

CHAPTER 2.4.17.

TRYPANOSOMOSIS (tsetse-transmitted)

SUMMARY

Definition of the disease: Tsetse-transmitted trypanosomosis is a disease complex caused by several species of protozoan parasites of the genus *Trypanosoma*, mainly transmitted cyclically by the genus *Glossina* (tsetse flies), but also transmitted mechanically by several biting flies (tabanids, stomoxes, etc.). The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis, is particularly important in cattle. It is mainly caused by *Trypanosoma congolense*, *T. vivax* and, to a lesser extent, *T. brucei brucei*.

Description of disease: Tsetse-transmitted trypanosomosis is a classically acute or chronic disease that causes intermittent fever and is accompanied by anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms.

Identification of the agent: Several parasite detection techniques can be used, including the microscopic examination of the wet or dry-stained thick or thin blood films. Diagnostic sensitivity is increased significantly by concentrating the parasites prior to examination in combination with a phase-contrast or dark-ground microscope. The centrifugation parasite concentration techniques have the added advantage that the packed cell volume, and hence the level of anaemia, can be determined at the individual animal and/or herd level. A highly specific and more sensitive test, used in an increasing number of laboratories, is the polymerase chain reaction, which can identify parasites at the genus, species or subspecies level, depending on the cases.

Serological tests: Two trypanosomal antibody detection tests, the indirect fluorescent antibody test and the antibody-detection enzyme-linked immunosorbent assay (ELISA), are routinely used for the detection of antibodies in cattle. They have high sensitivity and genus-specificity but can only be used for the presumptive diagnosis of trypanosomosis. The antibody-detection ELISA, in particular, lends itself to automation and will allow a high degree of standardisation when recombinant antigens have been developed and validated. However, they are at the present time carried out with native soluble antigens of trypanosomes grown in rodents with satisfying sensitivity and specificity.

Requirements for vaccines: No vaccines are in use at the present time.

A. INTRODUCTION

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of their hosts. The genus *Trypanosoma* belongs to the protozoan branch, order Kinetoplastida, family Trypanosomatidae. Tsetse-transmitted trypanosomes belong to the salivarian section, subgenus *Nannomonas* for *T. congolense*, *Duttonella* for *T. vivax*, and *Trypanozoon* for *T. brucei* ssp.

Tsetse-transmitted trypanosomosis is a disease complex caused by several of these species, mainly transmitted cyclically by the genus *Glossina* (tsetse flies), but also mechanically by biting flies. Tsetse infest 10 million square kilometres and affect 37 countries, mostly in Africa, where the disease is known as 'nagana'. The disease infects various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle (also referred as tsetse-fly disease in southern Africa). It is mainly caused by *Trypanosoma congolense*, *T. vivax* and, to a lesser extent, *T. brucei brucei*. *Trypanosoma uniforme*, and *T. simiae* are other, less common tsetse-transmitted species. *Trypanosoma vivax* is also transmitted mechanically

by biting flies, among which tabanids and stomoxes are presumed to be the most important, as exemplified by its presence in South and Central America, but also as observed in some areas of Africa free or cleared of tsetse (Ethiopia, Chad, etc.). Tsetse-transmitted trypanosomosis can affect camels and is a natural barrier preventing the introduction of this mammalian species into the southern Sahel region of West Africa. Horses are also highly sensitive. Very rare human cases have been observed caused by animal *Trypanosoma* species. However, tsetse-transmitted trypanosomosis also affects humans, causing sleeping sickness, through infection with either *T. brucei gambiense* or *T. brucei rhodesiense*. A large range of wild and domestic animals can act as reservoirs of these human parasites; particular care must be taken for people handling biological material that can contain infective human parasites, for example in livestock or wild fauna.

Clinical signs of tsetse-transmitted trypanosomosis may include intermittent fever, oedema, abortion, decreased fertility and emaciation. Anaemia usually develops in affected animals and is followed by loss of body condition, reduced productivity and often mortality. Post-mortem signs may include emaciation, enlarged lymph nodes, enlarged liver and spleen, excessive fluid in the body cavities, and petechial haemorrhages. In animals that died during the chronic phase of the disease, the lymphoid organs are usually no longer enlarged and severe myocarditis is a common finding. Neither clinical nor post-mortem signs of tsetse-transmitted trypanosomosis are pathognomonic. Therefore, diagnosis must rely on direct techniques that confirm the presence of trypanosomes either by microscopic visualisation or by indirect serological techniques or by polymerase chain reaction (PCR). Clinically, trypanosomosis can be confused with babesiosis, anaplasmosis, theileriosis, hemonchosis and even ehrlichiosis, rabies or plant intoxications. Differential diagnosis is oriented by clinical observations, evolution, epidemiological context, but it is essentially based on laboratory diagnosis.

B. DIAGNOSTIC TECHNIQUES

A variety of diagnostic tests are available (Toure, 1976) and researchers are still trying to improve existing tests and to develop new ones. Current diagnostic tests vary in their sensitivity and specificity, the ease with which they can be applied and their cost (Paris *et al.*, 1982). The choice of a particular test will be guided by economic principles and the availability of expertise, but especially by the diagnostic requirement. For example, different degrees of sensitivity and specificity are applied to the confirmation of the infection in an individual animal as compared to the detection of infection at a herd level. Similarly, the diagnostic test(s) to establish the parasitological prevalence of trypanosomosis are different from those required to establish the presence or absence of the disease in an area. Reliable diagnosis may be achieved by combining appropriate diagnostic tests. Reliable interpretation of results from diagnostic tests will depend on test validity as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted (see the Table 1).

Table 1. Test methods available for the diagnosis of tsetse-transmitted trypanosomosis and their purpose

Purpose/ Method	Type of area					
	Non-infected area			Enzootic area		Both areas
	Confirmation & identification of suspected case	Population freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Specific characteristics or interest
Agent identification¹						
Thin stained blood smear	+++	–	–	++	+	Parasite morphology
DNA detection/PCR	+++	+++	+++	+++	+++	Sensitive & specific molecular identification
Detection of active infection						
Wet blood film	++	–	–	++	+	Fast follow-up (experimental) infection
Thick stained blood film	–	–	–	+	+	Cheap technique

¹ A combination of agent identification methods applied on the same clinical sample is recommended.

Purpose/ Method	Type of area					
	Non-infected area			Enzootic area		Both areas
	Confirmation & identification of suspected case	Population freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Specific characteristics or interest
Haematocrit centrifuge technique (HCT, Woo)	+++	+++	+++	+++	+++	Active infection & haematocrit value
Buffy coat technique (Murray)	+++	–	+	+++	++	Active infection & haematocrit value
Anion exchange columns	+++	–	+++	–	–	Small individual test or large parasite production
Parasite propagation						
Rodent inoculation	+++	+	+++	–	–	Sensitive parasite isolation or production
<i>In-vitro</i> culture	–	–	–	–	–	Parasite production
Serological diagnosis						
IFAT	+++	++	–	–	++	Small-scale studies
ELISA	+++	+++	+++	–	+++	Population studies

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Agent id. = agent identification; PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

Parasite detection techniques are highly specific, but their sensitivity is relatively low (i.e. the proportion of false-negative results recorded is high). Sensitivity is especially low when results are considered at the individual animal level rather than the herd level. Sensitivity is highly variable during the course of the infection: (i) in the early phase, the sensitivity is high as parasites are actively multiplying in the blood in the absence of immunological control; (ii) during the chronic phase the sensitivity is low as, due to the immune response of the host, parasites are scanty and rarely seen in the blood; (iii) finally the sensitivity is almost nil in healthy carriers, where parasites are never seen. At the population level these variations mean that parasite detection techniques are highly sensitive during epizootic outbreaks (when most of the animals are in the early stages of infection), and are of low or very low sensitivity in enzootic areas (most of the animals are in the chronic stages of infection), especially during subclinical phases of the infection (healthy carriers). Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis is a little or much lower than the true parasitological prevalence. The low diagnostic sensitivity also makes it difficult to detect trypanosomosis when present at low parasitological prevalence and it is impossible to establish the absence of the disease with a high degree of confidence. Moreover, in areas where trypanocidal drugs are used extensively, parasites may not be detected.

Several parasite detection techniques are available, each with varying sensitivity. The choice will depend on the laboratory facilities available and the aim of the diagnosis.

1.1. Direct examination techniques

The simplest techniques are examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail. Amongst the direct examination techniques, stained thin blood

films are generally regarded as more specific but less sensitive than the other two. The actual specificity and sensitivity of these techniques is directly dependent on the volume of blood actually examined and the skill and experience of the microscopist.

1.1.1. Wet blood films

These are made by placing a droplet of blood (about 2 µl) on a clean microscope slide and covering with a cover-slip (22 × 22 mm). The blood is examined microscopically at ×400 total magnification with condenser aperture, phase-contrast or interference contrast. Approximately 50–100 fields are examined. Trypanosomes can be recognised by their movement among the red blood cells (RBCs).

The method is simple, inexpensive and gives immediate results. Depending on the trypanosome size and movements a presumptive diagnosis can be made of the trypanosome species. Final confirmation of the species is made by the examination of the stained preparation.

The diagnostic sensitivity of the method is generally low but depends on the examiner's experience and the level of parasitaemia. Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent such as sodium dodecyl sulphate (SDS).

1.1.2. Thick blood films

These are made by placing a drop of blood (5–10 µl) on a clean microscope slide and spreading it over an area of approximately 2 cm in diameter, using the corner of another slide. The thickness of the resultant film should be such that, when dry, the figures on a wristwatch dial can just be read through it. The film is dried thoroughly by rapidly waving in the air and, without fixation, is dehaemoglobinised by immersion in distilled water for a few seconds and dried before staining. A dry smear should be kept dry and protected from dust, heat, flies and other insects. It is stained for 30 minutes with 4% diluted Giemsa stain in phosphate buffered saline, pH 7.2. Staining time and stain dilution may vary with stain and individual technique. Therefore, it is important to start with the manufacturer's directions and to vary staining time and stain concentration to obtain the optimal result. The stained smear is then washed with buffered water and examined at ×500 to ×1000 total magnification.

The method is simple and relatively inexpensive, but results are delayed because of the staining process; however commercial kits are available for quick staining. Trypanosomes are easily recognised by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the species.

1.1.3. Thin blood smear films

Thin blood smears are made by placing a small drop of blood (about 5 µl), for example from a microhaematocrit capillary tube, on a clean microscope slide approximately 20 mm from one end (allowing for space to apply the thick smear) and spreading with the edge of another slide. This slide is placed at an angle of approximately 30° to the first slide and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide in a fairly rapid but smooth motion. If the correct amount of blood is used, the slide should be covered with a film of blood with no surplus before the end of the slide is reached, and the smear should take the shape of a bullet. Ideally, thin films should be prepared so that the RBCs are fairly close to each other but not overlapping. The slide is dried quickly by waving in the air and protected from dust, flies and other insects. The slide is fixed for 3 minutes in methanol, and stained as for thick blood smears. After staining, the slide is washed gently under tap water and allowed to dry. A variation of this method is to fix in methanol for 2 minutes, apply May–Grünwald stain for 2 minutes, then add an equal volume of buffered water, pH 7.2, leave for a further 8 minutes and drain off. Approximately 50–100 fields of the stained thin smear are examined, with a ×50 or ×100 oil-immersion objective lens, before the specimen is considered to be negative. Even after a trypanosome has been detected, approximately 20 extra fields are investigated to determine if more than one species is present. The sharp extremity of the smear must be extensively explored as, because of their capillary properties, trypanosomes may be concentrated at this place (especially true for large species like *T. brucei* and *T. vivax*).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes.

Usually, both a thin and thick smear is made from the same sample. Thick smears contain more blood than thin smears and, hence, have a higher diagnostic sensitivity. Thin smears on the other hand allow *Trypanosoma* species identification. Trypanosome species can be identified by the following morphological characteristics:

- i) *Trypanosoma vivax*: 20–27 µm long, undulating membrane is medium or not obvious, free flagellum present at the anterior end, posterior end rounded, kinetoplast large and terminal.
- ii) *Trypanosoma brucei* is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e. a long slender form and a short stumpy form. Often, intermediate forms, possessing characteristics of both the slender and stumpy forms, are observed. The cytoplasm often contains basophilic granules in stained specimens.
 - a) *Trypanosoma brucei* (long slender form): 17–30 µm long and about 2.8 µm wide, undulating membrane is conspicuous, free flagellum present at the anterior end, posterior end pointed, kinetoplast small and subterminal.
 - b) *Trypanosoma brucei* (short stumpy form): 17–22 µm long and about 3.5 µm wide, undulating membrane is conspicuous, free flagellum absent, posterior end pointed, kinetoplast small and subterminal.
- iii) *Trypanosoma congolense*: 8–25 µm (small species), undulating membrane not obvious, free flagellum absent, posterior end rounded, kinetoplast is medium sized and terminal, often laterally positioned. Although *T. congolense* is considered to be monomorphic, a degree of morphological variation is sometimes observed. A number of morphotypes have been described so far; from the slender to the stumpiest: hyperleptomorph (rodhaini-form, very long and slender, with a free flagellum), leptomorph (simiae-form, slender, with a free flagellum), isomorph (congolense-form, short, without a free flagellum), pachymorph (montgomeryi-form, short and stout; $0.25 < \text{WLR} < 0.34$, without a free flagellum), and hyperpachymorph ('hyper-montgomeryi-form', short and very stout; $0.35 < \text{WLR} < 0.7$, without a free flagellum) (Desquesnes et al., 2012). Additionally, sphaeromorph and rosettes have also been described. Within *T. congolense*, different types or subgroups exist (savannah, forest, kilifi or Kenya coast) that have a different pathogenicity (Bengaly et al., 2002); also there is a large variation in pathogenicity within the savannah subgroup. These types can only be distinguished using PCR.
- iv) *Trypanosoma theileri*: (large species), typically 60–70 µm but individual organisms can range from 19 to 120 µm, undulating membrane is conspicuous, long free flagellum present, posterior end pointed and rigid, kinetoplast is large and positioned near the nucleus and in a marginal position. *Trypanosoma theileri* is normally nonpathogenic, but its presence can confuse the parasitological diagnosis. In Western Europe, *T. theileri* is the only trypanosome species occurring in cattle.

1.2. Parasite concentration techniques

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood examined and the level of parasitaemia. The amount of blood examined with direct examination techniques is low and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be improved by increasing the volume of blood to be examined and by concentrating the trypanosomes.

1.2.1. Microhaematocrit centrifugation technique (Woo method)

The microhaematocrit centrifugation technique, or the Woo method (1970), is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood sample depending on their specific gravity. The method is as follows:

- i) Fresh, usually ear vein blood (about 70 µl) is collected into heparinised capillary tubes (75 × 1.5 mm).
- ii) One end of the capillary tube is sealed with cristaseal or by heating, ensuring that the column of blood is not charred by the flame.
- iii) The sealed capillary tubes are placed in a microhaematocrit centrifuge with the sealed ends pointing towards the outside. To ensure good balance, the tubes are loaded symmetrically.
- iv) The rotary cover is screwed on and the centrifuge lid is closed.

- v) The capillary tubes are centrifuged at 9000 *g* for 5 minutes.
- vi) A tube carrier is made from a slide on which two pieces of glass 25 × 10 × 1.2 mm have been fixed, 1.5 mm apart, to form a groove.
- vii) The tube is placed in the groove, a cover-slip is placed on top and the interface is flooded with water. Alternatively, examination can be done without flooding the interface with water, but in such case, the light condenser must be placed in such a way that cells become refringent.
- viii) The plasma or white blood cell interface (buffy coat) is examined by slowly rotating the tube. Trypanosome movement can first be detected using the ×10 objective lens with reduced condenser aperture; the trypanosomes can be seen more clearly using the ×40 objective lens preferably with a long working distance to allow adequate depth of focus through the capillary tube.

The microhaematocrit centrifugation technique is more sensitive than the direct examination techniques (Kratzer & Ondiek, 1989). In the case of *T. vivax* infections, the sensitivity of the Woo methods approaches 100% when the parasitaemia is >700 trypanosomes/ml blood. Sensitivity decreases to 50% when parasitaemia varies between 60 and 300 trypanosomes/ml blood. Trypanosomes become very difficult to detect when the parasitaemia is lower than 60 trypanosomes/ml blood (Desquesnes, 2004). Identification of trypanosome species is difficult. As the specific gravity of *T. congolense* is similar to that of RBCs, parasites are often found below the buffy coat in the RBC layer. To improve the separation of RBCs and parasites, and increase the sensitivity for *T. congolense*, the specific gravity of RBCs can be increased by the addition of glycerol.

A modification of the Woo method is the quantitative buffy coat method (QBC) (Bailey & Smith, 1992). The method has been used for the diagnosis of *T. b. gambiense* infections; it is generally too expensive for the routine large-scale use in animal trypanosomosis surveys.

1.2.2. Dark-ground or phase-contrast buffy coat technique (Murray method)

The buffy coat technique or Murray method (Murray *et al.*, 1977) represents an improved technique for the detection of trypanosomes and is widely used. It is carried out following steps (i) to (v) above, after which the capillary tube is cut, with a diamond tipped pencil, 0.5 mm below the buffy coat, to include the top layer of RBCs. The buffy coat and the uppermost layer RBCs are extruded on to a clean microscope slide (check that the buffy coat is not sticking to the capillary tube; it should be visible on the slide before covering it with a cover-slip [22 × 22 mm]). Approximately 200 fields of the preparation are examined for the presence of motile trypanosomes with a dark-ground or a phase-contrast microscope with a ×40 objective lens. Trypanosome species can be identified by reference to the following criteria:

- i) *Trypanosoma vivax*: Large, extremely active, traverses the whole field very quickly, pausing occasionally.
- ii) *Trypanosoma brucei*: Various sizes, rapid movement in confined areas; undulating membrane traps the light into 'pockets' moving along the body.
- iii) *Trypanosoma congolense*: Small, sluggish, adheres to RBCs by anterior end.
- iv) *Trypanosoma theileri*: More than twice the size of pathogenic trypanosomes, tends to rotate; the posterior end is clearly visible, very long, sharp and rigid.

As with the microhaematocrit centrifugation technique, the buffy coat technique is more sensitive than direct examination techniques. The sensitivity of the buffy coat method can be improved by using the buffy coat double-centrifugation technique (Kratzer & Ondiek, 1989). A total amount of 1500–2000 µl of blood is centrifuged, after which the buffy coat is aspirated into a microhaematocrit capillary tube and centrifuged again. The buffy coat is examined. However, collection of the buffy coat after the initial centrifugation is a delicate step and results may vary from one technician to another.

Compared with the microhaematocrit centrifugation technique, the buffy coat technique has the added advantage that preparations can be fixed and stained for more accurate identification of species and for retention as a permanent record.

Both the microhaematocrit centrifugation and buffy coat techniques give direct results and can be used for screening large numbers of animals. They require specialised equipment and an electricity supply making the test more expensive compared with the examination of the wet

blood film. However, this is compensated for by increased sensitivity. Both parasite concentration techniques rely on the detection of motile, live, trypanosomes. Because trypanosomes can lose their vigour and die rather quickly once the blood sample is drawn, samples collected in capillary tubes should be cooled immediately and not be allowed to overheat in the microhaematocrit centrifuge or on the microscope stage. Samples should be examined as soon as possible after collection, preferably within a couple of hours.

The microhaematocrit centrifugation and buffy coat techniques are particularly useful in that the packed cell volume (PCV) can be assessed at the same time. To determine the PCV after centrifugation, the microhaematocrit capillary tube (containing ear vein or jugular vein blood) is placed in a haematocrit reader. The length of the packed RBC column is expressed as a percentage of the total volume of blood. Measuring the PCV is useful for determining the degree of anaemia. Anaemia can be caused by factors other than tsetse-transmitted trypanosomosis. It remains, however, one of the most important indicators of trypanosomosis in cattle. As trypanosomosis is a herd problem, the PCV-profile of a herd is influenced by the number of trypanosome-infected animals and can be used to indicate differences in disease challenge. The average PCV is also influenced by the age and level of genetic susceptibility of cattle.

1.2.3. Anion exchange

The miniature anion-exchange chromatography technique (m-AECT) is widely used for the diagnosis of human sleeping sickness caused by *T. b. gambiense* (Lumsden *et al.*, 1979). Blood is passed through a diethyl amino-ethyl (DEAE)-cellulose column equilibrated with a phosphate buffered saline (PBS) solution of an ionic strength suited to the blood of the animal species under examination. As the RBCs are more negatively charged than the trypanosomes, they are held in the column and the trypanosomes pass through with the eluate, which is collected, centrifuged to concentrate the trypanosomes and examined under the microscope.

Large volumes of blood can be examined from each animal and, therefore, the method has high sensitivity. However, the technique is cumbersome and is not suitable for the examination of a large number of animals because it is very expensive and time consuming.

1.2.4. *In-vitro* cultivation

A procedure for the *in-vitro* cultivation of *T. brucei* has been described, but success has been irregular over many years. Moreover, the method needs sophisticated equipment, produces results after a considerable delay and is certainly not suitable for large-scale use. A kit for *in-vitro* isolation of trypanosomes has proven to be promising in isolating and amplifying all species of *T. brucei* in humans, domestic and game animals (Truc *et al.*, 1992). The test's value in isolating *T. congolense* and *T. vivax* is still unknown. As it is based on the cultivation of procyclic forms of trypanosomes, species differentiation is not possible; however a recent method has been described for a complete *in-vitro* life-cycle of *T. congolense* (Coustou *et al.*, 2010). It should be noted that cultivation is a highly efficient and sensitive method for the detection of tabanid-transmitted *T. theileri*, the prevalence of which is often found to be close to 100% using this technique. In the case of mixed infections, *T. theileri* easily overgrows *T. b. brucei* (Verloo *et al.*, 2000).

1.3. Animal inoculation

Rodent inoculation is expensive, diagnosis is not immediate, and the method should be avoided as much as possible as it raises serious animal welfare concerns. However, the inoculation of blood into rodents, usually mice or rats, is more sensitive than the Haematocrit centrifuge technique and sometimes PCR as well, thus it is particularly useful in revealing subpatent infections, which may be especially important in non-endemic areas.

The laboratory animals are injected intraperitoneally with 0.1–0.5 ml (depending on the size of the rodent) of freshly collected blood. Artificial immunosuppression of recipient animals by irradiation or drug treatment (cyclophosphamide 200 mg/kg) will greatly increase the chances of isolating the parasite. A drop of blood is collected from the tip of the rodent's tail three times a week. The blood is examined using the wet film method. If an infection occurs, it generally shows after 3–10 days, however the rodents must be followed for at least 1 month.

In cases of suspected trypanosome infection, the success rate of this method depends on the *Trypanosoma* species involved: it is highly sensitive for detection of *Trypanozoon* infections, of medium sensitivity for *T. congolense* strains, and generally poor but in rare cases effective for *T. vivax*.

The modern use of rodent inoculation should therefore be restricted to (i) massive parasitic antigen-production for serological diagnosis, and (ii) attempts at parasite demonstration and isolation when a trypanosome infection is suspected in animals living in or travelling towards a non-endemic area.

1.4. Test to detect trypanosomal antigen

Antigen-detection enzyme-linked immunosorbent assays (ELISA) for trypanosomosis have been described (Nantulya & Lindquist, 1989). Field evaluations of the tests have given inconsistent results (International Atomic Energy Agency [IAEA], 1993). Additional works have been done under controlled conditions, which led to the conclusion that the sensitivity and specificity of these tests are not suitable for the diagnosis of trypanosomosis (Desquesnes, 1996; 2004; Eisler *et al.*, 1998).

1.5. DNA amplification tests

A PCR method has been developed as a tool for the diagnosis of infections with African trypanosomes in humans and animals, as well as tsetse flies. Specific repetitive nuclear DNA sequences can be amplified for *T. vivax* and three types of *T. congolense* (Desquesnes & Davila, 2002; Masiga *et al.*, 1992). A common primer set is available for detection of the three *T. brucei* subspecies. The primer sets available for different trypanosome subgenus, species and types are referred to as follows: Trypanozoon subgenus – TBR1 and TBR2; *T. congolense* (savannah type) – TCN1 and TCN2; *T. congolense* (forest type) – TCF1 and TCF2; *T. congolense* (Kenya Coast type) – TCK1 and TCK2; and *T. vivax* – TVW1 and TVW2. Due to the multiplicity of these taxon-specific primers in tsetse flies or cattle, a full trypanosome species identification requires that five PCR test be carried out per sample, which considerably increases the cost of diagnosis. Recently PCR restriction fragment length polymorphism (RFLP) assays and ITS1 of ribosomal DNA amplification have been developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (Delespaulx *et al.*, 2003; Desquesnes & Davila, 2002; Desquesnes *et al.*, 2001; Geysen *et al.*, 2003); however these tests are not yet suitable for routine diagnosis. Loop-mediated isothermal amplification is also under development for trypanosome diagnosis (Kuboki *et al.*, 2003).

Standard monovalent PCR amplifications are carried out in a reaction mixture containing Tris/HCl, MgCl₂, KCl, each of the four deoxyribonucleotide triphosphates, primers, DNA template and Taq DNA polymerase. Samples are incubated during several cycles at varying temperatures. The PCR products are electrophoresed through agarose. Gels are stained with ethidium bromide and visualised under UV light for the presence of specific weight products.

The procedure is extremely sensitive, but false-positive results may occur as a result of contamination of samples with other DNA. The test requires specialised equipment and highly trained personnel, so it is not suitable for use in many laboratories. False-negative results may occur when the parasitaemia is very low (< 1 trypanosome/ml of blood), which occurs frequently in chronic infections; they may also occur when the specificity of the primers is too high, so that not all isolates of a particular trypanosome species are recognised. Sample collection has been simplified by adapting the test using blood or buffy coats spotted on to filter paper (Geysen *et al.*, 2003; Katakura *et al.*, 1997). A large number of samples can be processed at one time, making it potentially suitable for large-scale surveys. However, at the moment, the cost of PCR analyses is prohibitive for the routine use of the test.

2. Serological tests

Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomosis, with variable sensitivity and specificity. The methods of choice are the indirect fluorescent antibody test (IFAT) (Katende *et al.*, 1987) and the trypanosomal antibody-detection ELISA (Hopkins *et al.*, 1998; Luckins, 1977). The identification of major antigens of trypanosomes, and their production as recombinant molecules or synthetic peptides, should hopefully lead to the development of new tests based on the use of defined molecules. Thus, in the future, it may be possible to improve the specificity of serological tests to allow the detection of species-specific antibodies, and to reach a high level of standardisation that is currently not achieved by the use of total parasite extracts.

2.1. Indirect fluorescent antibody test

The technique for the preparation of trypanosomal antigens (Katende *et al.*, 1987) involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal saline.

2.1.1. Test procedure

- i) Prepare thin smears from heavily parasitaemic blood or from a trypanosome suspension. Air-dry and fix in acetone for 5 minutes.
- ii) Mark circles of 5 mm diameter on glass slides using nail varnish.
- iii) Using a pipette, place a test serum, diluted 1/40, in each circle, ensuring that the area in each circle is completely covered.
- iv) Incubate the antigen/test serum preparation at 37°C for 30 minutes in a humid chamber.
- v) Wash the preparation three times in PBS for 5 minutes each time at 4°C, with gentle agitation. Air-dry the slides.
- vi) Apply conjugate: rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate.
- vii) Incubate and wash as above. Rinse in distilled water. Air-dry the slides.
- viii) Mount the slides in PBS or buffered glycerol and examine for fluorescence.

2.2. Antibody-detection enzyme-linked immunosorbent assay

The original antibody ELISA (Luckins, 1977) has recently been further developed for use in large-scale surveys of bovine trypanosomosis (Desquesnes, 1997; Hopkins *et al.*, 1998). Recommendations have been made that allow antigen production and standardisation of the test on a local basis (Desquesnes, 1997; 2004; Greiner *et al.*, 1997; Wright *et al.*, 1993).

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes. Trypanosomes are purified by DEAE anion-exchange chromatography of parasites from whole blood of infected rats (Lanham & Godfrey 1970). Antigens are prepared as a soluble fraction with lysis (with the addition of anti-enzyme) using seven freeze–thaw cycles and centrifugation at 10,000 *g* for 10 minutes. Antigens obtained from *in-vitro* propagated procyclic trypanosome forms can also be used (Greiner *et al.*, 1997). Soluble antigens must be added with a protease inhibitor cocktail and be stored at –80°C or –20°C for long and short periods, respectively, but they may also be lyophilised for conservation at room temperature. ELISAs using *T. congolense* or *T. vivax* precoated microtitre plates have been developed that have the advantage that a standardised denatured antigen is used that can be stored for long periods at room temperature (Rebeski *et al.*, 2000).

Both the IFAT and antibody-detection ELISA have been adapted for the analysis of blood samples collected on filter paper. Blood contained in one heparinised microhaematocrit centrifuge capillary tube is extruded on to a filter paper (Whatman® No. 4). Samples are air-dried out of direct sunlight and placed in a plastic bag with self-indicating silica gel desiccant. The bag is sealed and should be kept as cool as possible until specimens are refrigerated or frozen.

Each ELISA-microplate is run with strong positive, weak positive and negative reference sera, which are required to comply with pre-set values for quality assurance. The absorbance of each ELISA-sample tested is expressed as a percentage (percentage positivity: PP) of the strong positive reference standard (Wright *et al.*, 1993), or the positive and negative reference standards (Desquesnes, 1997); results are, therefore, quantifiable. The cut-off value is determined using known positive and negative field or experimental samples (Desquesnes, 1997; 2004).

Both antibody-detection tests have high sensitivity and genus specificity. Their species specificity is generally low, but may be improved by using a standardised set of the three species-specific tests (Desquesnes, 2004). They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. However, persistence of antibodies after a curative treatment or a self-cure is estimated to be on the average of 3–4 months in young and adult cattle infections (Desquesnes, 2004); although it might take up to 13 months before all antibodies have disappeared in some animals (Van den Bossche *et al.*, 2000) consequently, proper sampling and knowledge of trypanocidal use will give more acute information.

Immunodiagnosis needs expensive, sophisticated equipment and expertise, which is not always available. It has to be performed in specialised laboratories and there is a substantial delay between the actual sampling and the availability of the results. Nevertheless, the antibody ELISA lends itself to a high degree of automation and standardisation. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of tsetse-transmitted trypanosomosis.

C. REQUIREMENTS FOR VACCINES

No vaccines are in use at the present time.

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NB: There is an OIE Reference Laboratory for Trypanosomosis

(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:

<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/> <http://www.oie.int/>).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Trypanosomosis (tsetse-transmitted)