CHAPTER 3.4.16.

ANIMAL TRYPANOSOMOSES
(including tsetse-transmitted, but excluding surra and dourine)

SUMMARY

Description and importance of the disease: *Animal trypanosomosis of African origin is a disease complex caused by several species of protozoan parasites of the genus* Trypanosoma*, transmitted mainly cyclically by the genus* Glossina (*tsetse flies*) in sub-Saharan Africa (latitudes 10° North to 20–30° South) and some pockets of the Arabian peninsula, *but also mechanically transmitted by several biting flies* (*tabanids,* Stomoxys*, etc.*) in Africa and some other parts of the world. *The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle, where the disease is referred to as nagana. It is mainly caused by* Trypanosoma congolense (*subgenus Nannomonas*)*,* T. vivax (*subgenus Duttonella*) *and, to a lesser extent,* T. brucei brucei (*subgenus Trypanozoon*). *However, other hosts such as horses, donkeys, camels, goats, sheep, pigs and dogs may be affected, and, other* Trypanosoma *species must be considered, such as* T. simiae (*mostly found in pigs*), *and the two zoonotic subspecies* T. b. gambiense *and* T. b. rhodesiense *notably found in humans, cattle and pigs. Other* Trypanozoon *species derived from* T. brucei *lineage that are not transmissible by tsetse flies, e.g.* T. evansi (*responsible for "surra", mechanically transmitted by biting insects*) *and* T. equiperdum (*responsible for "dourine", venereally transmitted amongst equids*) *are presented in chapters 3.1.21 and 3.5.3, respectively. Because some of these parasites are not necessarily transmitted by tsetse and have spread outside Africa, with the exception of the two latter species, this chapter is devoted to animal trypanosomosis of African origin.*

Animal trypanosomosis of African origin 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 or nervous signs with emaciation and eventually death in chronic forms. Subclinical or healthy carriers of the parasites are frequently observed in enzootic areas, however, there are seasonal variations in transmission and clinical emergence.

Identification of the agents: *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* (*PCR*), *which can identify parasites at the genus, species, subspecies or type level, depending on the cases. Highly specific primers or sequencing of PCR products allow the zoonotic* T. brucei *spp. to be identified, which brings new information on the role of domestic and wild fauna in maintenance of some sleeping sickness foci. Additionally, in some geographical areas where nagana, surra and dourine may occur, there is a need to identify the non-tsetse transmitted* Trypanozoon *at the species level as control measures might be different from those of nagana.*

Serological tests: *The indirect fluorescent antibody test and the antibody-detection enzyme-linked immunosorbent assay* (*ELISA*) *are routinely used for the detection of antibodies against* Trypanosoma *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 should allow a high degree of standardisation when recombinant antigens or, better, in-vitro*-produced blood forms of the parasites have been developed and validated, work which is currently in progress. However, ELISA for antibodies to* T. congolense*,* T. viva*x and* T. brucei brucei *are at the present time carried out with native soluble antigens of trypanosomes grown in rodents and give reasonable 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* and *T. simiae*, Duttonella for *T. vivax* and *T. uniforme*, and *Trypanozoon* for *T. brucei brucei, T. brucei gambiense, T. brucei rhodesiense, T. evansi & T. equiperdum.*

Animal trypanosomosis of African origin is a disease complex caused by one or several of these *Trypanosoma* spp., transmitted mainly cyclically by the genus *Glossina* (tsetse flies), but also mechanically by biting flies such as tabanids and *Stomoxys* (Baldacchino *et al.,* 2014). Tsetse infest 10 million square kilometres and affect 37 countries, in sub-Saharan Africa (between latitude 10° North and 20–30° South) and in some pockets of the Arabian Peninsula. The disease, known as 'nagana', affects various species of wild and domestic mammals but, from an economic point of view, African trypanosomosis is particularly important in cattle (also referred to as tsetse-fly disease in southern Africa). It is mainly caused by *Trypanosoma congolense,* especially Savannah type, the most pathogenic one (Bengaly *et al.,* 2002), *T. vivax*, the most prevalent, and, to a lesser extent, *T. brucei brucei.*

Other hosts such as horses, donkeys, camels, goats, sheep, pigs, dogs and even humans may be affected, and other *Trypanosoma* species must be considered as well as other means of transmission. *Trypanosoma congolense* type forest and Kenya coast are mild pathogens for cattle, but their epidemiology is not fully elucidated. *Trypanosoma uniforme*, and *T. simiae*, a pig parasite, are other less common tsetse-transmitted *Trypanosoma* spp. *Trypanosoma vivax* is also mechanically transmitted by biting flies, among which tabanids and *Stomoxys* 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 (in Ethiopia, Chad, Senegal, Sudan, etc.). Tsetse-transmitted trypanosomosis can affect horses and camels and is a natural barrier preventing the introduction of camelids into the southern Sahel region of West Africa. Very rare human cases have been observed caused by animal *Trypanosoma* spp. such as *T. congolense* and *T. brucei brucei*, but also *T. evansi* (including *T. lewisi*, a cosmopolitan rat parasite; Truc *et al.,* 2013). Tsetse-transmitted trypanosomosis causes chronic or acute sleeping sickness in humans, through infection with *T. brucei gambiense* and *T. brucei rhodesiense,* respectively. A large range of wild and domestic animals, including cattle and pigs, can act as reservoirs of these human parasites, especially for the latter. Consequently, laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*) especially when handling cattle, pig or wild fauna samples. Under the expanding "One health"concept, identification of these zoonotic agents becomes a need for the control of human African trypanosomosis (Holmes, 2015). Similarly, in Latin America, and even in the USA, special care should be taken when handling animal samples due to the potential presence of *T. cruzi* (in cattle, sheep, horse dogs etc.), as recently shown in a horse exhibiting neurological clinical signs in Texas (Bryan *et al.,* 2016).

Another two *Trypanozoon* species derived from *T. brucei* lineage are not transmitted by tsetse flies: (i) *T. evansi*, the causative agent of "surra", especially pathogenic to camels and horses in Africa, but also to horses, cattle, buffaloes and others in Latin America and Asia*;* it is mechanically transmitted by tabanids and *Stomoxys*, but may be found in the same hosts and sometimes the same areas as the agents of nagana; only molecular diagnosis allows distinction of the agent of surra from other *Trypanozoon* involved in nagana; and (ii) *T. equiperdum*, the causative agent of dourine, a worldwide venereal disease transmitted to horses and mules. Diagnostic procedures for these parasites are presented in chapters 3.1.21 and 3.5.3, respectively.

Clinical signs of animal trypanosomosis of African origin may include intermittent fever, anaemia, 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), completed in some cases by sequencing. Clinically, African trypanosomosis can be confused with babesiosis, anaplasmosis, theileriosis, haemonchosis and even ehrlichiosis, rabies, plant intoxications or *T. cruzi* infection in Latin America. Final diagnosis is aided by clinical observations, and the 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 working to improve existing tests and 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 with the detection of infection at the 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 Table 1).

Table 1. *Test methods available for the diagnosis of animal trypanosomoses and their purpose*

Method	**Purpose**					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Thin stained blood smear	–	+	–	+++	+	n/a
DNA detection/PCR	+++	+++	+++	+++	+++	n/a
Wet blood film	–	–	–	++	–	n/a
Thick stained blood film	–	+	–	+	+	n/a
Haematocrit centrifuge technique (HCT, Woo)	+++	+++	+++	+++	+++	n/a
Buffy coat technique (BCT, Murray)	–	–	++	++	++	n/a
Anion exchange columns	–	+	++	–	–	n/a
Rodent inoculation[2]	+	++	++	++	–	n/a
In-vitro culture	–	–	–	–	–	n/a

1 A combination of agent identification methods applied on the same clinical sample is recommended.
2 Test valuable only if *T. evansi* infection is suspected.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
IFAT	++	++[3]	++[3]	–	++[3]	n/a
ELISA	+++	+++	+++	–	+++	n/a

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method but may need further validation;
+ = may be used in some situations, but cost, reliability, or other factors severely limits its application;
– = not appropriate for this purpose; n/a = purpose not applicable.
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 (subgenus), but their sensitivity is relatively low (i.e. the proportion of false-negative results recorded is high), which give them a low negative predictive value (NPV). 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 stable enzootic areas (most of the animals are in the chronic stages of infection), especially during subclinical phases of the infection, which makes the animals appear like 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 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 during the post-treatment period.

Several parasite detection techniques are available, exhibiting variable sensitivity. The choice will depend on the laboratory facilities available and the aim of the diagnosis accordingly to the recommendations indicated in Table 1.

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. Examination of Giemsa-stained blood smear (GSBS) remains the classic and most certain reference diagnostic test for trypanosome infection.

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).

3 CATT (card agglutination test for *T. evansi*) should be used if a *T. evansi* infection is suspected.

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 (GSBS).

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.

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 de-haemoglobinised 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 subgenus.

1.1.3. Thin blood smear films: Giemsa-stained blood smear

Thin blood smears are made by placing a small drop of blood (about 3 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. The blood is thus pulled (by capillary action) by the spreader slide. 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, incubate for a further 8 minutes and drain off. Finally some rapid methods use staining by 4–5 dips of 1 second each, serially into methanol, eosinophilic and basophilic solutions. Approximately 50–100 fields of the stained thin smear are examined, with a ×50 or ×100 oil-immersion objective lens (total magnification ×500–×1000), 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 or for oedema fluids. It can also help with differential diagnosis from other haemoparasites such as *Anaplasma, Babesia* and *Theileria*.

Usually, both a thin and thick smear are 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* subgenus identification. Trypanosome subgenera or species can be identified by the following morphological characteristics:

i) *Duttonella*: *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. *Trypanosoma uniforme* presents the same characteristics although it is smaller (12–20 µm long);

ii) *Trypanozoon*: *Trypanosoma brucei* (e.g. *T. brucei brucei*, *T. b. gambiense* & *T. b. rhodesiense*) 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. *Trypanosoma evansi* and *T. equiperdum* can be confused with the slender form of *T. brucei* in Equidae.

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) *Nanomonnas*: *Trypanosoma congolense* is 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 monomorphous, a degree of morphological variation is sometimes observed. In *Nanomonnas*, 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 (this is *T. simiae*-form, slender, with a free flagellum), isomorph (*congolense*-form, short, without a free flagellum), pachymorph (montgomeryi-form, short and stout; 0.25<WLr<0.34, without a free flagellum), and hyperpachymorph ('hyper-montgomeryi-form', short and very stout; 0.35<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. Finally, the pig and monkey parasite, *T. simiae,* is pleomorphic, appearing from hyperleptomorph to pachymorph, most often like a long parasite (leptomorph), with well developed undulating membrane, occasionally exhibiting a free flagellum, it may also appear like a classical *T. congolense*.

iv) *Megatrypanum:* Megatrypanum are not tsetse transmitted; they are Stercorarian parasites, cyclically transmitted by tabanids; they are however, regularly found in bovine blood samples and must be distinguished from pathogenic *Trypanosoma* spp. *Trypanosoma theileri* is typically 60–70 µm (large species), 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 and Japan, *T. theileri* is the only trypanosome species occurring in cattle. As a consequence of its cyclical transmission by tabanids, highly cosmopolite and abundant vectors, this parasite is very common worldwide and has a very high prevalence in bovines. Detection is rare however, due to very low parasitaemia. Other related species, such a *T. ingens* (found in bovines and antelopes), can be distinguished by a typical unstained transversal band inside the nucleus on GSBS.

v) Other species: in the area of distribution of animal trypanosomosis of African origin, other *Trypanosoma* spp. may be found in blood samples and should be identified. Although they may be highly polymorphic, the most characteristic identification criteria of two common parasites are described hereafter. *T. lewisi* (rat parasite) can be found in rodents and sometimes in primate samples including humans; it is characterised by a large size (30 µm), a posterior nucleus, a free flagellum, a large sub-terminal kinetoplast and a very sharp posterior extremity in the C shape adult form. *T. cruzi* is a medium-sized parasite (16–25 µm) with central nucleus, free flagellum, very large and protruding sub-terminal kinetoplast, and a C-or S-shape adult form; it should be noted that amastigote parasites may be found, notably in spinal fluid and muscles.

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. Amongst these methods, the haematocrit centrifuge technique (HCT) is the classic and most certain reference diagnostic test for detection of living trypanosomes.

1.2.1. Haematocrit centrifuge technique (HCT, Woo method)

The haematocrit centrifuge technique (HCT), or (Woo, 1970), is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood 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); when the blood is collected from a larger vein in an anti-coagulant tube, a dry capillary tube can be filled.

ii) One end of the capillary tube is sealed with cristaseal.

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. The capillary tubes are centrifuged at 9000 ***g*** for 5 minutes.

iv) 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. The capillary 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 lowered in such a way that cells become refringent.

v) The interface of the plasma and buffy coat (platelets and white blood cells [WBCs]) is examined by slowly rotating the tube 6–7 times for about 60 degrees of angle. 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 HCT is more sensitive than the direct examination techniques. 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 WBCs, parasites are often found inside the buffy coat. To improve the separation of blood cells and parasites, and increase the sensitivity for *T. congolense*, the specific gravity of blood cells can be increased by the addition of glycerol.

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

The buffy coat technique (BCT) or Murray method (Murray *et al*., 1977) represents an improved technique for the detection of trypanosomes. It is carried out following steps (i) to (iii) above (Section B.1.2.1), 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 (it is important to 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 (×400 total magnification). 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 obviously rigid.

As with the HCT, the BCT is more sensitive than direct examination techniques. The sensitivity of the BCT can be improved by using the buffy coat double-centrifugation technique. A total amount of 1500–2000 µl of blood is centrifuged, after which the buffy coat is aspired into a microhaematocrit capillary tube and centrifuged again. The upper 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 HCT, the BCT has the added advantage that preparations can be fixed and stained for more accurate identification of species and for retention as a permanent record. However, repeatability of the method is lower than HCT as the procedure for dropping the buffy coat from the capillary tube to the slide is uncertain and consequently its success varies from one technician to another. Most often the buffy coat sticks to the wall of the capillary tube and thus may be missed and the examination is negative. Additionally, BCT is more time consuming than HCT. For these reason, preference is given to HCT as a reference method in routine use.

Both the HCT and BCT give direct results but HCT can better 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. As 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. Capillary tubes should be kept vertically just after centrifugation, to avoid spreading of the buffy coat. Blood samples should, be tested as soon as possible after collection, preferably within a couple of hours.

The HCT and BCT 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 + buffy coat 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, however, it remains 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 (Lanham & Godfrey, 1970). 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 variable to low. As it is based on the cultivation of procyclic forms of trypanosomes, species differentiation is not possible; however recent method have been described for a complete *in-vitro* life-cycle of *T. congolense*. 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). For the reasons stated above, *in-vitro* culture of trypanosomes cannot be recommended for diagnostic purposes.

1.3. Animal inoculation

Rodent inoculation is expensive and should be restricted to massive parasitic antigen-production for serological diagnosis, and only in exceptional circumstances for parasite demonstration and isolation. 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.

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

1.4. 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 highly repetitive nuclear DNA sequences (also called satellite DNA, presenting 10,000–20,000 serial repeats in the genome) can be amplified for *T. vivax* and three types of *T. congolense* (Desquesnes & Davila, 2002). A common primer set is available for detection of all *Trypanozoon* taxa, including the three *T. brucei* spp., *T. evansi* and *T. equiperdum*. DNA preparation is a determining step; several methods including commercial kits are available (Penchenier *et al.,* 1996). Similarly to parasitological examinations, a concentration technique by centrifugation allows enrichment of blood samples; it is therefore recommended to carry out the DNA preparation step on buffy coats.

Well validated primer sets available for the different trypanosome subgenera, species and types are referred to as follows (Table 2): *Trypanozoon* subgenus – TBR1 and TBR2; *T. congolense* savannah type – TCS1 and TCS2; *T. congolense* forest type – TCF1 and TCF2; *T. congolense* Kenya Coast type – TCK1 and TCK2; *T. simiae*– TSM1 and TSM2, and *T. vivax*– TVW1 and TVW2 (Masiga *et al*., 1992). Other sets of primers are available to distinguish *T. evansi* and its types A & B (Claes *et al.,* 2004; Njiru *et al.,* 2006). More specific methods are also available to identify *T. b. gambiense* and *T. b. rhodesiense* (Njiru *et al.,* 2004; Picozzi *et al.,* 2008; Radwanska *et* 2002a; 2002b), which allow investigations of the animal reservoir of sleeping sickness (Hamill *et al.,* 2013; Karshima *et al.,* 2016). Due to the multiplicity of these taxon-specific primers in tsetse flies or cattle, a complete *Trypanosoma* species identification requires three to six or more PCR tests be carried out per sample, which considerably increases the cost of diagnosis. In the USA, primers for detection of *T. cruzi* –TCZ1 &TCZ2 might also be used (Moser *et al*., 1989).

Amplifications of ITS1 of ribosomal DNA have also been developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (Desquesnes *et al*., 2001, Desquesnes & Davila, 2002; Njiru *et al.,* 2005). These tests are useful for screening, however sizing of the PCR product(s) on gels is sometimes not reliable, thus, sequencing is most often required to confirm species identification, which is not suitable for routine diagnosis. Loop-mediated isothermal amplification has also been developed for trypanosome diagnosis (Kuboki *et al*., 2003), however so far its limited use did not allow full validation for veterinary purposes.

Table 2*. Well validated primer sequences for animal Trypanosomes identification*

Specificity	Primer sequences	References
T. brucei s.l.	TBR1: 5'-CGA-ATG-AAT-ATT-AAA-CAA-TGC-GCA-G-3'	Moser *et al.,* 1989
	TBR2: 5'-AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC-3'	
T. congolense type savannah	TCS1: 5'-CGA-GCG-AGA-ACG-GGC-AC-3'	Masiga *et al.*, 1992
	TCS2: 5'-GGG-ACA-AAC-AAA-TCC-CGC-3'	
T. congolense type forest	TCF1: 5'-GGA-CAC-GCC-AGA-AGG-TAC-TT-3'	Masiga *et al.*, 1992
	TCF2: 5'-GTT-CTC-GCA-CCA-AAT-CCA-AC-3'	
T. congolense type "Kilifi"	TCK1: 5'-GTG-CCC-AAA-TTT-GAA-GTG-AT-3'	Masiga *et al.*, 1992
	TCK2: 5'-ACT-CAA-AAT-CGT-GCA-CCT-CG-3'	

Specificity	Primer sequences	References
T. simiae	TSM1: 5'-CCG-GTC-AAA-AAC-GCA-TT-3'	Masiga *et al.*, 1992
	TSM2: 5'-AGT-CGC-CCG-GAG-TCG-AT-3'	
T. vivax	TVW 1: 5'-CTG-AGT-GCT-CCA-TGT-GCC-AC-3'	Masiga *et al.*, 1992
	TVW 2: 5'-CCA-CCA-GAA-CAC-CAA-CCT-GA-3'	
T. cruzi	TCZ1: 5'-CGA-GCT-CTT-GCC-CAC-ACG-GGT-GCT-3'	Moser *et al.*, 1989
	TCZ2: 5'-CCT-CCA-AGC-AGC-GGA-TAG-TTC-AGG-3'	
Specificity	Other commonly used Primer sequences	References
T. evansi	TEPAN1: 5'-AGT-CAC-ATG-CAT-TGG-TGG-CA-3'	Panyim *et al.*, 1993
	TEPAN2: 5'-GAG-AAG-GCG-TTA-CCC-AAC-A-3'	
T. evansi	ESAG6/7F: 5'-ACA-TTC-CAG-CAG-GAG-TTG-GAG-3'	Holland *et al.*, 2001
	ESAG6/7R: 5'-CAC-GTG-AAT-CCT-CAA-TTT-TGT-3'	
T. evansi (type A)	RoTat1.2F: 5'-GCG-GGG-TGT-TTA-AAG-CAA-TA-3'	Claes *et al*., 2004
	RoTat1.2R: 5'-ATT-AGT-GCT-GCG-TGT-GTT-CG-3'	
*T. evansi (*type B)	EVAB1: 5'-CAC-AGT-CCG-AGA-GAT-AGA-G-3'	Njiru *et al.*, 2006
	EVAB2: 5'-CTG-TAC-TCT-ACA-TCT-ACC-TC-3'	
T.brucei gambiens	Tgs-GP F: 5'-GCT-GCT-GTG-TTC-GGA-GAG-C-3'	Radwanska *et al.*, 2002a
	TgsGP R: 5'-GCC-ATC-GTG-CTT-GCC-GCT-C-3'	
T.brucei rhodesiense	Tbr F: 5'-ATA-GTG-ACA-AGA-TGC-GTA-CTC-AAC-GC-3'	Radwanska *et al.*, 2002b
	Tbr R: 5'-AAT-GTG-TTC-GAG-TAC-TTC-GGT-CAC-GCT-3'	
Pan-tryp.: *T.brucei, T.vivax, T.congolense* forest, savannah, Kilifi, *T. evansi, T. lewisi*	TRYP1S: 5'-CGT-CCC-TGC-CAT-TTG-TAC-ACA-C-3'	Desquesnes *et al.*, 2002
	TRYP1R: 5'-GGA-AGC-CAA-GTC-ATC-CAT-CG-3'	
Pan-tryp.: *T.brucei, T.vivax, T.congolense* forest, savannah, Kilifi, *T. evansi*	ITS1 CF: 5'-CCG-GAA-GTT-CAC-CGA-TAT-TG-3'	Njiru *et al.*, 2005
	ITS1 BR: 5'-TTG-CTG-CGT-TCT-TCA-ACG-AA-3'	

Monovalent PCR amplifications are carried out in a reaction mixture containing Tris/HCl, $MgCl_2$, KCl, each of the four deoxyribonucleotide triphosphates, primers, DNA template and Taq DNA polymerase. Samples are incubated during 30 cycles at varying temperatures. The PCR products are electrophoresed through agarose. Gels are stained with ethidium bromide or other nucleic acid gel stain 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 trypanosome 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. False negative results 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 (Katakura *et al*., 1997); such methods are greatly favoured, nowadays, especially for international shipment of samples. A large number of samples can be processed at one time, making it potentially suitable for large-scale surveys.

Specific DNA reference samples for PCR can be obtained from the OIE Reference Laboratory for trypanosomes of African origin (CIRAD, Montpellier) as well as from the OIE Reference Laboratory for surra (ITM, Anvers).

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. Alternatively, improving techniques for *in-vitro* production of blood stages of various *Trypanosoma* spp. are highly promising as they will allow production of standardised whole cell lysate soluble antigens, which guaranties a high sensitivity because of the rich panel of native antigens they exhibit. In 2017, ELISA-*T. congolense* savannah, ELISA-*T. vivax* and ELISA-*T. b. brucei* are the recommended methods for detection of anti-trypanosome antibodies in most of the host species affected by nagana.

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.

Interpretation of the fluorescence remains subjective and the procedure is not adapted to large-scale studies; therefore the IFAT is generally used for individual diagnosis as an alternative to ELISA.

2.2. Antibody-detection enzyme-linked immunosorbent assay (ELISA)

The original antibody ELISA (Luckins, 1977) has 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 produced in laboratory rats. 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 after lysis (with the addition of anti-enzyme) using five to seven freeze–thaw cycles (or sonication) and centrifugation at 10,000 ***g*** for 10 minutes; the supernatant contains the soluble antigens that must be added with a protease inhibitor cocktail and be stored at –80°C or –20°C for long and short periods, respectively. Alternatively, soluble antigens may be lyophilised for conservation at room temperature or international shipment of standardised reagents. ELISAs using *T. congolense* or *T. vivax* precoated microtitre plates have been developed that have the advantage of a standardised denatured antigen, which can be stored for long periods at room temperature (Rebeski *et al*., 2000), however sensitivity and specificity of the test are lower. A well standardised method with good performance is expected in the near future with *in-vitro* production of blood-form trypanosomes.

Both the IFAT and antibody-detection ELISA have been adapted for the analysis of blood samples collected on filter paper. 30–100 µl of serum or plasma sample is deposited on a filter paper. 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. Further validation of this technique is being undertaken as such methods greatly facilitate the international shipment of samples.

Each ELISA-microplate is run with duplicate strong, medium and 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 *et al*., 2011). 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 accurate information.

Immunodiagnosis requires the production of native antigens, needs expensive and sophisticated equipment and expertise, which are 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, as well as for post-treatment follow-up.

Specific antigens for ELISA and reference samples (positive and negative reference serum samples) can be obtained from the OIE Reference Laboratory for animal trypanosomes of African origin.

Recommended tests for the diagnosis of trypanosomosis are the following: GSBS, HCT, PCR using satellite primers (Masiga *et al.,* 1992) and ELISA using soluble antigens from whole cell lysates of *T. congolense* savannah, *T. vivax* and *T. brucei brucei*.

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 Trypanosomoses of African origin
(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/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for
Trypanosomoses (tsetse-transmitted)/Trypanosomoses of African origin.

NB: FIRST ADOPTED IN 1991 AS TRYPANOSOMIASIS; MOST RECENT UPDATES ADOPTED IN 2018.