people living in sleeping sickness areas, strongly interferes with trypanosome detection. A more sensitive technique is the mini Anion Exchange Centrifugation Technique, developed by Lumsden et al. (1979). The mAECT is based on the separation of trypanosomes from the host blood through an anion exchange gel. The technique works best with the three subspecies of *T. brucei* and with *T. evansi*. The elution buffer may need adaptation to the host species. Some improvements to the original techniques using modern material have been published (Zillmann et al. 1996). For sleeping sickness diagnosis, a concentration technique originally developed for malaria, the Quantitative Buffy Coat (Bailey et al. 1992) has been introduced and is now applied by those who can pay for it. The technique combines facilitated visualization of trypanosomes by their concentration at the expanded buffy coat level, their motility and the staining of their nucleus and kinetoplast by acridine orange. Detection of trypanosomes in fresh thin blood preparations using this dye has been proposed long ago (Carrie1981) but has never been applied in the field. A major drawback of acridine orange is the simultaneous staining of the white blood cell nuclei. Therefore, we are currently studying techniques for species specific fluorescent staining of the trypanosomes in (fixed) blood samples by monoclonal antibodies. However, the small amounts of examined blood will always limit the sensitivity of such tests. Techniques based on expansion of trypanosomes through culture in laboratory animals or *in vitro* exist but their value is limited to experimental studies. It is generally accepted that not much progress can be expected in parasite detection techniques, at least not for direct diagnosis. This has serious consequences on the evaluation of indirect diagnostic tests due to the lack of a 'gold standard'.

ANTIBODY DETECTION

Indirect evidence of infection with African trypanosomes can be obtained through the presence of specific circulating antibodies. Sometimes, it is of interest to combine antibody detection with hematocrit estimation. Particularly in trypanosusceptible animals such

as camels and some breeds of ruminants, low PCV values together with the presence of specific antibodies are highly predictive for active infection. A wealth of different antibody detection systems exists ranging from direct and indirect agglutination, immunofluorescent assays and ELISA. They are adapted for testing blood (fresh or dried on filter paper), serum, plasma and cerebrospinal fluid from all kind of host species. They are extremely useful to study the prevalence of African trypanosomiasis in a population and particularly in *T.b. gambiense* sleeping sickness, the introduction of a simple, quick direct agglutination test, the CATT (Magnus et al. 1978) has been a major breakthrough in the control of this disease by selecting the seropositive individuals for parasite examination to confirm the infection. The test is based on detection of antibodies against predominant surface antigens of *T.b. gambiense*. A similar test exists for *T. evansi* (CATT/*T.evansi* (Bajyana Songa et al. 1988). Unfortunately, the antigenic variation of *T.b. brucei* and *T.b. rhodesiense* is too large to allow the development of CATT tests for these subspecies. For the latter subspecies, another direct agglutination test exists (Liu et al. 1989) which recently has been simplified by using fixed instead of live procyclic trypanosomes (Ngaira et al. 1999). Knowledge about the antigenic repertoires of *T. congolense* and *T. vivax* is almost non-existent. Antibody detection tests for these species make use of more or less purified but still crude antigens leaving room for aspecific cross-reactions. The situation is less problematic for *T. congolense* than for *T. vivax* and *T. brucei*. In regions where *T. vivax* and *T. brucei* or *T. evansi* occur as mixed infections it is almost impossible to make the distinction at the level of circulating antibodies. As mentioned earlier, almost every research or surveillance group produces its own antigens on a day by day basis leaving us with poorly standardized tests. A remarkable exception is the effort of IAEA, Vienna, to standardize an ELISA test for *T. vivax* and *T. congolense* in bovine and to have it validated and introduced in many African laboratories (Rebeski et al. 1999b, 2000). This work is a fine example of difficulties that may be encountered during development of ELISAs for African trypanosomes ranging from the choice of the microtiter plates, the standardized production of the native antigens, the choice of appropriate controls and conjugates, the stabilization of the test kit and the analysis of the data. Unfortunately, the IAEA project came to an end and the continuation of the production is not guaranteed, partly due to the low economical value. It is interesting to observe that many valuable antigen candidates for antibody detection have been described such as Invariable Surface Glycoproteins (Ziegelbauer et al. 1992) and cytoskeleton proteins

from *T. brucei* (Imboden et al. 1995) and a cysteine protease from *T. congolense* (Authié et al. 1993) but that this not necessarily leads to the development of diagnostic tests. Reasons for this must be found in the fact that sufficient amounts of pure native antigen are difficult to prepare and that groups involved in fundamental research have neither the experience nor the interest to develop diagnostic tests. However, efforts to replace native antigens by recombinant ones in order to simplify production and to increase species specificity of antibody detection tests still continue e.g. at ILRI, Nairobi. These attempts, seldom published, are hindered by the well known polyclonal activation of B-cells in trypanosome infected hosts, leading to undesired cross-reactions when the recombinant antigen contains impurities. Probably only the use of synthetic peptides may lead to the desired sensitivity and specificity. In principle, such peptides may be applied in different test systems, as there are latex agglutination, ELISA, immunofluorescence or lateral flow devices. Considering the price of lateral flow devices it is difficult to believe that they ever will be produced for African trypanosomiasis, be it in animals or in man. One exception may be *T. equiperdum* by the mere fact that Europe and North America are threatened by its re-introduction. Only for *T. equiperdum*, our laboratory has been able to raise sufficient funding within the European context to start development of a new generation of antibody tests. *T. equiperdum* is believed to be the causative agent of dourine, a sexually transmitted disease in equidae. The trypanosome is very difficult to detect in the infected host. Therefore, antibody detection is used to screen for a possible infection. It appears that the only officially approved test by the OIE is the Complement Fixation Test, although it is generally accepted that this test cannot discriminate between *T. evansi* and *T. equiperdum*.

Development of antibody detection tests might seem relatively simple, their evaluation is far more difficult, particularly in the absence of a 'gold standard'. As long as a diagnostic test is evaluated on an experimentally infected population along with a naive control population, data are easy to analyze. However, for evaluation on naturally infected populations, other statistical techniques must be used such as the 'latent class analysis' or Bayesian methodology (EnŶe et al. 2000). This approach has not yet been followed in African trypanosomiasis diagnosis and should be encouraged by all means.

ANTIGEN DETECTION

For quite a long time, it was believed that antigen detection might overcome the problems encountered with parasite or antibody

detection. Much research by different groups has been conducted on the development of antigen detection ELISAs (Liu and Pearson 1987; Nantulya and Lindqvist 1989; Olaho-Mukani et al. 1993) dipstick assays (Kashiwazaki et al. 1994) and latex agglutination (Nantulya 1994, 1997). Unfortunately, at present no antigen detection test with proven efficiency exists. Multiple explanations have been put forward such as the intrinsic low amount of circulating antigen which in addition may be bound to immune complexes, the nature of the capturing antibodies and their target antigen (Rebeski et al. 1999a) Other causes of failure could be the occurrence of anti-idiotypic antibodies that mimic the target antigen and circulating anti-mouse antibodies, generated by the polyclonal activation of B-cells, which may interfere with the antigen capturing. The existence of cryptic infections, which cannot be revealed by parasite detection techniques, has been postulated as well but for obvious reasons this cannot be tested. For those who are not convinced of the failure of antigen detection tests in African trypanosomiasis, it may be useful to consider specificity studies on animal or human samples from Europe where these infections are totally absent.

MOLECULAR DIAGNOSIS

Molecular diagnosis, mainly based on detection of trypanosome DNA, opens new perspectives for diagnosis of African trypanosomiasis when parasite detection fails. Studies on the genome of trypanosomes have led to the development of species and subspecies specific primers for PCR. To increase the sensitivity and the specificity, PCR is sometimes combined with hybridization (Clausen et al. 1999). Specific primers exist for *T. congolense* and *T. vivax* although for the latter species it is not certain that all strains are recognized by these primers. For the *Trypanozoon* group, the picture is less clear. Most of the primer sets cannot make the distinction between *T. evansi* and *T. brucei* s.l. nor between the subspecies of *T. brucei* as there are *T.b. brucei*, *T.b. gambiense* and *T.b. rhodesiense* (Pereira de Almeida 1999). In most instances, this is not a problem but when one is interested for example in the animal reservoir of human infective trypanosomes, PCR may not be useful until gambiense and rhodesiense specific primer sets are used. The fact that *T.b. gambiense* itself can be divided in at least two different groups further complicates the situation. For *T.b. gambiense* group I, specific primers already have been identified (Bromidge et al. 1993; Mathieu-Daudé et al. 1994; Biteau et al. 2000). Most probably, in the near future more and better primer sets will become available.

In principle, all types of biological host samples can be assayed for trypanosome DNA (blood, lymph, cerebrospinal fluid, tissue). A multitude of sample collection and DNA preparation methods exist ranging from the classical chloroform/phenol extraction through purification with the help of resins or silica. Many of these methods have already been tested in African trypanosomiasis without unexpected problems. However, it should be kept in mind that, depending on the technique, the risk of contamination during sample preparation under field conditions could be high.

In addition, it appears that the extreme low detection limit obtained with PCR on DNA directly purified from trypanosome cultures (less than 1 trypanosome per ml) is never obtained when starting with trypanosome containing blood from clinical samples. In those samples, the detection limit of PCR is comparable to that of the most sensitive parasitological tests (Kabiri et al. 1999; Omanwar et al. 1999). Furthermore, attention should be paid to the conservation of the samples. In our hands, the stabilization of blood samples in the AS1 buffer included in the Qiagen mini blood kit gave excellent results (Holland et al. 2000) while important loss of DNA was observed when plain blood samples are frozen or dried on filter paper. The availability of filter paper especially designed for DNA collection (e.g. FTA paper from Whatman) may bring the solution although the amount of blood that can be applied may be too small.

Apart from the fact that the current price and technical requirements of molecular diagnostics are prohibitive for their generalized application in developing countries, more and more evidence becomes available that also molecular tests suffer from unexplained false negative (Kabiri et al. 1999; Simo et al. 1999) and false positive results. PCR can remain positive long after successful cure (Kirchhoff 1998; Pereira de Almeida 1999) and almost half of a human control population in Côte d'Ivoire without any clinical, serological or parasitological evidence of infection was found positive in PCR (Garcia et al. 2000). Furthermore, repeated sampling and DNA extraction of the same individuals can yield contradictory results (Solano, personal communication). It is clear that still much work has to be done on the development and validation of molecular diagnostic tests for African trypanosomiasis starting with studies on the specificity of the existing primer sets against a large collection of trypanosome strains and other pathogenic organisms followed by specificity testing on clinical samples from non-endemic regions and sensitivity testing on confirmed infections. Again, the absence of a 'gold standard' should be taken into account and one should not be too confident that a PCR positive result is a definite proof of infection.

As parasitologists are natural optimists and can hardly wait for the validation of newly developed tests, investigations into the applicability of alternative molecular tests such as PCR-ELISA, multiplex PCR, PCR-RFLP and PNA have already been initiated. As mentioned in the introduction, international institutions may play a leading role in supporting multicenter studies with one major goal, which is the improvement of African trypanosomiasis diagnosis for the benefit of the end user.

STAGE DETERMINATION AND FOLLOW-UP IN SLEEPING SICKNESS

In sleeping sickness, the drug used for treatment depends on whether the parasite has reached the central nervous system. For treatment of the second or meningo-encephalitic stage, drugs that pass the blood-brain barrier in sufficient amounts should be used. Since there are no exclusive clinical signs indicating the evolution from the haemo-lymphatic to the meningo-encephalitic stage, the only way to determine the disease stage is by examination of the cerebrospinal fluid (CSF) obtained by lumbar puncture, assuming that the changes observed in the CSF reflect the events going on in the central nervous system (CNS). The same applies for follow-up after treatment: CSF should be obtained and examined regularly for up to two years before a patient can be considered cured. According to the recommendations of the WHO for stage determination and follow-up, the CSF has to be examined on white blood cell number, total protein concentration and presence of trypanosomes. Since there is no close relationship between these parameters, ideally all of them should be examined. Abnormal values, indicating central nervous system infection are as follows: > 5 cells/µl, presence of trypanosomes, protein concentration > 250 mg/l (trichloroacetic acid precipitation) or > 370 mg/l (dye binding method) or > 450 mg/l (sulfosalicylic acid precipitation). For people with some experience in the field, it is clear that the determination of these CSF parameters poses some problems: cell counting at the detection limit of the classical counting chambers suffers from low repeatability, 'normal' cell count strongly depends on age of the patient, elevated cell counts may be caused by other infections, 'normal' protein concentration depends on the method and the standard solution used, trypanosomes may be difficult to detect etc. In addition, little is known about the evolution of these parameters after treatment, except from the fact that it may take two years before the parameters have returned to normal. Rather recently, a limited number of research groups have renewed interest into the

development of improved techniques for stage determination with some remarkable results.

For example, the replacement of classical cell counting chambers by disposable plastic counting chambers like the KOVA Glasstick slides from ICL retaining a fixed volume of CSF, greatly improves the reproducibility of the technique by reducing manipulation errors to a minimum (Lejon et al. 1998a). For detection of trypanosomes in the CSF, a modified single centrifugation technique (Miézan et al. 2000) has been proven to be much simpler and as sensitive as the double centrifugation technique. For more than 30 years, it is known that CSF of second stage sleeping sickness patients contains high amounts of IgM but for technical reasons it was impossible to quantify this IgM in African rural health centres. Only recently, a simple, rapid and stable agglutination test (LATEX/IgM) for IgM quantification in CSF has become available for application in the field (Lejon et al. 1998b). Detection of trypanosome specific antibodies in CSF has also become possible with a simple agglutination test, the LATEX/*Tb gambiense* (Büscher et al. 1999). For research purposes, an ELISA system has been developed for simultaneous measurement of trypanosome specific IgM and IgG in the CSF and in the serum thus allowing to calculate intrathecal antibody production as a marker for CNS infection (Lejon et al. 1999a). Other CSF markers for CNS inflammation and destruction are currently being studied such as anti-galactocerebroside antibodies (Bisser et al. 2000), neurofilament and glial fibrillary acidic protein (Lejon et al. 1999b) and cytokines (MacLean et al. 1999; Lejon et al. 1999c). The main interest of tests based on these markers probably lies in their potential to shorten the follow-up period after treatment.

Reports on the use of molecular diagnostic techniques for stage determination and follow-up are still rare (Kirchhoff 1998; Truc et al. 1999; Kyambadde et al. 2000)but application of these techniques on CSF samples is straightforward and may offer new tools, particularly to clinical treatment studies for cure rate assessment. Once again, prior validation of DNA detection techniques will be required.

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