CHAPTER 3.4.14.

NAGANA: INFECTIONS WITH SALIVARIAN TRYPANOSOMOSES (EXCLUDING *TRYPANOSOMA EVANSI* AND *T. EQUIPERDUM*)

SUMMARY

Description and importance of the disease: *Nagana is a disease complex caused by several species of protozoan parasites of the genus* Trypanosoma*, section salivaria, transmitted mainly cyclically by flies of 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 though other hosts such as horses, donkeys, camels, goats, sheep, pigs and dogs may be affected. Nagana is mainly caused by* Trypanosoma congolense (*subgenus Nannomonas*)*,* T. vivax (*subgenus Duttonella*) *and, to a lesser extent,* T. brucei brucei (*subgenus Trypanozoon*) *however, 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 the* 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 3.6.3, respectively.*

Animal infections with these salivarian 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 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.

Detection 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 *sub-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 using recombinant antigens or, better, in-vitro-produced blood forms of the parasites, once their development and validation are completed. However, ELISA for antibodies to* T. congolense, T. vivax *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. In areas where several species of trypanosomes are present (*including* T. cruzi, T. evansi *and* T. equiperdum) *mixed infections may not be detected because cross-reactions amongst pathogenic trypanosomes may occur with any serological test employed, and an agent detection test may provide false negative results; in this situation the exact status of an animal with regard to the* Trypanosoma *species it is carrying cannot be established.*

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. It is divided into two sections, Stercorarian (which implement their cyclical development in the posterior part of the digestive tract of their vector), to which belongs *Trypanosoma cruzi* a zoonotic parasites present in America, and Salivarian (which implement their cyclical development in the anterior part of the digestive tract of their vector), to which belong all animal trypanosomes originating from Africa (subgenera and species or subspecies): *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 and T. equiperdum.*

Nagana is a disease complex caused by one or several of these trypanosomes, transmitted mainly cyclically by flies of the genus *Glossina* (tsetse flies), but also mechanically by biting flies such as tabanids and *Stomoxys* spp. (Baldacchino *et al.,* 2014). Tsetse flies 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). The most prevalent and pathogenic Trypanosome species in African cattle is *Trypanosoma congolense* (Savannah sub-type); however *T. vivax* and *T. brucei brucei* are also important causative agents (Bengaly *et al.,* 2002).

Other hosts such as horses, donkeys, camels, goats, sheep, pigs, dogs, cats, non-human primates 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 flies (in Ethiopia, Chad, Senegal, Sudan, etc.). Tsetse-transmitted trypanosomosis can affect horses and camels and act as 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. of African origin such as *T. congolense* and *T. brucei brucei*, but most of them are due to *T. evansi* (other human infections by animal trypanosomes are due to *T. lewisi*, a cosmopolitan rat parasite). Infection with *T. brucei gambiense* and *T. brucei rhodesiense* cause chronic or acute sleeping sickness in humans, 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 is essential 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).

Two other *Trypanozoon* species, derived from the *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, and to horses, cattle, buffaloes and others in Latin America and Asia*;* it is mechanically transmitted by tabanids and *Stomoxys* spp., but may be found in the same hosts and sometimes in the same areas as the agents of nagana; only highly specific molecular diagnosis allows distinction of the agent of surra from other *Trypanozoon* involved in nagana; and (ii) of *T. equiperdum*, the causative agent of dourine, a venereal disease with a relatively global distribution, transmitted to horses and mules. Diagnostic procedures for these parasites are presented in chapters 3.1.21 and 3.6.3, respectively.

In the present chapter, the following animal infections with salivarian trypanosomes are considered: *T. congolense, T. brucei*, *T. vivax* and *T. simiae* – the agents of Nagana.

Clinical signs of Nagana 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 animal infections with these salivarian trypanosomes 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), complemented in some cases by sequencing and sequence analysis. Clinically, infections of animals with salivarian trypanosomes can be confused with babesiosis, anaplasmosis, theileriosis, haemonchosis and even ehrlichiosis, rabies, plant intoxications or *T. cruzi* infection in Latin America (Bryan *et al.,* 2016; Desquesnes 2004). 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 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. 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). Detailed diagnosis techniques (including figures) of all tests described in section B can be found in the "Compendium of standard diagnostic protocols for Animal Trypanosomoses of African Origin", available online[1].

Table 1. Test methods available for the diagnosis of animal trypanosomosis 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
Detection of the agent[(a)]						
GSBS	–	+	–	+++	+	–
DNA detection/PCR	+++	+++	+++	+++	+++	–
Wet blood film	–	–	–	++	–	–
TGSBF	–	–	–	+	+	–
HCT (Woo)	+++	+++	+++	+++	+++	–
BCT (Murray)	–	–	++	++	++	–
AECT	–	+[(b)]	++	++[(a)]	–	–

1 http://www.oie.int/nttat/Attached%20files/A16-REC-COMPENDIUM_PROTOCOLES_TRYPANO-En.pdf

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[c],[d]	++	++	++	–	++	–
ELISA[d]	+++	+++	+++	–	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
GSBS = thin Giemsa stained blood smear; PCR = polymerase chain reaction; TGSBS = thick Giemsa stained blood film;
HTC = haematocrit centrifuge technique; BCT = buffy coat technique; AECT = anion exchange chromatography technique;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification tests applied on the same clinical sample is recommended.
[b]Although expensive and time consuming, the mini-AECT can be useful for the detection of low parasitaemic animals.
[c]CATT (card agglutination test for *T. evansi*) and IFAT or ELISA *T. evansi* should be used if a *T. evansi* infection is suspected (as such or in a mixed infection).
[d]Choice of the antigen(s), *T. vivax, T. congolense* type savannah and/or *T. brucei brucei* (± *T. evansi*) must be adapted to the epizootiological situation; 1 to 4 IFATs or ELISAs may be justified.

1. Detection of the agent

Identification of the agents of nagana can be made by visualisation of the parasite with a microscope or by demonstration of its DNA. Molecular techniques are highly sensitive and their specificity can be very high; they are increasingly used and they have considerably improved the diagnosis of African trypanosomosis. Parasitological methods however, remain the most employed; they are easy and inexpensive to carry out, and parasite visualisation remains the best method for diagnostic certainty.

Parasite detection techniques are subgenus specific, 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, thus 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 can be slightly 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.

Rodent inoculation is expensive and of ethical concern. It should no longer be used as a diagnostic method, and should be restricted to antigen production for use in serological diagnostic tests

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).

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 must be 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 (the test may be positive when the parasitaemia is above 10^4 parasites/ml). Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent.

Due to its very low sensitivity, this technique is generally used to follow-up experimental infections (in which high parasitaemias are expected), rather than to detect infections in field samples.

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 recognised by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the subgenus. The test is positive when the parasitaemia is above 10^4–10^5/ml. Thin blood smears are generally preferred to thick ones, because of the lower specificity of the latter.

1.1.3. Thin blood smear films: Giemsa-stained blood smear (GSBS)

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 when both techniques are applied) and spreading with the edge of another slide (spreader). 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 when 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 pure methanol for 2 minutes, apply May–Grünwald stain for 2 minutes, then add an equal volume of

buffered water, pH 7.2, incubate for 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 in this area (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*.

Although it is more time-consuming, both a thin and a thick smear may be 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; the test is positive when the parasitaemia is around or above 10^5/ml. 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*.

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) Nannomonas: *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 *Nannomonas*, 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 is

long, sharp-pointed and rigid, kinetoplast is large and positioned near the nucleus and in a marginal position. *Trypanosoma theileri* is normally non-pathogenic, 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 cattle [not pathogenic] and wild ruminants [reservoir hosts])[2], can be distinguished by a typical unstained transversal band inside the nucleus on GSBS.

v) Other species: in the area of distribution of salivarian trypanosomes, 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. *Trypanosoma 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. *Trypanosoma cruzi* is a medium-sized parasite (16–25 μm) with central nucleus, free flagellum, a 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; *T. cruzi* can be found in all mammal species in Latin America and the southern part of the USA.

As stated above, these criteria allow identification of the parasites on GSBS at the subgenus level by microscopic visualisation. In a number of epizootiological contexts, they allow the species to be identified; for example a Duttonella is almost always linked to *T. vivax*, a Megatrypanum to *T. theileri*, a Nannomonas to *T. congolense* savannah in cattle, etc. However, only molecular techniques allow identification of the species with certainty (as opposed to microscopic visualisation, which brings diagnostic certainty at the genus level).

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 (2–10 μl) 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 method, 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 density and shape. The method is as follows:

i) Usually about 70 μl of fresh blood, preferably from the ear vein, 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 (plasticine).

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

2 FAO Guide, Table 2: http://www.fao.org/3/X0413E/X0413E02.htm

done without flooding the interface with water, but in such case, the light condenser must be lowered in such a way that cells become refringents.

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). Sensitivity is higher with large trypanosomes such as Trypanozoon, and lower with small ones such as Nannomonas. Overall, HCT is considered to be positive when the parasitaemia is around or above 10^2–10^3. Identification of trypanosome species is difficult. As the specific decantation parameters of *T. congolense* are very close to those of WBCs and platelets, 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 density 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 derives from the Woo method. 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 of 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 HCT and 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 directly examined (HCT) or collected and examined (BCT). 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 species identification 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 time to time, and 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 reasons, 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 live, motile 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 trypanosomosis, however, it remains one of the most important indicators of the disease 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 the pellet is collected, spread on a slide and examined under the microscope. The positivity threshold is around 10^2–10^3 parasites/ml of blood.

Large volumes of blood can be examined from each animal and, therefore, the method may exhibit higher 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. It can be occasionally used when diagnostic certainty is required.

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*; a common primer set is available for detection of all *Trypanozoon* taxa, including the three *T. brucei* spp., *T. evansi* and *T. equiperdum* (Desquesnes & Davila, 2002). DNA preparation is a determining step; several methods including commercial kits are available, however, a resin preparation method is generally recommended (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 to increase the sensitivity of parasite DNA detection. Using the correct DNA preparation method on buffy coat samples, and satellite DNA primers, PCRs are generally positive when the parasitaemia is around or above 1–10 trypanosomes/ml of blood.

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* type A (RoTat1.2 primers) and B (EVAB primers) from *T. brucei* sspp. and *T. equiperdum*, but some strains are cross-reacting (Claes *et al.,* 2004; Njiru *et al.,* 2006). More specific methods are also available to identify *T. b. gambiense* and *T. b. rhodesiense* (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 may require three to six or more PCR tests be carried out per sample, which considerably increases the time and cost of diagnosis. In the USA and Latin America, primers for detection of *T. cruzi* – TCZ1 & TCZ2 (Moser *et al*., 1989) might also be required for trypanosome identification, notably in horses (Bryan *et al.,* 2016).

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. The ITS1 sequence being repeated 500–800 times only in a genome, the sensitivity of this PCR is lower than that of the satellite DNA primers; the test is generally positive when the parasitaemia is above 50–100 trypanosomes/ml of blood. 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 (5' → 3')	References
Trypanozoon (*T. brucei brucei, T. b. gambiense, T. b. rhodesiense, T. evansi & T. equiperdum*)	TBR1: CGA-ATG-AAT-ATT-AAA-CAA-TGC-GCA-G	Masiga *et al*, 1992; Moser *et al.,* 1989
	TBR2: AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC	
T. congolense type savannah	TCS1: CGA-GCG-AGA-ACG-GGC-AC	Masiga *et al.*, 1992
	TCS2: GGG-ACA-AAC-AAA-TCC-CGC	
T. congolense type forest	TCF1: GGA-CAC-GCC-AGA-AGG-TAC-TT	Masiga *et al.*, 1992
	TCF2: GTT-CTC-GCA-CCA-AAT-CCA-AC	
T. congolense type "Kilifi" (or Kenya coast)	TCK1: GTG-CCC-AAA-TTT-GAA-GTG-AT	Masiga *et al.*, 1992
	TCK2: ACT-CAA-AAT-CGT-GCA-CCT-CG	
T. simiae	TSM1: CCG-GTC-AAA-AAC-GCA-TT	Masiga *et al.*, 1992
	TSM2: AGT-CGC-CCG-GAG-TCG-AT	
T. vivax	TVW 1: CTG-AGT-GCT-CCA-TGT-GCC-AC	Masiga *et al.*, 1992
	TVW 2: CCA-CCA-GAA-CAC-CAA-CCT-GA	
T. cruzi	TCZ1: CGA-GCT-CTT-GCC-CAC-ACG-GGT-GCT	Moser *et al.*, 1989
	TCZ2: CCT-CCA-AGC-AGC-GGA-TAG-TTC-AGG	
Specificity	**Other commonly used Primer sequences (5' → 3')**	**References**
*T. evansi**	TEPAN1: AGT-CAC-ATG-CAT-TGG-TGG-CA	Panyim *et al.*, 1993.
	TEPAN2: GAG-AAG-GCG-TTA-CCC-AAC-A	
*T. evansi**	ESAG6/7F: ACA-TTC-CAG-CAG-GAG-TTG-GAG	Holland *et al.*, 2001.
	ESAG6/7R: CAC-GTG-AAT-CCT-CAA-TTT-TGT	
T. evansi (type A)**	RoTat1.2F: GCG-GGG-TGT-TTA-AAG-CAA-TA	Claes *et al*., 2004
	RoTat1.2R: ATT-AGT-GCT-GCG-TGT-GTT-CG	
*T. evansi (*type B)	EVAB1: CAC-AGT-CCG-AGA-GAT-AGA-G	Njiru *et al.*, 2006
	EVAB2: CTG-TAC-TCT-ACA-TCT-ACC-TC	

Specificity	Other commonly used Primer sequences (5' → 3')	References
T. brucei gambiense	Tgs-GP F: GCT-GCT-GTG-TTC-GGA-GAG-C	Radwanska *et al.*, 2002a
	TgsGP R: GCC-ATC-GTG-CTT-GCC-GCT-C	
T. brucei rhodesiense	Tbr F: ATA-GTG-ACA-AGA-TGC-GTA-CTC-AAC-GC	Radwanska *et al.*, 2002b
	Tbr R: AAT-GTG-TTC-GAG-TAC-TTC-GGT-CAC-GCT	
Pan-tryp.: *T. vivax, Trypanozoon, T.congolense* savannah forest, Kilifi, *T. lewisi,* etc.	TRYP1S: CGT-CCC-TGC-CAT-TTG-TAC-ACA-C	Desquesnes *et al.*, 2002
	TRYP1R: GGA-AGC-CAA-GTC-ATC-CAT-CG	
Pan-tryp.: *T. vivax, Trypanozoon, T. congolense* savannah forest, Kilifi, *etc.*	ITS1 CF: CCG-GAA-GTT-CAC-CGA-TAT-TG	Njiru *et al.*, 2005
	ITS1 BR: TTG-CTG-CGT-TCT-TCA-ACG-AA	

*May also amplify other Trypanozoon (Holland *et al.,* 2001)
**May not amplify all *T. evansi* Type A (Njiru *et al.,* 2006), but may also amplify some *T. equiperdum* strains, while other primers such as Te664 may amplify some but not all strains of *T. evansi T. equiperdum* and *T. brucei* (Claes *et al.,* 2004).

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 WOAH Reference Laboratory for animal trypanosomes of African origin (CIRAD, Montpellier, France) as well as from the WOAH Reference Laboratories for surra[3].

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 2019, 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 Rodent inoculation for antigen preparation

The laboratory animals are injected intraperitoneally with 0.1–0.5 ml (depending on the size of the rodent) of freshly collected infected 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 blood film method. If an infection occurs, it generally shows after 3–10 days, however the rodents must be followed for at least 1 month.

3 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.2. Indirect fluorescent antibody test (IFAT)

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.

2.2.1. 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.

2.3. 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).

2.3.1. Soluble antigens from whole cell lysate

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes produced in laboratory rats. Trypanosomes are separated by DEAE anion-exchange chromatography from whole blood of infected rats (Lanham & Godfrey 1970), washed by centrifugation and suspended in 1% PSG (phosphate saline glucose). The parasite suspension is treated with protease inhibitor cocktail. Antigens are prepared as a soluble fraction after lysis using five to seven freeze–thaw cycles and ultrasonicated three times for 2 minutes on ice to ensure complete disintegration of the organisms, and centrifuged at 4°C and 10,000 ***g*** for 10 minutes. The supernatant that contains the soluble antigens is collected and the protein concentration estimated by UV readings at 260 and 280 nm or by colorimetry, and 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 and performing method is expected in the near future with *in-vitro* produced blood-form trypanosomes.

2.3.2. Test procedure

i) *Sensitisation:* flat bottom, nonspecific binding 96-well polystyrene micro-plates[4] are used, with antigens diluted in a 0.01 M carbonate/bicarbonate coating buffer, pH 9.6.

4 The polystyrene plate of choice must not adsorb the proteins in a peculiar orientation so that all antigenic epitopes be available to bind the antibodies; hydrophobic microplates are preferred to hydrophilic plates where protein binding is structurally oriented, thus hiding some of the antigenic epitopes.

Trypanosome soluble antigen is diluted to a final concentration of 5 µg/ml, dispensed in 100 µl per well, incubated overnight at 4°C or for 2 hours at 37°C on a shaker at 300 rpm.

ii) *Blocking:* plates are emptied by inversion and 150 µl of blocking buffer (BB = 0.01 M PBS 5% skim milk, 0.1% Tween20) is added per well; plates are placed on a shaker-incubator at 300 rpm for 30 minutes at 37°C.

iii) *Serum predilution:* a predilution at 1/10 of serum or plasma is performed in a round-bottom polypropylene plate (U), using BB.

iv) *Transfer:* the plates are emptied by inversion, rinsed once with PBS, and 90 µl BB is added. 10 µl serum diluted 1/10 is transferred rapidly using an eight-channel pipette into two horizontally neighbouring wells (final dilution 1/100); the plates are placed on a shaker at 300 rpm for 30 minutes at 37°C.

v) *Washing:* the plates are emptied by inversion and the wells filled with washing buffer (WB = PBS with 0.1% Tween20); the plates are emptied and washed four additional times, and emptied and drained by banging the plates vigorously on absorbent paper towels.

vi) *Conjugate:* 100 µl of horseradish peroxidase-conjugate diluted in BB (dilutions adapted to the conjugate) is added to each well; the plates are placed on a shaker-incubator at 300 rpm for 30 minutes at 37°C.

vii) *Washing:* same washing and draining procedure as previously in step v).

viii) *Substrate-chromogen:* 100 µl of substrate-chromogen is added to each well (TMB, or a mixture of citric acid 25 ml + 125 ml ABTS + 100 µl H_2O_2). Plates are kept in the dark for 30 minutes at room temperature.

ix) *Reading:* the optical densities (absorbance) of the wells are measured, at the appropriate wavelength for the chromogen (TMB: 620 nm; ABTS: 405 nm).

Each ELISA-microplate is run with duplicate strong, medium and weak positive and negative reference sera (total of 6 control sera in duplicate), which are required to comply with pre-set values for quality assurance. The absorbance of each sample tested in duplicate is expressed as a relative percentage of positivity (RPP) of 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 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.

ELISA performs better than IFAT in terms of sensitivity and specificity (Luckins, 1977), and can achieve a high degree of automation and standardisation; it is thus the recommended test. ELISA antibody-detection tests have high sensitivity (on average above 90%) and genus specificity (on average above 95%), but their subgenus and species specificity is generally low. Inter-species cross-reactions amongst *T. vivax*, *T. brucei* and *T. congolense* (and even *T. cruzi*) have been described and measured, but they do not allow a true species-specific diagnosis and may be improved by using a standardised set of the three species-specific tests (Desquesnes *et al*., 2011). However, animals infected with Megatrypanum, such as *T. theileri*, do not cross react in IFATs and ELISAs for *T. vivax*, *T. brucei*, *T. evansi* and *T. congolense* (Desquesnes 2004; Luckins, 1977).

In areas where several species of trypanosomes are present (including *T. cruzi*, *T. evansi* and *T. equiperdum*), mixed infections may not always be detected because cross-reactions among pathogenic trypanosomes may occur with any serological test employed, and agent detection tests may provide false negative results (due to low parasitaemia). Thus, once an animal is seropositive, it is not possible to ascertain whether it harbours one or several trypanosome species.

Serological tests 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. Positive seroconversion in ELISA generally occurs within 1 to 6 weeks after infection, thus, a negative results in a field sample must be interpreted in the light of other information or test results.

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 animal trypanosomosis, as well as for post-treatment follow-up or elimination campaigns.

Specific antigens for ELISA and reference samples (positive and negative reference serum samples) can be obtained from the WOAH Reference Laboratory for animal trypanosomes of African origin; lyophilised reagents are now available, that considerably ease the shipment from the Reference laboratory to regional laboratories.

3. Test applications

Clinical signs including fever, anaemia, oedema, loss of weight, abortion and nervous signs can only lead to a suspicion of animal infection with salivarian trypanosomes; to complete the clinical diagnosis, recommended tests are (alternative tests into brackets): GSBS (/TGSBF), HCT (/BCT), PCR (for primers, see below) and ELISA (/IFAT) (for species antigens used in serology, *T. vivax, T. brucei* and *T. congolense*, see below). Rather than a single test, a combination of several recommended tests must be implemented, according to the context, as indicated below, and in line with the *Terrestrial Code* recommendations.

3.1. Characteristics and performances of recommended tests for diagnosis of animal trypanosomosis

Parasitaemia being highly variable, the sensitivity of the agent detection tests is highly variable.

i) GSBS: low sensitivity (10^5–10^6 trypanosomes/ml of blood); subgenus and sometimes species specific; the species can also be deduced from epizootiological information. When positive, the GSBS brings diagnostic certainty. Parasitaemia being highly variable, the sensitivity of the test is highly variable and generally considered as low.

ii) HCT: medium sensitivity (10^2–10^3 trypanosomes/ml of blood); genus and sometimes subgenus specific; examinations must be carried out within a short time after blood sampling (preferably 1–2 hours). When positive, HCT must be complemented with GSBS and/or PCR (+/- ELISA), to confirm the diagnosis.

iii) PCR: Sample preparation for PCR must be done on blood, or preferably on buffy coat, after blood centrifugation, using a commercial DNA purification kit or a Chelex resin preparation method (Penchenier *et al.*, 1996). Recommended primers (Gold standard) are those targeting the satellite DNA (Masiga *et al.,* 1992); primers predominantly used are: TVW (*T. vivax*), TBR (Trypanozoon) and TCS (*T. congolense* type savannah); PCRs using these primers are highly sensitive and species-, sub-genus- or type-specific, respectively. When positive, PCR alone is not a diagnostic certainty; it must be complemented by other tests or information (see below), because it may give false positive results due to sample contaminations. When negative, PCR alone cannot ascertain the absence of infection due to false negative results obtained from animals with low parasitaemia.

Antibody-detection tests (ELISA and IFAT) become positive 1–6 weeks after infection, thus with an incubation period of 2 weeks on average, and the persistence of antibodies after parasite elimination can last from 1 to 13 months, and is 3 months on average.

iv) ELISA: Three ELISAs using soluble antigens from whole cell lysates of animal trypanosomes are recommended: ELISA *T. vivax,* ELISA *T. brucei brucei* and ELISA *T. congolense* type savannah; depending on the context (America/Africa), 1 to 3 tests can be recommended; they all cross react. ELISAs exhibit high sensitivity (> 90%) and genus specificity (>95%), but subgenus specificity is not consistent, and none of them is species-specific. A positive sample reveals an immune response in the host to the parasite, but it does not indicate an active infection due to the persistence of antibodies after parasite elimination (as stated above), thus: (i) it must be complemented by other

tests or information (see below) if active infection is to be confirmed, and (ii) once an animal is seropositive to one or several ELISAs (or IFATs), it is not possible to determine whether it is or has been harbouring one or several trypanosome species. Even when a positive serology is associated with a positive species-specific agent detection test, other *Trypanosoma* species may be suspected in a mixed infection.

Providing it is associated with negative results to sensitive agent detection tests, when negative, ELISA is reliable, due to its high genus specificity. Both tests must be repeated at 30-day intervals due to the delay in seroconversion; however, longer delays may be observed occasionally.

Alternatively, IFATs can be used in the same conditions as ELISAs.

3.2. Association of recommended tests for the diagnosis of animal trypanosomosis

3.2.1. Recommended method for sensitive agent detection:

A combination of GSBS, HCT and PCR is recommended for agent detection.

i) GSBS: when positive, it brings diagnostic certainty, but when negative, it must be complemented with more sensitive test: HCT and/or PCR.

ii) HCT: when positive, HCT brings genus- or subgenus-specific diagnostic certainty, but it must be complemented with GSBS and PCR for agent identification. When negative, it should be complemented with the more sensitive PCR.

iii) PCR: routine diagnosis must use the satellite DNA primers that detect the most prevalent species responsible for Nagana: TVW, TBR and TCS in Africa, and TVW and TBR in America. When positive, PCR must be complemented with a positive GSBS, HCT, ELISA, or clinical examination to ascertain the infection.

In Africa, if PCR with the above primers is negative, complementary primers must be added: TCF, TCK and TSIM.

If TBR primers give positive response, and *T. evansi* might be suspected, complementary primers must be used to ascertain the species: RoTat1.2 & EVAB; however, their sensitivity is lower, which may lead to inconclusive outcome when they provide negative results.

In Africa, if TBR primers give positive response, and human *Trypanosoma* sspp. are suspected, complementary primers must be used: Tgs-GP and Tbr F/R however, their sensitivity is lower, which may lead to inconclusive situation when they provide negative results.

If PCR with the above primers are negative in a context of trypanosomosis suspicion (clinical signs, ELISA or HCT positive), other complementary primers must be used for parasite identification: TRYP1 or ITS1-CF/BR, and, in America and Latin America, TCZ (for *T. cruzi* DNA detection).

Due to possible low or even no parasitaemia, agent detection tests can confirm an infection when they are positive, but they cannot confirm the absence of infection when they are negative; they need to be associated with negative antibody detection test for this purpose.

3.2.2. Recommended method for antibody detection

ELISAs using soluble antigens extracted from whole trypanosome lysate are recommended tests, to be applied according to the epizootiological context. In Africa the recommended method to get high sensitivity and cover the most prevalent *Trypanosoma* spp. is to use all three: ELISA *T. vivax*, ELISA *T. brucei brucei* and ELISA *T. congolense* type savannah. In Latin America, the recommended method is to use only ELISA *T. vivax*, preferably in association with ELISA *T. evansi* (or ELISA *T. brucei brucei*) when Trypanozoon parasites are also investigated.

A seropositive sample is “suspect for active infection” but it is not a confirmed diagnosis; it must be complemented with positive results to GSBS or PCR, or associated with clinical signs of trypanosomosis or epidemiologically linked to a confirmed trypanosomosis case. In Africa, infections with *T. evansi* and *T. equiperdum* may interfere in the serodiagnosis of nagana. In

America, infections with *T. evansi*, *T. equiperdum* and *T. cruzi*, as well as *Leishmania* spp., may interfere in the serodiagnosis of *T. vivax*. Appropriate primers must be used in PCR to ascertain the infection and identify the parasite.

Finally, in areas where several species of trypanosomes are present (including T. *cruzi, T. evansi* and *T. equiperdum*), since cross-reactions may occur with any serological test employed, the exact status of a seropositive animal regarding active infection with one or several *Trypanosoma* species cannot be established.

Alternatively, IFATs can be used, in the same conditions described above for ELISAs.

C. REQUIREMENTS FOR VACCINES

No vaccines are in use at the present time.

REFERENCES

BALDACCHINO F., DESQUESNES M., MIHOK S., FOIL L.D., DUVALLET G. & JITTAPALAPONG S. (2014). Tabanids: neglected subjects of research, but important vectors of disease agents! *Infect. Genet. Evol.,* **28**, 596–615.

BENGALY Z., SIDIBE I., GANABA R., DESQUESNES M., BOLY H. & SAWADOGO L. (2002). Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. *Vet. Parasitol.,* **108**, 1–19.

BRYAN L.K., HAMER S.A., SHAW S., CURTIS-ROBLES R., AUCKLAND L.D., HODO C.L., CHAFFIN K. & RECH R.R. (2016). Chagas disease in a Texan horse with neurologic deficits. *Vet. Parasitol.,* **216**, 13–17.

CLAES F., RADWANSKA M., URAKAWA T., MAJIWA P.A., GODDEERIS B. & BUSCHER P. (2004). Variable Surface Glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. *Kinetoplastid Biol. Dis.,* **3**, 3.

DESQUESNES M. (1997). Standardisation internationale et régionale des épreuves immuno-enzymatiques: méthode, intérêts et limites. *Rev. Sci. Tech. Off. Int. Epiz.,* **16**, 809–823.

DESQUESNES M. (2004). Livestock Trypanosomoses and their Vectors in Latin America. OIE and CIRAD (Centre de coopération internationale en recherche agronomique pour le développement). World Organisation for Animal Health (WOAH), Paris, France, 192 pp.

DESQUESNES M. & DAVILA A.M.R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes; a review and perspectives. *Vet. Parasitol.,* **109**, 213–231.

DESQUESNES M., BENGALY Z., MILLOGO L., MEME Y. & SAKANDE H. (2011). The analysis of the cross-reactions occurring in antibody-ELISA for the detection of trypanosomes can improve identification of the parasite species involved. *Ann. Trop. Med. Parasitol.,* **95**, 141–155.

DESQUESNES M., MCLAUGHLIN G., ZOUNGRANA A. & DAVILA A.M.R. (2001). Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *Int. J. Parasitol*., **31**, 610–614.

DESQUESNES M., RAVEL S. & CUNY, G. (2002). PCR identification of *Trypanosoma lewisi,* a common parasite of laboratory rats. *Kinetoplastid Biol. Dis.,* **1**, 2.

DESQUESNES M., RAVEL S., DESCHAMPS J.-Y., POLACK B. & ROUX F. (2012). Atypical hyperpachymorph *Trypanosoma* (Nannomonas) *congolense* forest-type in a dog returning from Senegal. *Parasite,* **19**, 239–247.

GREINER M., KUMAR S. & KYESWA C. (1997). Evaluation and comparison of antibody ELISAs for serodiagnosis of bovine trypanosomosis. *Vet. Parasitol.,* **73**, 197–205.

HAMILL L., KAARE M., WELBURN S. & PICOZZI K. (2013). Domestic pigs as potential reservoirs of human and animal trypanosomiasis in Northern Tanzania. *Parasit. Vectors,* **6**, 322.

HOLLAND W.G., CLAES F., MY L.N., THANH N.G., TAM P.T., VERLOO D., BUSCHER P., GODDEERIS B. & VERCRUYSSE J. (2001). A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. *Vet. Parasitol.,* **97**, 23–33.

HOLMES P. (2015). On the road to elimination of Rhodesiense human African trypanosomiasis: first WHO meeting of stakeholders. *PLoS Negl. Trop. Dis.,* **9**, e0003571.

HOPKINS J.S., CHITAMBO H., MACHILA N., LUCKINS A.G., RAE P.F., VAN DEN BOSSCHE P. & EISLER M.C. (1998). Adaptation and validation of the antibody trapping ELISA using dried blood spots on filter paper, for epidemiological surveys of tsetse transmitted trypanosomosis in cattle. *Prev. Vet. Med.,* **37**, 91–99.

KATAKURA K., LUBINGA C., CHITAMBO H. & TRADA Y. (1997). Detection of *Trypanosoma congolense* and *T. brucei* subspecies in cattle in Zambia by polymerase chain reaction from blood collected on a filter paper. *Parasitol. Res.,* **83**, 241–245.

KATENDE J.M., MUSOKE A.J., NANTULYA V.M. & GODDEERIS B.M. (1987). A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanosomiasis. *Trop. Med. Parasitol.,* **38**, 41–44.

KARSHIMA S.N., AJOGI I. & MOHAMMED G. (2016). Eco-epidemiology of porcine trypanosomosis in Karim Lamido, Nigeria: prevalence, seasonal distribution, tsetse density and infection rates. *Parasit. Vectors*, **9**, 448.

KUBOKI N., INOUE N., SAKURAI T., DI CELLO F., GRAB D.J., SUZUKI H., SUGIMOTO C. & IGARASHI I. (2003). Loop-mediated isothermal amplification for detection of African trypanosomes. *J. Clin. Microbiol.,* **41**, 5517–5524.

LANHAM S.M. & GODFREY D.G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-Cellulose. *Exp. Parasitol.*, **28**, 521–534.

LUCKINS A.G. (1977). Detection of antibodies in trypanosome infected cattle by means of a microplate enzyme–linked immunosorbent assay. *Trop. Anim. Health Prod.,* **9**, 53–62.

LUMSDEN W.H.R., KIMBER C.D., EVANS D.A. & DOIG S.J. (1979). *Trypanosoma brucei*: miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. *Trans. R. Soc. Trop. Med. Hyg.,* **73**, 312–317.

MASIGA D.K., SMYTH A.J., HAYES P., BROMIDGE T.J. & GIBSON W.C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *Int. J. Parasitol*., **22**, 909–918.

MOSER D.R., KIRCHHOF L.V. & DONELSON J.E. (1989). Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction. *J. Clin. Microbiol.,* **27**, 1477–1482.

NJIRU Z. K., CONSTANTINE C.C., GUYA S., CROWTHER J., KIRAGU J.M., THOMPSON R.C. & DAVILA A.M. (2005). The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitol. Res.,* **95**, 186–192.

NJIRU Z.K., CONSTANTINE C.C., MASIGA D.K., REID S.A., THOMPSON R.C. & GIBSON W.C. (2006). Characterization of *Trypanosoma evansi* type B. *Infect. Genet. Evol.,* **6**, 292–300.

PANYIM S., VISESHAKUL N., LUXANANIL P., WUYTS N. & CHOKESAJJAWATEE N. (1993). A PCR method for highly sensitive detection of *Trypanosoma evansi* in blood samples. Proceedings of EEC contractants workshops, "Resistance or tolerance of animals to diseases and veterinary epidemiology and diagnostic methods", Rethymno, Greece, 2–6 November 1992; ed CIRAD-EMVT, 138–143.

PENCHENIER L., DUMAS V., GREBAUT P., REIFENBERG J.-M. & CUNY G.(1996). Improvement of blood and fly gut processing for PCR diagnosis of trypanosomosis. *Parasite,* **4**, 387–389.

RADWANSKA M., CLAES F., MAGEZ S., MAGNUS E., PEREZ-MORGA D., PAYS E. & BUSCHER P. (2002a). Novel primer sequences for polymerase chain reaction-based detection of *Trypanosoma brucei gambiense*. *Am. J. Trop. Med. Hyg.,* **67**, 289–295.

RADWANSKA M., CHAMEKH M., VANHAMME L., CLAES F., MAGEZ S., MAGNUS E., DE BAETSELIER P., BUSCHER P. & PAYS E. (2002b). The serum resistance-associated gene as a diagnostic tool for the detection of *Trypanosoma brucei rhodesiense*. *Am. J. Trop. Med. Hyg*., **67**, 684–690.

REBESKI D.E., WINGER E.M., OKORO H., KOWALIK S., BURGER H.J., WALTERS D.E., ROBINSON M.M., DWINGER R.H. & CROWTHER J.R. (2000). Detection of *Trypanosoma congolense* antibodies with indirect ELISAs using antigen-precoated microtitre plates.*Vet. Parasitol*., **89**, 187–198.

TRUC P., AERTS D., MCNAMARA J.J., CLAES Y., ALLINGHAM R., LE RAY D. & GODFREY D.G. (1992). Direct isolation *in vitro* of *Trypanosoma brucei* from man and other animals, and its potential value for the diagnosis of Gambian trypanosomiasis. *Trans. R. Soc. Trop. Med. Hyg.*, **86**, 627–629.

VAN DEN BOSSCHE P., CHIGOMA D. & SHUMBA W. (2000). The decline of anti-trypanosomal antibody levels in cattle after treatment with trypanocidal drugs and in the absence of tsetse challenge. *Acta Trop*., **77**, 263–270.

WRIGHT P.F., NILSSON E., VAN ROOIJ E.M.A., LELENTA M. & JEGGO M.H. (1993). Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Rev. sci. tech. Off. int. Epiz.,* **12**, 435–450.

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NB: There is a WOAH Reference Laboratory for Nagana (infections with salivarian trypanosomoses) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Nagana.

NB: FIRST ADOPTED IN 1991 AS TRYPANOSOMIASIS. MOST RECENT UPDATES ADOPTED IN 2021