OIE Reference Laboratory for Animal Trypanosomoses of African origin

COMPENDIUM OF STANDARD DIAGNOSTIC PROTOCOLS FOR ANIMAL TRYPANOSOMOSES OF AFRICAN ORIGIN

Montpellier, October 2017.

Marc Desquesnes,

with contributions by Zakaria Bengaly, David Berthier, Géraldine Bossard, Bila Cene, Hamidou Ilboudo, Vincent Jamonneau, Jacques Kaboré, Youl Mémé, Léopold Millogo, Sophie Ravel, Hassane Sakandé, Lassina Sanogo, Sophie Thévenon, Wilfrid Yoni and Adrien Zoungrana (and Nick Barton for translation).

Foreword

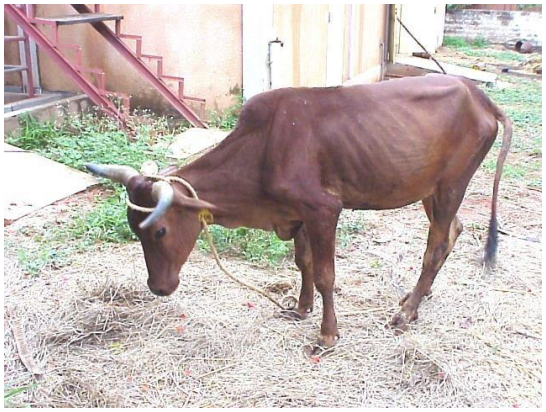
Animal trypanosomoses of African origin are a group of diseases caused by several species of protozoan parasites of the genus *Trypanosoma*, mainly transmitted cyclically by the genus *Glossina* (tsetse flies), but also transmitted mechanically by several biting insects (tabanids, stomoxes, etc.). The diseases can affect various species of mammals but, from an economic point of view, are particularly important in livestock, including cattle, pigs, horses, sheep and goats. They are caused mainly by *Trypanosoma congolense*, *T. vivax* and, to a lesser extent, *T. brucei brucei*, but also by *T. evansi*. The ethiological agent of dourina, *T. equiperdum*, is a peculiar case of trypanosomose, exclusively transmitted venerealy in equids; in the present compendium, it will be considered only for differential diagnosis.

Animal trypanosomoses of African origin are classically acute or chronic diseases that cause intermittent fever and are accompanied by anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms.

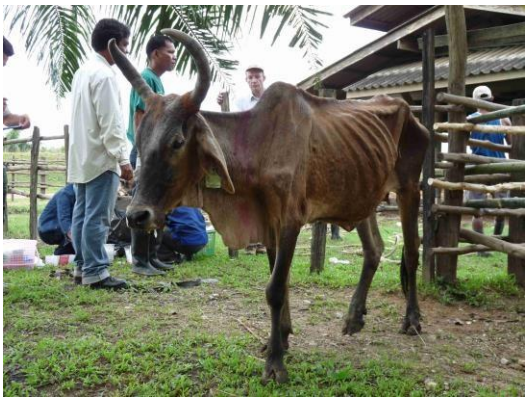
A prerequisite for trypanosomosis control, as with any infectious disease, is the ability to detect the agent and the immune response in the host. Furthermore, diagnostic tests in the vector and the host provide the basis for all epidemiological investigations and monitoring of control programmes.

Within the framework of the OIE Reference Laboratory for Animal Trypanosomoses of African Origin Twinning Project (2012-2017), CIRAD, in its capacity as the 'Parent' Reference Laboratory, with its facilities in Montpellier and Bangkok, and CIRDES,

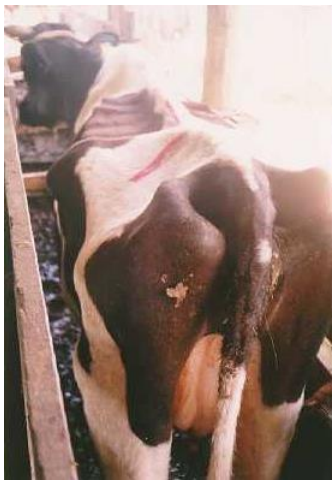
Bobo-Dioulasso, Burkina Faso, the Candidate Laboratory, have been working together for the past 6 years to standardise, optimise and harmonise diagnostic methods and banks of samples and reference strains, with the aim of improving the methods and enabling CIRDES to become an OIE Reference Laboratory for Animal Trypanosomoses of African origin.



Bovine infected with *T. congolense,* West Africa



Bovine infected with *T. evansi* South-East Asia



Cattle infected with *T. vivax*, South America
(M. Desquesnes)

Acknowledgements

To all participants contributing to the implementation of the CIRAD-CIRDES twinning in the framework of the « Reference laboratory for animal trypanosomoses of African origin », from the training of the contributors to the spreading of the present compendium, including all stages of its production, and, with the hope that it will be useful to general knowledge and practices.

Contents

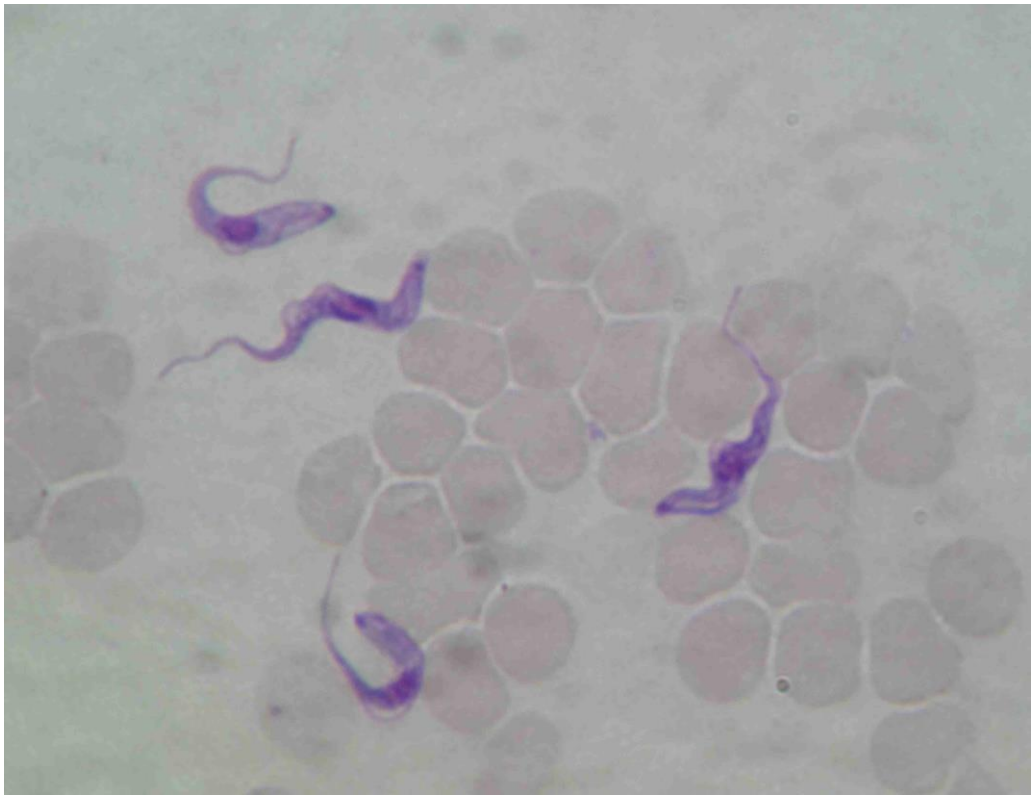
Introduction

This Compendium has been compiled to serve as a reference tool for the diagnostic methods for trypanosomoses used at the OIE Reference Laboratory for Animal Trypanosomoses of African Origin, CIRAD, Montpellier, France, and its partner laboratory, CIRDES, Bobo-Dioulasso, Burkina Faso.

The Compendium describes the standard parasitological, serological and molecular biology methods recommended by the OIE for the diagnosis of African trypanosomoses, as published in Chapter 2.4.17 'TRYPANOSOMOSIS (tsetse-transmitted)' of the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (hereafter, the *Terrestrial Manual*). It describes the main characteristics of these methods, enabling users to correctly interpret the results of the tests. It describes all the protocols in detail so that other regional or national laboratories can use these methods to carry out tests independently of OIE Reference Laboratories. However, it is recommended that permanent links are maintained with these Reference Laboratories for quality control purposes. The Compendium includes a description of the quality control methods needed to ensure the continued validity of the tests carried out.

The chapter on tsetse-transmitted trypanosomosis in the OIE *Terrestrial Manual* is included in Part 2 OIE Listed Diseases and Other Diseases of Importance, section 2.4. Bovinae (which includes cattle, antelopes, sheep, goats, etc). Nevertheless, trypanosomoses affect species of other families and may also need to be diagnosed, especially in the case of equidae and carnivores. Diagnostic considerations for these species will therefore also be taken into account. For the purpose of detecting trypanosome infection, no assumptions are made regarding the trypanosome species involved, and all species must therefore be considered regardless of whether they are tsetse-transmitted, which implies, at least for diagnosis purpose, to consider as well *T. cruzi*, *T. evansi* and *T. equiperdum*.

The diagnostic methods described below can be used either to complement clinical findings or as epidemiological survey tools. In addition to the purely technical sections, dealing with the collection and labelling of samples, parasitological diagnosis, diagnosis using DNA detection and serological diagnosis, the Compendium provides recommendations on using trypanosomosis diagnostic tests and means of ensuring the quality of diagnosis, and covers topics ranging from maintaining laboratory records, maintaining a bank of samples and reference strains, quality control, and recommendations on inter-laboratory standardisation, and packaging and transport of biological samples. The Compendium ends with a bibliography and technical Annexes describing each technique in detail.



Trypanosoma vivax on Giemsa stained blood smear (M. Desquesnes)

1. Collection, labelling and storage of samples

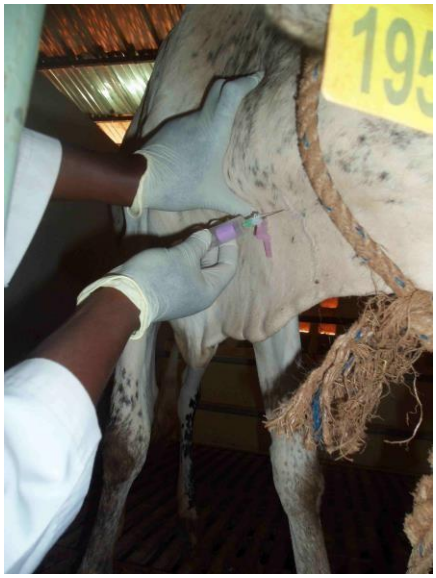
In mammals, laboratory diagnosis of trypanosomosis is usually performed on blood, but other biological samples may be tested, such as cerebrospinal fluid (CSF), lymph, joint fluid, and ocular or genital serous or mucous discharge.

In bovids, blood samples are normally taken, using a sterile needle, from the jugular, caudal or ear vein. The ear vein allows a more sensitive direct examination than the others and in an individual animal scarification of the vein can be used; nevertheless, when sampling a number of animals and/or for practical reasons (e.g. to obtain a sufficient volume for other examinations), jugular or tail venipuncture is generally preferred in cattle, and jugular venipuncture in sheep and goats. In dogs, blood is collected from the cephalic vein (foreleg) or saphenous vein (hind leg), whereas in horses the upper part of the jugular vein is preferred.

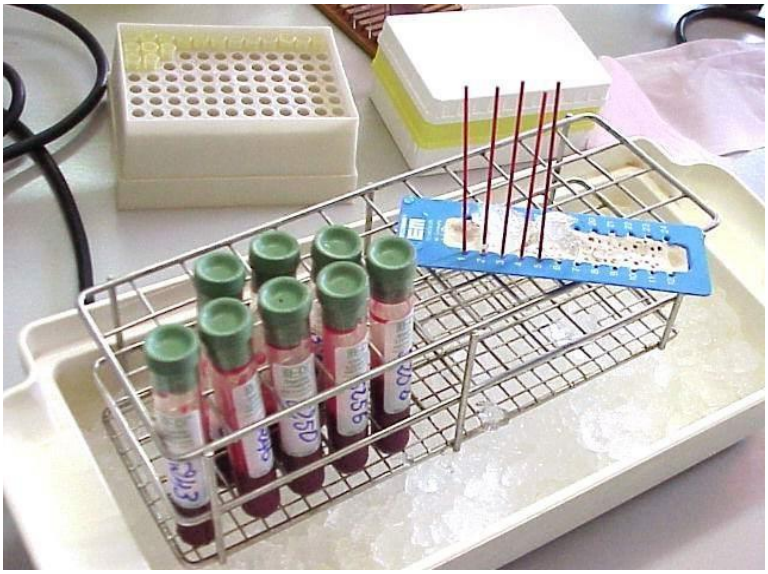
Biological samples not requiring anticoagulant (CSF, lymph, etc.) are placed in dry tubes (conical microtubes) and kept chilled (0-4°C).

The usual precautions should be taken when collecting samples, namely use of boots, overall and gloves. The blood is either collected directly in dry vacutainer tubes (without anticoagulant) or vacutainer tubes containing anticoagulant (heparin, EDTA, etc., depending on the protocol), or collected with a syringe and distributed to the tubes. Dry tubes should be kept cool for 6 to 10 hours (15-20°C) and should not be refrigerated or kept in direct contact with ice to avoid clotting – always a possibility in the event of rapid cooling –, and can also lead to a degree of lysing of red blood cells and alter the quality of the sera to be collected. The dry tubes should therefore be kept in the shade, in a cool place during the day, and then be placed in a refrigerator 6 to 10 hours later, to allow the retraction of clots to continue. Serum can be collected by pipette 24 to 48 h later, preceded by centrifugation if necessary.

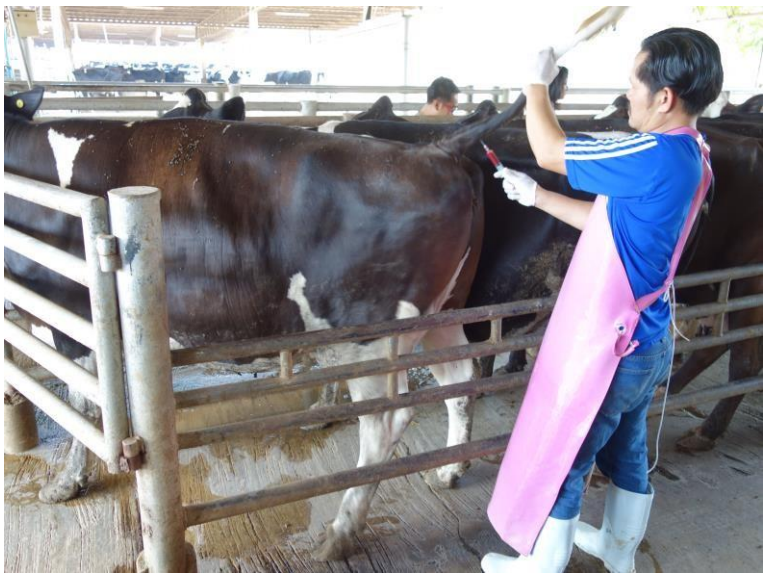
Tubes with anticoagulant should immediately be chilled (in a cooler at 0-4°C) to await testing or further preparations.

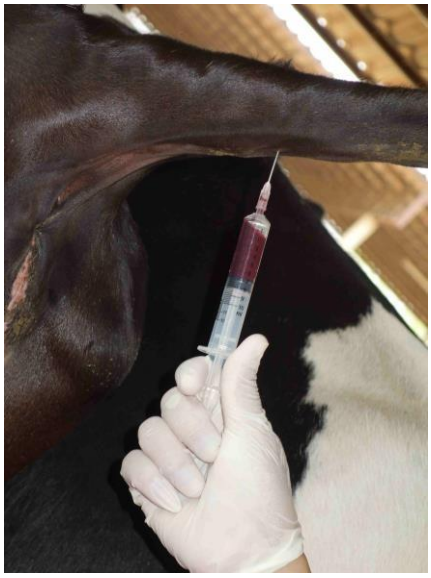


Collecting blood from the jugular vein using a vaccum tube



Preparation of capillary tubes





Using a syringe to collect a blood sample from the caudal vein, in dairy cows
(M. Desquesnes)

The labelling of tubes during sample collection must be harmonised to ensure that labels are universally understood. Ideally, the codes used should be the same as those used for biological samples stored in banks, but differences in habits and in languages from one country to another means that, in practice, there is often considerable freedom in the choice of labelling methods in the field and they are not always strictly applied. Every effort should therefore be made to copy, at least as closely as possible, the codes defined for sample banks, failing which the changeover from field labelling to laboratory labelling can lead to transcription errors.

Tubes are usually labelled in the following manner:

Two letters are used to identify the animal species sampled and, if possible, the type (e.g. BC for beef cattle or BV for *bovin à viande*, depending on the language of the country); it is advisable to adopt the English form so that all partners will be using the same codes. The universal codes for the various species are given herefater.

For the follow-up of tested animals: 2 letters to identify the animal species, followed by the animal's reference number and, after a dash (or on the following line), the date dd/mm/yy; example for beef cow No. 224 sampled on 8 February 2012: BC224 - 08/02/12

or BC224
08/02/12

For samples taken from herds in the field, indicate the host species followed by the identification number of the animal or the serial number of the sample; then the name of the locality abbreviated to the first three letters (or, if necessary, another code but preferably no more than 5 letters); for example, the 15th dairy cow (dairy cattle: DC) sampled at Kou on 28 February 2013: DC14 - KOU - 28/02/13.

For samples other than blood, the nature of the sample must be specified separately from the rest of the data (articular liquid: AL; cerebrospinal fluid: CSF, etc.).

Labelling of tubes at the laboratory must be harmonised to ensure a common understanding. It is generally done as follows for the most common types of samples, namely sera, plasma, blood and buffy coat.

These 4 types of samples must be kept in different boxes to avoid confusion between serum and plasma or blood and buffy coat. The sample type is also indicated on the cryotube box. Furthermore, different cryotubes are used for whole blood, which is kept in cryotubes of 1.5 ml (or larger), and for buffy coats, which are kept in smaller cryotubes, for example 0.5 ml. However, as different types of sample may have to be handled at the same time, a letter is placed on the cap of the cryotubes: "S" for serum, "P" for plasma, "B" for blood and "C" for buffy coat.

When a sample enters a bank of samples, it is assigned a serial number preceded by 2 letters to identify the host; this alphanumeric number is its permanent unique identifier. A code often consisting of a single letter can also be used.

Universal codes to be used for the various species

Beef cattle	BC	*Mus musculus* (mouse)	MM (or M)
Dairy cattle	DC	Horse	HH (or H)
Bos taurus	BT	Donkey	DD (or A)
Bos indicus	BI	Mule	DH
Water buffalo (*Bubalus bubalis*)	BB (or B)	Pig (*Sus scrofa*)	SS (or P)
Sheep (ovine)	OV (or S)	Dog	CN (or D)
Goat (caprine)	CP (or G)	Asian elephant	EM (or E)
Other: indicate the initials of the species		Rat	RN

2. Microscopic examination

2.1 Direct examination techniques

The microscopes used should have magnifications of at least X400 or 500 and X1000 and preferably be equipped with a dark-field condenser and phase contrast objectives (X40 or X50) for direct examination of fresh samples. Gloves should be worn to protect from the risk of infection and to avoid getting grease on the slides.

2.1.1 Wet blood films

Place 2-3 microliters of blood on a microscope slide, then cover it with a cover-slip (22 x 22 mm) then apply light pressure with absorbent paper to spread the blood evenly and absorb any excess. Examine the blood microscopically at X400 magnification, without a light condenser, or better still, with dark-field phase contrast or interference contrast, or else by lowering the light condenser to create artificially refraction of the plasma membranes. Approximately 40 to 50 fields are examined. Trypanosomes can be recognised by their movement among the red blood cells. Based on the size, shape (posterior extremity, undulating membrane) and movements and displacement of the trypanosome, a specific diagnostic hypothesis can be made. Final confirmation of the species is done by examining a stained section or by PCR. The following criteria of morphology and motility can be used:

- Very large trypanosome (but variable in size from one specimen to another), posterior extremity pointed and rigid, movements generally regular but sluggish, very low parasitaemia: these findings are compatible with *T. theileri*.

- Large, long and slender trypanosome (sometimes wide in the stumpy form) with large movements and a “light capture” phenomenon in the undulating membrane that accompanies the movements along the body, conspicuous free flagellum, posterior extremity pointed but not rigid: these findings suggest a *Trypanozoon (T. brucei* sspp*, T. evansi, T. equiperdum)*.

- Medium-sized trypanosome with rapid movements and ability to cross the microscope field, “light capture” phenomenon sometimes visible but moderate, conspicuous free flagellum, rounded posterior extremity: these findings suggest *T. vivax*.

- Small, vermiform trypanosome, with sluggish movements, absence of free flagellum: these findings are characteristic of *T. congolense*.

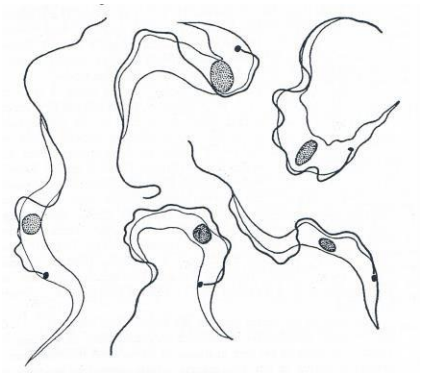
- Lastly, though a parasite of rats, this form may be encountered during cultivation in laboratory animals: Medium-sized trypanosome with extremely rapid movements, with the capacity to cross the microscope field very rapidly (stage 2) or move in regular circles within the microscope field (stage 1): these findings suggest *T. lewisi*.

Species identification in a fresh sample is only possible if one has acquired extensive experience and observed numerous trypanosome species in a wide range of conditions if it is to be reliable; it therefore remains the prerogative of a few specialised technicians.

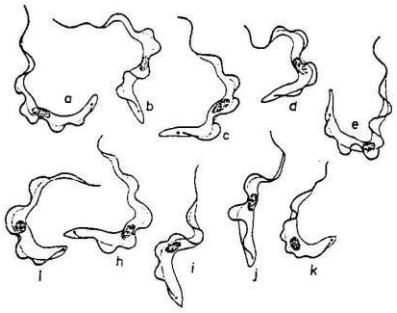
2.1.2 Thick blood films

These are made by placing a drop of blood (6-8 µl) on a microscope slide and spreading it concentrically over an area approximately 2 cm in diameter using the corner of another slide. The thickness of the film should be such that, when dry, one should be able to read printed text through the slide. The film is dried thoroughly by rapidly waving the slide in the air and, without fixation, is dehaemoglobinised by immersion in distilled water for a few seconds and dried before staining. A dry smear should be kept dry and protected from dust, heat, flies and other insects. It is stained with Giemsa solution (0.4% pure commercial solution diluted 1:20 in phosphate buffered saline (PBS), pH 7.2); the slide is immersed for 30 min, rinsed once in deionised water, then rinsed well in tap water. Staining time and stain dilution may vary according to the stain and individual technique. Consequently, it is important to follow the manufacturer's instructions and to vary staining time and stain concentration (1/10-1/20) to obtain the optimal result. The stained smear is then thoroughly dried before being examined at X500 or X1000 total magnification. See also (in French): http://www.sleeping-sickness.ird.fr/diagnostic42.htm

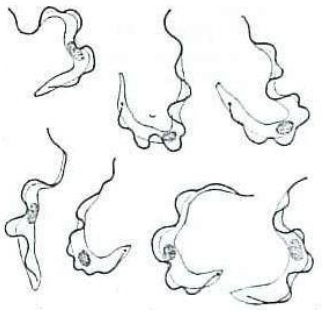
The method is simple and relatively inexpensive, but results are delayed because of the staining process. Trypanosomes are easily recognised by their morphology, but may be damaged during the staining process. This may make it difficult to identify the species. The figures below present the characteristics of the main species of trypanosomes.



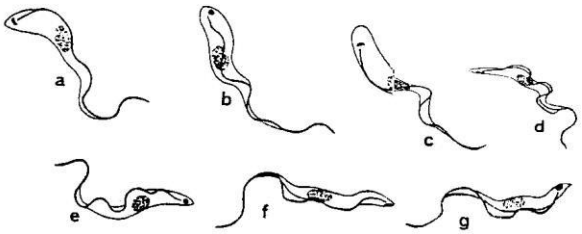
T. theileri



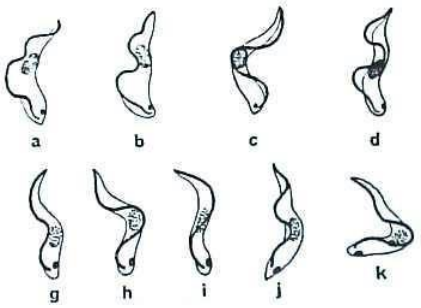
T. evansi



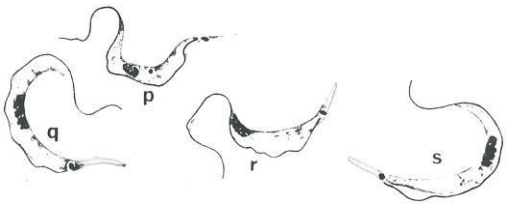
T. brucei

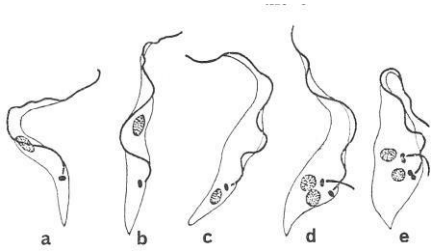


T. vivax



T. congolense





T. lewisi, adult, bloodstream form (left) and during multiplication in rat (right)

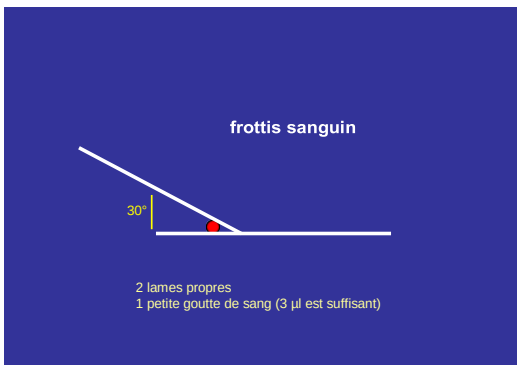
adapted from Hoare, 1972

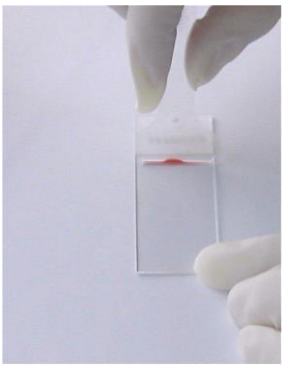
2.1.3 Thin blood smear films

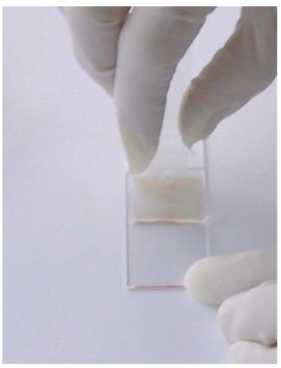
Clean microscope slides are crucial to the success of the following examinations. If greasy or dusty slides are used, the results will be unusable. It is essential to thoroughly degrease the slides before carrying out these examinations.

Thin blood smears are made by placing a small drop of blood (about 3-4 µl), for example from a microhaematocrit capillary tube or the cap of an EDTA tube, on a perfectly clean and degreased (with ethanol) microscope slide; if necessary, the drop of blood should be placed

approximately 20 mm from one end of the slide (allowing for space to apply the thick smear). The blood is spread with the perfectly clean and smooth edge of another slide (preferably with bevelled edges). 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 spread along the whole of the edge of the spreader, which is then pushed to the other end of the slide with a rapid and smooth movement; in this way the blood is spread by capillary traction. If the correct amount of blood is used, the slide should be covered with an even film of blood in the shape of a bullet (the quantity of blood in the middle of the slide being greater than that at the edges, it governs the shape of the smear). 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 (a smear dotted with small circles of about 1 mm in diameter is the sign of a blood meal by biting flies…). Using clean slides is crucial to the success of this examination (thoroughly degrease the slides with ethanol and dry them before making the smear). Clean the spreader slide immediately after use, before it has time to dry, otherwise the next smear will be streaky and unreadable.

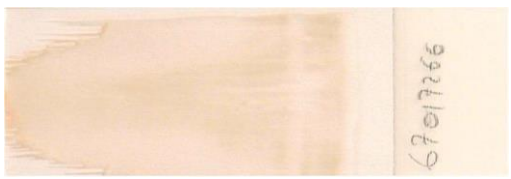




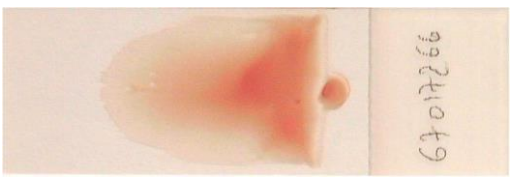


a

b



c



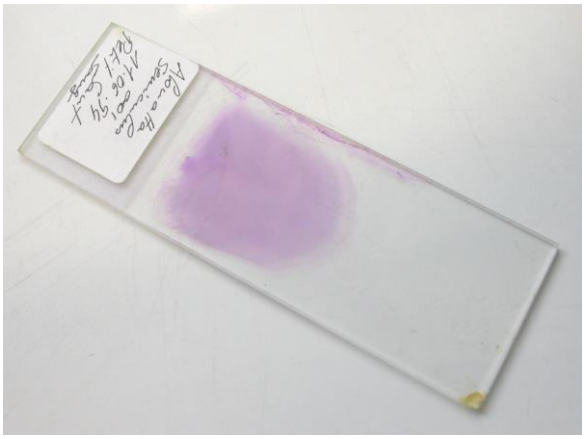
d

(a) Greasy slide, “vacuolisation” of the smear; (b) correct (c) excess blood and absence of “head” of the smear; (d) excess blood and inadequately spread; superimposed cells.

The smear is then fixed on the slide by immersion in methanol for 3 min and stained with the solution of Giemsa at 0.4% (pure commercial solution) diluted 1:10 or 1:20 in a PBS buffer,

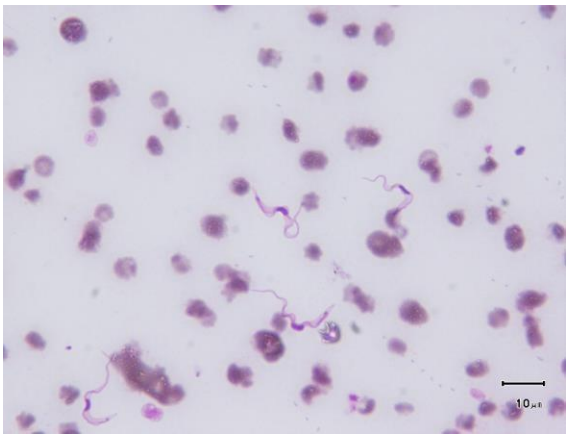
pH 7.45, freshly made using a mixture of 17.5% of Solution A (KH_2PO_4 [Merck Art. 4873] 9.08 g/l, 1/15 molar) and 82.5% of solution B (Na_2HPO_4 2 H_2O [Merck Art. 6580] 11.87 g/l, 1/15 molar), for 30 min, rinsed once in deionised water, then rinsed thoroughly in tap water. An RAL555 type rapid stain kit can also be used; the procedure consists of five successive 1-sec immersions in solutions of fixative (methanol), eosin and blue, followed by rinsing in tap water. In all cases, the stained smear is then dried thoroughly before being examined at X500 or X1000 magnification. It is important to dry the slide thoroughly, otherwise the mixture of water and immersion oil will render the slide unreadable due to the light interference created by the water and the air trapped under the oil. A well-prepared slide will show a light rim around the head of the smear; this zone is particularly rich in white cells (and possibly trypanosomes), due to the strong capillary forces linked to their large size. This “concentration” phenomenon is similar to that on which the haematocrit centrifugation method is based.

Staining kit



Stained smear

Objective and immersion oil



Trypanosomes on smear in immersion (x1000)

Approximately, 50–100 fields of the stained slide are examined at a magnification of X500 and then X1000 with immersion oil before a sample can be considered negative. Even after a trypanosome has been observed, about 20 more fields must be examined to determine if more than one species is present. The sharp extremity of the smear must be extensively

explored as it has the highest concentration of white cells, trypanosomes and any parasitized RBCs (due to their increased size and therefore their adherence to the spreader slide).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes. Usually, both a thin and a thick smear are made from the same sample. Thick blood films contain more blood than thin blood smears and consequently have a higher diagnostic sensitivity. Thin smears, on the other hand, allow *Trypanosoma* species identification. Trypanosome species can be identified on smears by the following morphological characteristics:

- *Trypanosoma vivax:* 20-27 µm long, 2-4 µm wide; undulating membrane is visible but poorly developed, free flagellum present at the anterior extremity, central nucleus, posterior extremity rounded, kinetoplast large (1.1 µm) and terminal.

- *Trypanosoma brucei* is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e., a long slender form and a short stumpy form. Often, intermediate forms possessing characteristics of both the slender and stumpy forms are observed. The cytoplasm often contains basophilic granules in stained specimens. The kinetoplast is small (0.5-0.7 µm).

- *Trypanosoma brucei* (long slender form): 17-30 µm long and 2.8 µm wide, undulating membrane is well developed and presents 3 to 5 convolutions, free flagellum present at the anterior extremity, central nucleus, posterior extremity pointed, kinetoplast small and subterminal. *T. evansi* presents almost exclusively in the long form of *T. brucei*.

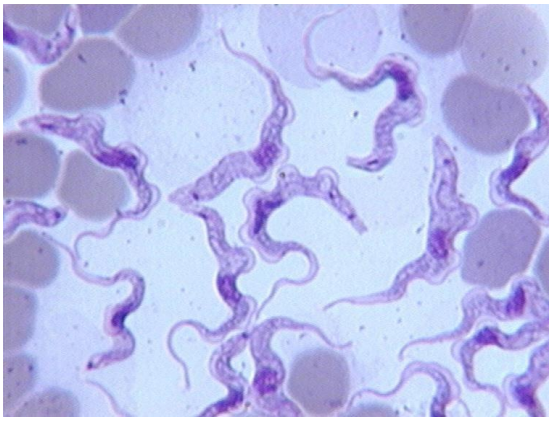
- *Trypanosoma brucei* (short stumpy form): 17-22 µm long and 3.5 µm wide, undulating membrane is conspicuous, central nucleus, free flagellum absent, posterior extremity pointed, kinetoplast small and subterminal.

- *Trypanosoma congolense:* 8-22 µm (small species), undulating membrane not obvious, central nucleus, free flagellum absent, posterior extremity rounded, kinetoplast is medium sized and subterminal, often laterally positioned. Although *T. congolense* is considered to be monomorphous, considerable morphological variation can be observed. The subgenus *Nannomonas* comprises 2 taxa of the species *T. simiae* and *T. congolense.* Within *T. congolense*, different groups and subgroups exist (savannah, forest, 'kilifi' or Kenya coast) that have a different pathogenicity. These types can only be distinguished using PCR. Seven different morphotypes of *T. congolense* have been described (including those of *T. simiae*), from the most slender to the most stumpy: hyperleptomorph (very long and slender, with a free flagellum), leptomorph (long and slender, with a free flagellum), isomorph (slender, with or without a free flagellum), pachymorph (short and stout, 0.25 < width / length < 0.34, without a free flagellum), hyperpachymorph (very short and stout, 0.35 < width / length < 0.70,

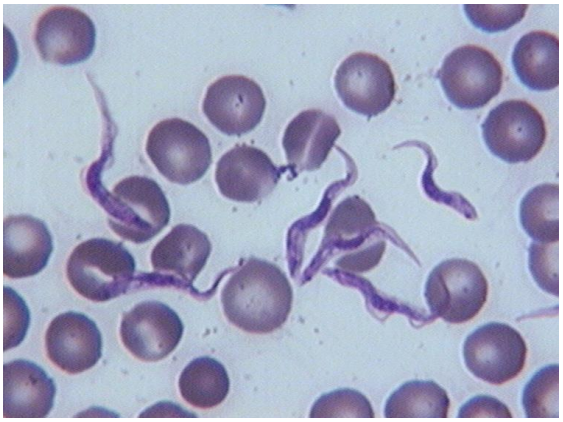
without a free flagellum) and sphaeromorph (globular, width / length > 0.8, with or without a free flagellum).

-*Trypanosoma theileri*: variable size, typically 15 to 70 µm and may reach 110 µm, but on average around 50 µm, with a considerable width (3-5 µm), kinetoplast is large (1.1 µm in diameter), rounded, located in a marginal position, distant from the posterior extremity (at a distance of 12-40 µm), close to the nucleus (KI = 2.4-5.8) which is centrally located. The undulating membrane is well developed and the flagellum is partially free. The posterior extremity is pointed and straight, or slightly curved, but never sinuous. It should be noted that a light band crosses the nucleus in *T. ingens*, a neighbouring Megatrypanum, found in bovines and antelopes. *Trypanosoma theileri* is normally nonpathogenic, but its presence may interfere with parasitological diagnosis. In Western Europe, *T. theileri* is normally the only trypanosome species observed in livestock, and is cosmopolitan.

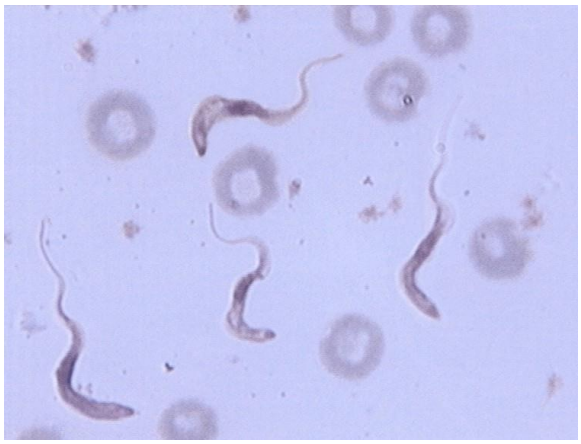
-*Trypanosoma lewisi* and *T. musculi:* although these parasites, of rat and mouse, respectively, are not specifically African (i.e. cosmopolitan) and do not occur in livestock, they may be encountered in studies involving laboratory rodents and one must therefore be able to identify them. They vary in length from 21 to 36 µm, are usually around 30 µm, are quite wide (1.5–2.2 µm), with a large, oval-shaped kinetoplast (0.7–1 µm in diameter) in a marginal position, distant from the posterior extremity (at a distance of 5-8 µm), and the nucleus is close to the anterior extremity. Large epimastigote and even amastigote forms may be observed. In the classic adult form, the cytoplasm is C-shaped with the undulating membrane on the outside, a long and conspicuous free flagellum, a large, oval kinetoplast distant from the posterior extremity and a dense nucleus in a very anterior position.



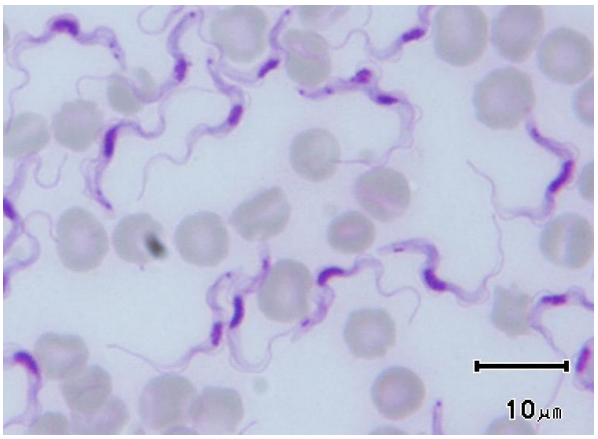
T. brucei (in a mouse)



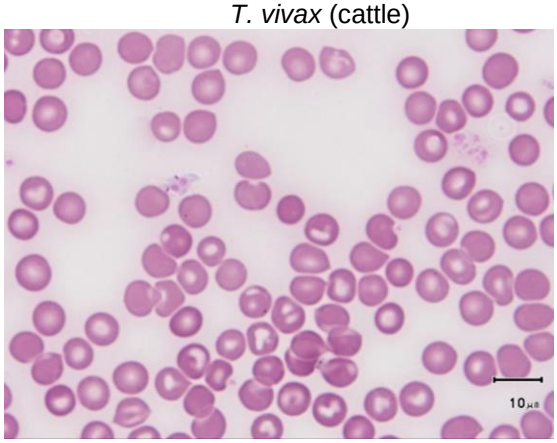
T. congolense (cattle)



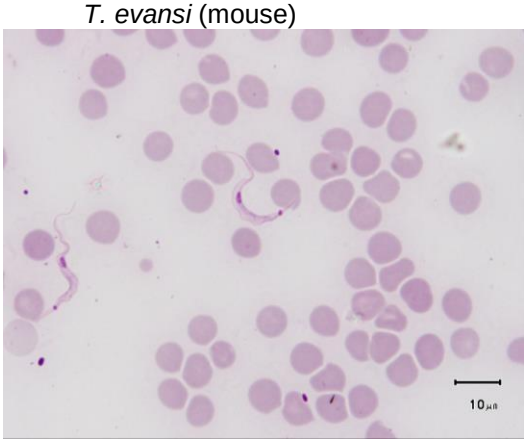
T. vivax (cattle)



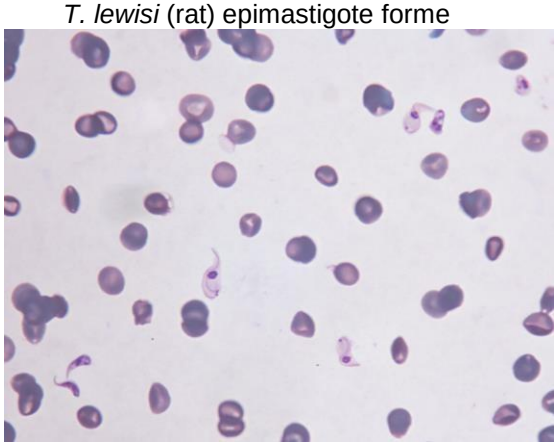
T. evansi (mouse)



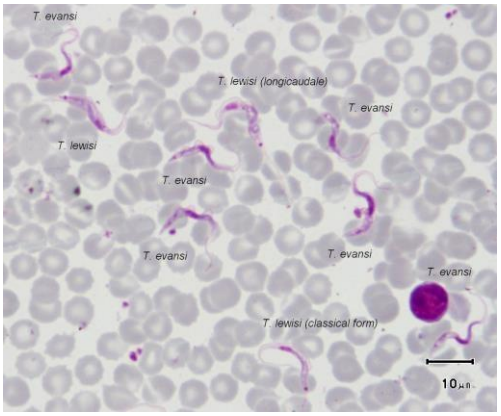
T. lewisi (rat) epimastigote forme



T. lewisi (rat) classical form



Forest *T. congolense* (dog): isomorphs (bottom left) hyperpachymorphs (centre) and sphaeromorphs right).



T. lewisi and *T. evansi* in a rat, (easily distinguished by size of the (top Kinetoplast)

(M. Desquesnes)

2.2 Parasite concentration techniques

The probability of detecting trypanosomes in a sample from an infected animal largely depends 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 examined and by concentrating the trypanosomes.

2.2.1 Microhaematocrit centrifugation technique (Woo method)

The microhaematocrit centrifugation technique, or Woo method (1970), is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood sample according to their size, shape and density. The method is as follows:

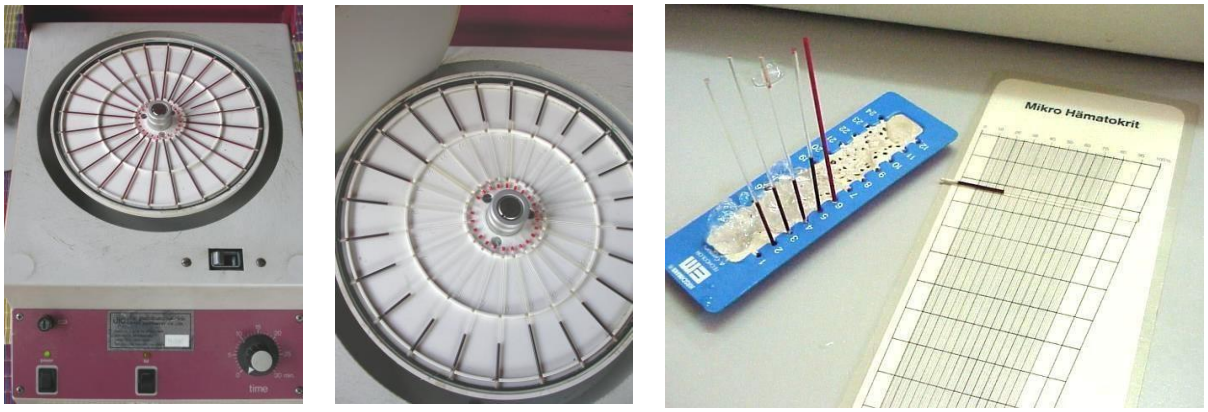
i) Fresh, ear vein blood (about 70 µl) is collected into heparinised capillary tubes (75 x 1.5 mm) or filled from a tube of blood on EDTA or heparin, in which case a dry capillary tube is used; while filling the capillary tube, make sure that no air bubbles interrupt the column of blood, otherwise the examination could fail; filling of the capillary tube should therefore be uninterrupted, and when the level is close (about 0.5 cm) to the dry end, cover this end with your finger before pushing it into plasticine.

ii) As the capillary tube is pushed into the plasticine, remove your finger from the dry end to allow some plasticine to enter the tube; the tube should be inclined and twisted to be sure of picking up some plasticine, then repeat this movement once more to ensure that the tube is properly sealed; failure to do so could result in the plasticine being ejected during the centrifugation process and the blood being expelled into the centrifuge. In warm conditions, if the plasticine becomes too soft, it can be cooled before use.

iii) The sealed capillary tube is then placed in one of the numbered grooves in a microhaematocrit centrifuge with the sealed end facing outwards and against the rubber band around the capillary centrifuge rotor. To ensure the centrifuge is correctly balanced, the tubes should be placed symmetrically.

iv) Screw down the rotor cover securely (without moving the tubes) before closing the centrifuge, otherwise the tubes could scatter and break when the centrifuge starts.

v) The capillary tubes are subjected to a centrifugal force of 9000 ***g*** for 5 min (about 12 000 rpm);



Legend. Capillary tubes before (left) and after (centre) centrifugation, reading the haematocrit value using a microhaematocrit capillary tube reader (right)

vi) After centrifugation, the haematocrit value is read using a microhaematocrit capillary tube reader and is expressed as % (packed cell volume: PCV). This information is important as it helps to select suspect samples with a low haematocrit level (<24% for example, in bovines) for further examination by PCR. Twenty-four percent is the value normally chosen in bovines; it corresponds to the mean haematocrit value in the species, minus 30% (35%-10.5% = 24.5%); other values will need to be defined for the other species.

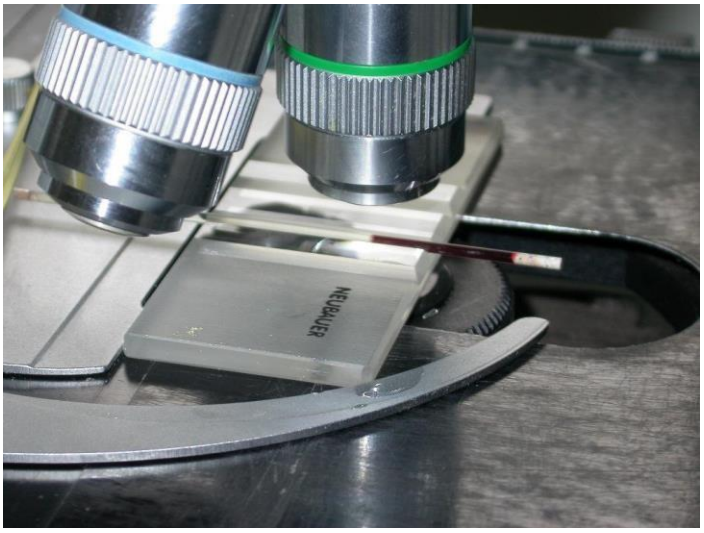
vii) A Neubauer cell can be used or alternatively a capillary tube holder consisting of a slide on which 2 pieces of glass (25 x 10 x 1.2 mm) are glued, 1.5 mm apart, to form a groove in which the capillary tubes are placed for examination.

viii) The tube inserted in the groove can be held in place by an elastic band around the microscope stage. The buffy coat is positioned roughly under the objective. The microscope's light condenser should be lowered, possibly with the diaphragm closed, to increase the refraction of cells and make them visible, as the trypanosomes and white blood cells are normally almost transparent. The appearance of a grey-black border around white blood cells indicates that the microscope is correctly adjusted for this examination.

ix) The plasma/white blood cell interface (buffy coat) is examined by slowly rotating the tube. Trypanosome movement can first be detected using the X40 objective at a distance that provides the appropriate depth of field through the capillary tube. To apply regular rotations to the capillary tube, a hexagonal pencil inserted under the elastic band on the left edge of the stage can be used; when the pencil is turned it rotates the capillary tube. In this way, all sides of the capillary tube can be explored, and by turning the micrometric screw, one can explore a part of the depth of the tube after each rotation movement (a rotation of about 90° applied to each movement allows the circumference of the tube to be explored in 4 partial rotations).

The microhaematocrit centrifugation technique is more sensitive than direct examination techniques. In the case of infections with *T. vivax*, the sensitivity of the Woo method is close to 100% when parasitaemia is above 700 trypanosomes/ml of blood. However, sensitivity falls to 50% when parasitaemia is between 60 and 300 trypanosomes/ml of blood. Trypanosomes are very difficult to detect when parasitaemia is less than 60 trypanosomes/ml of blood; it can therefore be considered that the technique is capable of detecting parasitaemia when it exceeds 200 trypanosomes/ml. Identifying the actual species of trypanosome is difficult. As the specific gravity of *T. congolense* is close to that of red blood cells, the parasites are often observed below the buffy coat or in the layer of white blood cells. To enhance the separation between RBCs and parasites and increase sensitivity, for *T. congolense*, the inherent density of the RBCs can be increased by adding glycerol.

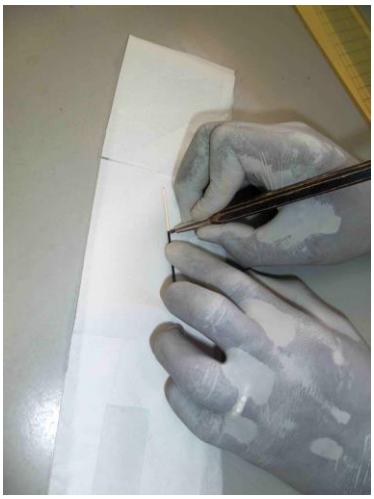
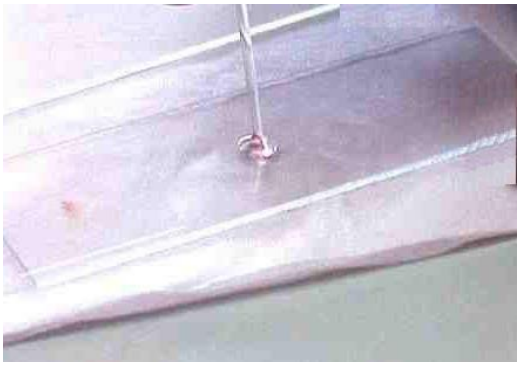
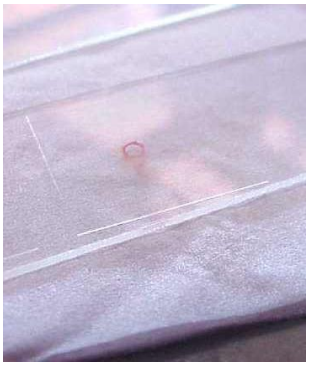
Microscope equipped with dark-field system



Arrangement for microscopic examination of the capillary a tube at the buffy coat/plasma interface

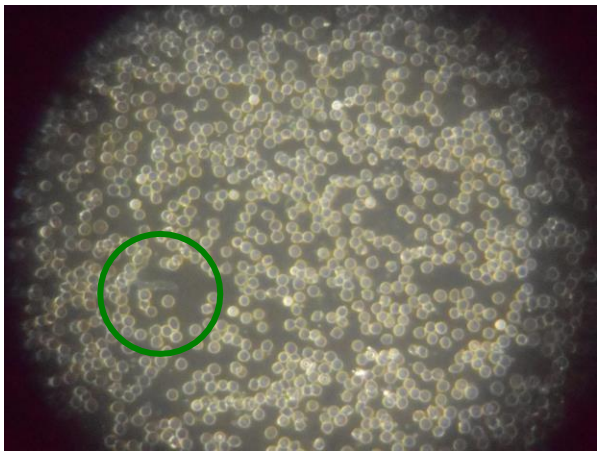
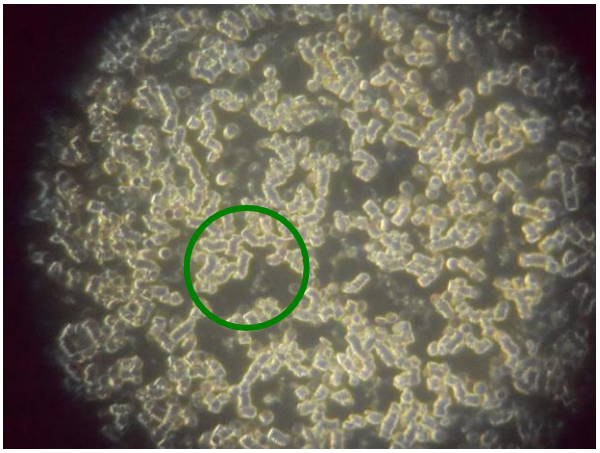
2.2.2 Dark-ground or phase-contrast buffy coat technique

The first steps of the buffy coat technique, or Murray method, are identical to steps i) to vi) described above, after which the 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 onto a microscope slide and covered with a cover-slip (22 x 22 mm). This step is crucial; to be successful, the buffy coat must be visible on the slide before it is covered with the cover-slip; the buffy coat is quite dense, forming a mass of cells that needs to be flattened with the cover-slip to keep it in the same plane. If the buffy coat is not visible it means that it has stuck to the side of the capillary tube; the examination cannot then take place. For these reasons, there is quite a strong 'technician' effect, some finding it easy to place the buffy coat while others find it more difficult; for the same reasons, the test has limited reproducibility.

Cut the tube with a diamond tipped pencil, place the buffy coat and cover with the cover-slip

The microscopic examination is performed with the light condenser in the dark ground position and using a X40 or X50 phase-contrast objective lens (PhaCo) to obtain a magnification of X400 or X500 with a X10 eyepiece.

T. lewisi: light parasitaemia *T. evansi*: very heavy parasitaemia

Approximately 200 fields of the preparation are examined for the presence of motile trypanosomes, at a X400 magnification using a dark-field or phase-contrast microscope. As with direct examination of wet blood films, trypanosome species can be identified based on the following criteria:

- *Trypanosoma vivax*: Large, extremely active, traverses the whole field very quickly, pausing occasionally.

- *Trypanosoma brucei*: Various sizes, rapid movement in confined areas; undulating membrane traps the light into ‘pockets’ moving along the body.

- *Trypanosoma congolense*: Small, vermiform, sluggish, adheres to red blood cells by its anterior extremity.

- *Trypanosoma theileri*: More than twice the size of pathogenic trypanosomes, tends to rotate or stay immobile as if stuck to the glass, whip-like movements with its tapering posterior end, the tip of which appears rigid.

Like the microhaematocrit centrifugation technique, the buffy coat technique is more sensitive than direct examination techniques. The sensitivity of the buffy coat method can be improved by using the double-centrifugation technique. A total amount of 1500 µl of blood is centrifuged, after which the buffy coat is aspired into the microhaematocrit capillary tube and centrifuged again. The “buffy coat of the buffy coat” is examined. However, this method is more costly and time-consuming, as the collection of the buffy coat after the first centrifugation is a delicate step and results may vary even more from one technician to another; the technique is therefore rarely used.

Compared to the Woo technique, the Murray technique has the added advantage that preparations can be fixed and stained for more accurate species identification and for retention as a permanent record. However, it is difficult to remove the cover-slip without losing or damaging the biological material, and reading the fixed sample is less sensitive than that of the fresh sample. A combined protocol can be applied, testing for the presence of the parasite with the Woo technique and, in the event of a positive result, using the buffy coat technique for staining and confirmation of the species identification.

Both the haematocrit centrifugation and buffy coat techniques give direct results and can be used for screening large numbers of animals. They require specialised equipment and an electricity supply, making these techniques more expensive than examination of wet blood films, but these minor drawbacks are amply compensated by the increased sensitivity. Both parasite concentration techniques rely on the detection of motile, live trypanosomes. Because trypanosomes can lose their vigour and die rather quickly once the blood sample is drawn, samples collected in capillary tubes should be cooled immediately and not allowed to overheat in the microhaematocrit centrifuge, on the microscope stage or during a long wait between centrifugation and examination. Samples should therefore be prepared and examined as soon as possible after collection: preparation preferably within 2 to 4 hours after collection and examination within minutes after centrifugation.

The microhaematocrit centrifugation and buffy coat techniques are particularly useful in that the haematocrit (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 red blood cell column is

expressed as a percentage of the total volume of blood. Measuring the PCV is in order to determine the degree of anaemia. Anaemia can be caused by factors other than trypanosomosis. It remains, however, one of the most important indicators of trypanosomosis in cattle. As trypanosomosis is a herd problem, the PCV profile of a herd is influenced by the number of trypanosome-infected animals and can be used to indicate differences in disease challenge. The average PCV is also influenced by the age and level of genetic susceptibility of cattle. For economic reasons, to reduce the number of samples to be studied by PCR, the individual haematocrit value can be used to identify suspect samples to be selected for PCR diagnosis. Depending on the case, the threshold below which infection is suspected can be set at a haematocrit value of between 24% and 20%. When the haematocrit value is determined before the microscopic reading of the tubes, it is imperative for the tubes to be immediately returned to a vertical position while awaiting the reading; if this is not done, the buffy coat will be disorganised and the microscopic reading impaired or even impossible.

2.2.3 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*. 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 species under examination. As the red blood cells 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 thus be examined from each animal and the method is highly sensitive. However, the technique is time-consuming and therefore not suitable for examining large numbers of animals. Furthermore, it is very expensive due to the high cost of the diethyl amino-ethyl cellulose column (DE52) and the time taken to prepare it.

See the following website (in French) http://www.sleeping-sickness.ird.fr/diagnostic61.htm

In veterinary medicine, the method is only used to separate trypanosomes cultivated in rat (or other rodent) to prepare the antigens used in the ELISA technique (see below, Part 3).

2.3 Cultivation techniques

The amplification technique *par excellence*, cultivation can be performed *in vivo*, preferably in laboratory animals (rats or mice), but sometimes in livestock hosts if the strains do not grow in rodents (e.g. *T. vivax* of Latin America) or *in vitro*, which is preferable for ethical reasons but not always feasible.

2.3.1 *In vitro* cultivation

In vitro cultivation of trypanosomes is often tricky, requires suitably adapted protocols and gives results that are highly variable according to the species and even the strains of trypanosomes (selection bias); it remains an expensive method. For these reasons, *in vitro* cultivation techniques are little used, especially in the veterinary field where the only species that presents a comparative advantage is *T. theileri*, a non-pathogenic species, present in very small quantities in blood but fairly easy to cultivate. Nevertheless, there have been research efforts aimed at adapting several species of trypanosome to cultivation.

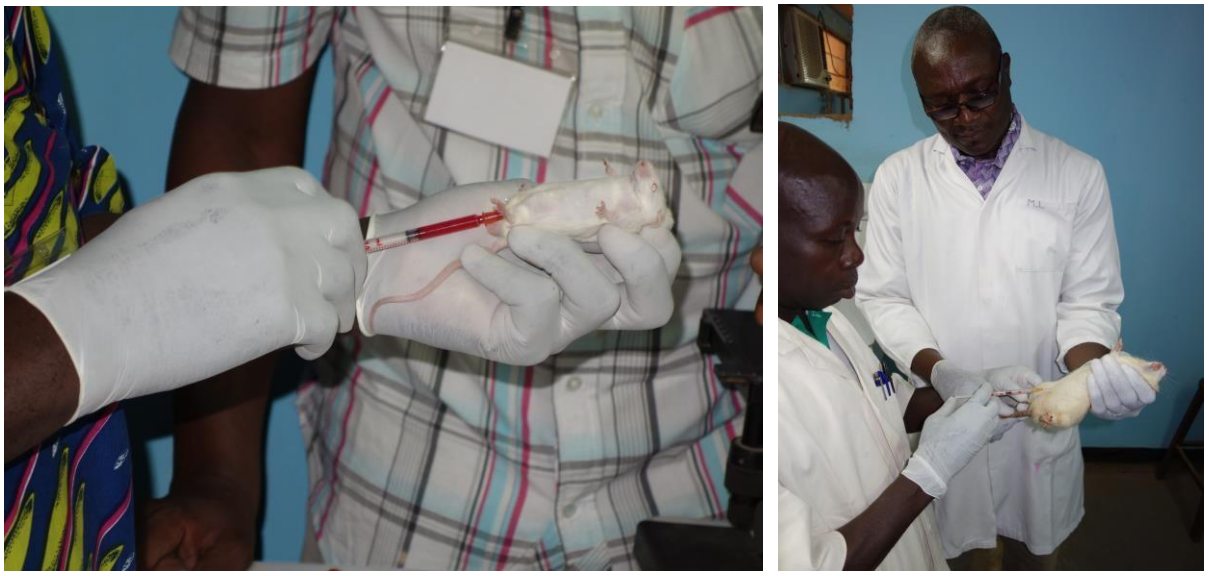
A procedure for the *in vitro* cultivation of *T. brucei* has been described, but its success is irregular, the method requires sophisticated equipment, produces results after a long delay and is not suitable for large-scale use. A kit for *in vitro* isolation (KIVI) of trypanosomes has proven to be promising in isolating and amplifying all species of *T. brucei* in humans and in domestic and game animals. The value of the test in isolating *T. congolense* and *T. vivax* is still unknown. As it is based on the cultivation of pericyclic forms of trypanosomes, species differentiation is not possible and its usefulness in veterinary medicine is consequently rather limited.

In routine diagnosis, only the species *T. theileri* could be detected with good sensitivity through *in vitro* cultivation; however, as this species is not considered pathogenic, it is rarely cultivated. It is worth noting that rodent trypanosomes *T. lewisi* and *T. musculi* can be cultivated *in vitro* for the production of antigens.

2.3.2 Animal inoculation

The inoculation of blood into rodents, usually mice or rats, is particularly useful in revealing subpatent infections. The laboratory animals are injected intraperitoneally with 0.1–0.5 ml (depending on the size of the rodent) of freshly collected blood on anticoagulant. Artificial immunosuppression of recipient animals by irradiation or drug treatment (e.g. cyclophosphamide 200 mg/Kg) (Endoxan®) 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 for 2 weeks to 2 months. The fresh blood is placed on a slide, covered with a cover-slip and

examined directly under the microscope (preferably by phase-contrast with a magnification of X400 or X500). The subgenus is easily identified, as indicated at point 1.1.1. Some species, such as *T. evansi*, may grow very quickly and kill the rodents within a few days; the inoculated animals should therefore be closely monitored, especially if the identity of the species that could be isolated is unknown.



Intraperitoneal inoculation of infected blood in a mouse (volume 0.1 to 1 ml) and a rat (0.5-1.5 ml) (M. Desquesnes)

Animal inoculation is more sensitive than direct examination of fresh blood samples. Nevertheless, the method is not practical; it is costly and the diagnosis is not immediate. The method is very sensitive for the detection of *T. b. brucei* infections. However, several *T. congolense* strains are not easily cultivated and *T. vivax* rarely infects laboratory animals, apart from a few strains that grow spontaneously. Furthermore, animal inoculation should be avoided as it raises ethical issues related to animal welfare.

In terms of diagnosis, this method should be strictly limited to:

- isolation of parasite strains of special interest (suspected drug resistance, pronounced pathogenicity, etc.);
- highly sensitive detection and identification of infection in suspected animals living in or travelling towards an uninfected area, or valuable animals that are regularly transported internationally (racehorses, for example).

The technique is, however, very useful for the massive production of parasites in rats for the preparation of antigens, whether for IIF, ELISA or CATT.

2.4 Comparison of the sensitivity of parasite detection tests

Parasitological detection tests have different analytical sensitivities (analytical sensitivity is the smallest detectable parasite in a sample, usually measured as the number of parasites / ml of blood). By way of illustration, Table 1 summarizes the characteristics and the orders of magnitude of the analytical sensitivity of the tests for the detection of parasitemias expressed as the number of trypanosomes per ml of blood. It specifies the relative costs of the tests and various other advantages or disadvantages (reproducibility, time, etc.). Analytical sensitivity may be calculated in relation to a given parasitaemia value. On the other hand, the sensitivity of a field test, which is the probability of obtaining a positive response by a diagnostic technique in an infected subject, will in addition depend on the stage of infection, the parasite species, the host susceptibility ... and should be re-estimated in the studied populations.

In the table that follows the analytical sensitivity is indicated by a threshold below which the examination will probably be negative.

Table 1: Comparison of parasite detection tests

Technique	Analytical Sensitivity (parasites/ml)	Specificity	Advantages	Disadvantages	Indications
Microscopical observations					
Direct blood examination	10^5- 10^6	low	simple, quick, economical	low sensitivity (Se)	*Trypanosoma* sp.
Thick blood film (Giemsa)	10^4-10^5	low	simple, economical	delay, low sensitivity	*Trypanosoma* sp.
Stained blood film	10^5-	subgenus	Specific (Sp), simple, economical	delay, low sensitivity	*Trypanosoma* sp.
HCT (Woo)	10^2- 10^3	subgenus	Se., Sp, rapid, PCV	must be done quickly	*Trypanosoma* sp.
BCM (Murray)	10^2- 10^3	subgenus	Se, Sp., PCV	urgent and time-consuming	*Trypanosoma* sp.
QBC Quantitative buffy coat	10^2- 10^3	low	PCV	costly no longer used	*Trypanosoma* sp.
Lysis / Centrifugation	10^2- 10^3	low	PCV	Long, costly no longer used	*Trypanosoma* sp.
DE52 minicolumn	10^2-10^3	subgenus	Parasite isolation	long and costly	*Trypanosoma* sp.
Centrifugation in silicon	10^2-10^3	subgenus	Parasite isolation	long, costly no longer used	*Trypanosoma* sp.
In vivo cultivation					
Rodent inoculation	10- 10^3	species or genus	Sensitive	uncertain, long, costly, ethical issues	*T. evansi, T. cruzi, T. equiperdum*?
In vitro cultivation					
Blood culture	10- 10^2	species or genus	Sensitive for some species	long, costly, delayed	*T. theileri T. cruzi*

Se= sensitivity; Sp= specificity; PCV = packed cell volume

3. Molecular detection

As well as direct observation of the parasites by microscopic examination, there are indirect methods to detect infection by trypanosomes by specifically evidencing the presence of trypanosome proteins or DNA. Demonstrating the presence of these molecules is not in itself proof of active infection (active infection is defined as the presence of live trypanosomes in the host at the time of sampling). Nonetheless, given the rapid elimination of parasite proteins and DNA from the blood after the death of the parasites (the elimination delay is estimated to be 24-48 h for DNA and several days for proteins), it can generally be considered that the detection of DNA is synonymous with active infection, especially if there is no history of recent trypanocide treatment. Subject to this caveat, both these methods can therefore be considered as techniques for detecting active infection.

3.1 Trypanosome antigen detection assays

Several enzyme-linked immunosorbent assay (ELISA) antigen detection methods, based on immune sera or monoclonal antibodies, have been described for trypanosomosis. However, field evaluations of these tests have given variable and generally unsatisfactory results. In particular, Ag-ELISA tests based on monoclonal antibodies presented a lack of specificity between trypanosome species, and there were also cross-reactions with other, non-identified parasitic agents, resulting in high rates of false-positives in non-endemic areas. Sensitivity also proved very disappointing, and was even below 15% in some cases of *T. vivax* infection. Antigen detection by ELISA is therefore not used in routine diagnosis of trypanosomosis (Desquesnes, 1996).

3.2 Tests to detect trypanosomal DNA

Several PCR methods for the detection of trypanosomal DNA have been developed as tools for diagnosing trypanosome infections in humans and other mammalian hosts, as well as in tsetse flies. Specific highly repetitive nuclear DNA sequences (10 000 to 20 000 repeats) called “satellite DNA” can be amplified; their amplification can be used to detect and identify *Trypanozoon* subgenera (*T. brucei* sspp, *T. evansi* and *T. equiperdum*), *T. vivax* species, several types of *T. congolense* (savannah, forest and Kenya coast) and other species of *Nanomonnas* (*T. simiae, T. godfreyi*). Table 2 lists the available primers for the different subgenera, species and types of trypanosomes of mammals. Certain primers are reportedly

able to distinguish *T. evansi* from *T. brucei*, but in the absence of exhaustive studies there is currently no set of primers universally regarded as allowing this distinction to be made; moreover, the distinction with *T. equiperdum* is even more delicate and controversial. Indeed, amplification of the gene coding for the Rhode trypanosome antigenic type protein RoTat1.2 has been proposed as a means of differentiating *T. evansi*, which possesses the gene, from *T. equiperdum*, which does not. Unfortunately, this distinction does not take into account the existence of *T. evansi* group B, identified in East Africa, which does not possess the gene. Some parasites may even possess the gene without expressing it. Lastly, a probe is available to detect the 3 subspecies of *T. brucei*, but the method is little used as it is cumbersome to implement.

Diagnostic methods were then developed using amplification of the internal transcribed spacer 1 (rDNA-ITS1) region of ribosomal DNA (rDNA). They can be used to identify the same taxa, either in single or mixed infections, using a single test. The major advantages are the cost, which is reduced for an unlimited number of species valences, and the possibility of amplifying all species of trypanosomes, and therefore even including unknown or non-targeted species. On the other hand, the sensitivity of the test is lower than that of monospecific PCRs and the observation certain products of imprecisely determined size can make interpretation difficult or even impossible.

More recently, a technique for amplifying DNA at a constant temperature (LAMP: loop-mediated isothermal amplification) has been developed and has been applied to several species of trypanosomes. However, such methods have not yet been sufficiently validated to be of practical use in the field.

A number of important steps are required before PCR techniques can be used, namely the collection, preservation and, where appropriate, concentration of parasites, and the preparation of samples and even the purification of DNA to allow the PCR reactions to take place.

3.2.1 Preparation of samples for PCR

As biological samples collected from hosts or vectors contain substances capable of inhibiting PCR reactions, sample preparation is an important preliminary step before DNA molecular amplification methods can be applied. This step is crucial and will vary according to the nature of the collected sample.

Samples may be derived from a mammal or an insect, be kept refrigerated or be placed on filter paper or in alcohol; numerous protocols exist and will need to be adapted according to the various subsequent stages in the diagnosis.

Tsetse samples:

The tsetse flies are identified and then dissected in several drops of physiological liquid and 3 organs are collected separately for microscopic examination and/or PCR.

Dissection of tsetse fly and preparation of proboscis, salivary glands and midgut

- Tsetse proboscis: the organs are dissected and placed in a drop of physiological liquid for microscopic examination and/or collected in 30 µl of distilled water; at this stage they can be frozen until preparation. After thawing, the sample is triturated using a tip, 30 µl of 5% Chelex is added and then the Chelex preparation protocole is applied (see below).

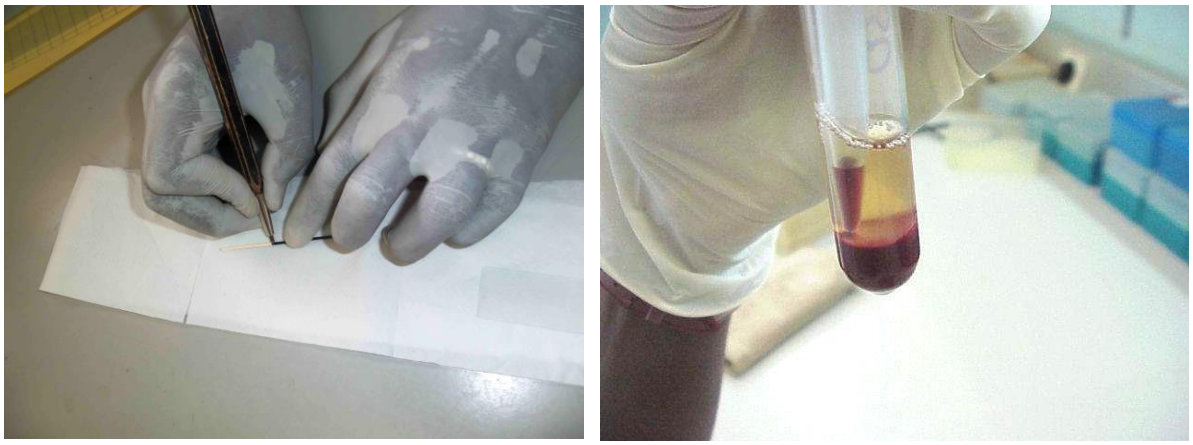
- Tsetse salivary glands and midgut: these are collected and placed in a drop of physiological liquid for microscopic observation and/or placed respectively in 30 µl and 50 µl of distilled water. These samples of moderate molecular richness should be triturated, homogenised and dilacerated and pipetted several times before the addition of an equivalent volume of 5% Chelex (30 µl for salivary glands and 50 µl for midgut). The Chelex preparation protocole (see below) can then be applied.

Chelex preparation protocole: the samples suspended 1:1 in 5% Chelex are vortex mixed, left for 1 h at 56°C, then vortex mixed and left for 30 min at 95°C, then vortex mixed and centrifuged for 2 minutes at 5000 rpm; the supernatant of the sample is pipetted to avoid debris particles and resin beads blocking the tip and/or interfering with the PCR.

If the PCR exams are not carried out immediately after the Chelex preparation, it is necessary to collect the entire supernatant by pipetting in surface (avoiding Chelex beads and debris) and place it in a new microtube to be stored at -20°C until PCR is carried outsa.

Blood samples:

Blood collected on anticoagulant is centrifuged at high speed (>9000 rpm, 5 minutes) to accelerate the decantation of parasites and blood corpuscles, as described for concentration methods prior to microscopic examination; the trypanosomes are thus concentrated above the buffy coat (leukocyte-platelet layer).



Sectioning of capillary tube to obtain buffy coat, or pipetting from a sampling tube

The buffy coats can be collected either from capillary tubes (approximately 10-15 µl of buffy coat samples placed on 30 µl of distilled water) or from tubes or microtubes (approximately 100-150 µl of buffy coat), and can then be prepared in various ways to optimise the sensitivity of the PCR tests.

- Blood or buffy coats on filter paper: the blood or buffy coats are spotted onto filter paper (a filter paper confetti, for example); they can be eluted with a Tris-EDTA (TE) buffer or, better still, directly, using Chelex (see below).

Sensitivity studies have been carried out to compare different methods of preparing infected blood samples with series of dilutions of parasites. Recent studies with a parasite of the subgenus *Trypanozoon* have shown that the classical phenol–chloroform method remains the best in terms of both the sensibility of the tests and the quality, integrity and duration of preservation of the extracted DNA (Pruvot et al 2013). However, in view of the

time-consuming nature of the method and the danger of handling toxic products (phenol and chloroform), other, faster and in some cases economical methods have proved effective, such as the use of Chelex (Walsh et al., 1991; Penchenier et al., 1996). There are also numerous commercial kits for preparing or harvesting DNA, but the cost is generally prohibitive, especially for routine diagnosis in large batches of samples. However, their diagnostic superiority has rarely been demonstrated, and the duration of preservation of DNA prepared in this way is quite short (several weeks) making it difficult to repeat the tests should the need arise.

Preparation with phenol–chloroform: Place 500 µl of denaturing solution (guanidine thiocyanate) in a 1.5-ml microtube and add 100 µl of buffy coat (or, if not available, blood), vortex mix at high speed for 5 minutes. Add 150 µl of chloroform and 150 µl of phenol, vortex mix at high speed for 5 minutes, then centrifuge at 13 000 rpm (15493 *g*) for 5 minutes. Collect the supernatant and place it in a new tube. Again add 150 µl of chloroform and 150 µl of phenol, vortex mix at high speed for 5 minutes, then centrifuge at 13 000 rpm for 5 minutes. Collect 400 µl of supernatant, transfer it to a new 1.5-ml tube and add 1 ml absolute ethanol and leave to precipitate overnight at -20°C. Centrifuge cold for 10 minutes at 13 000 rpm and discard the supernatant. Wash the pellet twice with 75% ethanol and centrifuge at 13 000 rpm for 5 minutes. Eliminate the supernatant. Allow the pellet to dry in the air. Re-suspend in 50 µl of Tris-EDTA (TE) buffer. This method enables the sample to be concentrated from an initial volume of 100 µl to a final volume of DNA in 50 µl.

Preparation with Chelex: blood or buffy coat samples are stored frozen. The sample is thawed and an equivalent volume of 5% Chelex-100 (suspension of 5% Chelex-100 in distilled water) is added. It is kept for 1 h at 56°C, then vortex mixed and kept for 30 minutes at 95°C, then vortex mixed and centrifuged for 2 minutes at 5000 rpm; the supernatant is pipetted to avoid debris particles and resin beads blocking the tip and/or interfering with the PCR.

This method can also be applied to dried blood collected on filter paper. Cut the filter paper into small pieces (of about 2-3 mm^2) and place in a microtube, add a volume of approximately 2 µl/mm^2 (i.e. 50 µl Chelex for 25 mm^2 of confetti, or 150-200 µl Chelex for a square centimetre of filter paper) and carry out the classical Chelex preparation (1 h at 56°C, then 30 min at 95°C, then centrifugation).

Similarly, buffy coats spotted onto filter paper in a volume of 10-20 µl (from a capillary tube) or 50-100 µl (from a 0.5-ml microtube) can be treated respectively with volumes of 100 and 200 µl of 5% Chelex-100.

Chelex preparation

Chelex beads in suspension

3.2.2 Monospecific PCR

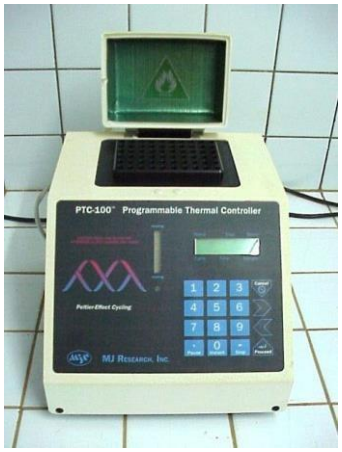
In addition to the importance of the method used to prepare the biological samples referred to above, the sensitivity of the PCR also depends on the affinity of the PCR primers for their target sequences, and more particularly on the highly repetitive nature, or otherwise, of these sequences. Thus the detection of satellite DNA sequences is very sensitive as they are repeated 10 000 to 20 000 times in the genome, whereas that of ITS1 is only moderately sensitive (200 to 500 repetitions) and that of other sequences may have a very low number of repeats or even no repeats.

DNA amplifications by standard PCR are performed using a mixture comprising Tris/HCl, MgCl2, KCl, all four deoxynucleoside triphosphates, the primers, the DNA matrix and the Taq polymerase. The samples are incubated for several cycles at different temperatures. The PCR products are subjected to agarose gel electrophoresis. The gels are then stained with ethidium bromide or with other stains enabling DNA to be visualised on the electrophoresis gels (e.g. GelStar and SYBR Green).

With monospecific primers, PCR is only positive if the amplification product is exactly the expected size. If other products are visible, they are considered nonspecific and the result negative (see sample circled in red on the photograph below). For example, with the TCF and TCS primers, nonspecific products are frequently observed with DNA of *T. congolense* savannah and *T. congolense* forest, respectively.

A classic master-mix composition is as follows: Tris buffer (50 mM Kcl, 1.5 mM $MgCl_2$) + 200 µM dNTP + 1 µM of each primer + 0.5 units of Taq Pol per reaction. A pre-programmed Excel spreadsheet can be used to calculate all the volumes of stock solution according to the reaction volumes and the required number of reactions (see appended laboratory worksheet).

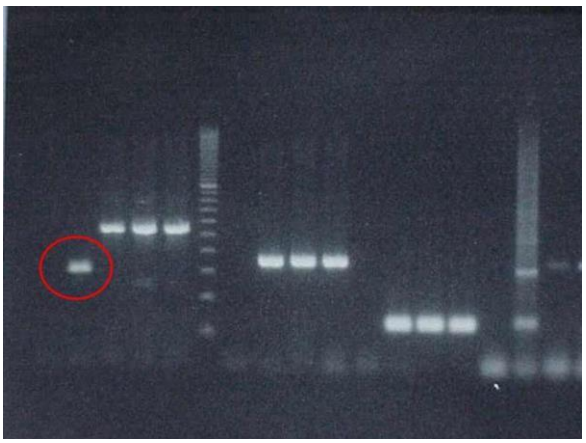
Preparation under a hood



Thermal cycler

Microwave and gel electrophoresis apparatus

Gel visualisation device (UV)



Electrophoresis gel with 3 different primers a nonspecific product (red circled) is visible on the gel

The procedure is extremely sensitive but false-positives may occur as a result of sample contamination with other DNAs. The assay requires specialist equipment and highly trained personnel, and many laboratories are not suitably adapted. False-negatives may be observed when parasitaemia is low (less than 1 trypanosome/ml of blood), which is frequently the case in chronic infections; it may also occur when the specificity of the amplified sequences is so high that none of the isolates of a particular species of trypanosome can be recognised (e.g. the sequence of VSG RoTaT1.2 which is not present in
T. evansi type B). Greater test sensitivity is achieved by preparing DNA from a sample that has been enriched by centrifugation (buffy coat). The collection of samples can be simplified by adapting the assay to blood or buffy coat spotted onto filter paper. A large number of samples can be analysed at the same time, thus making the PCR suitable for larger scale surveys. Nevertheless, the cost of PCR is currently still too high for routine use.

The various primers and thermal cycles used for specific diagnoses are shown in Table 2.

Table 2: Characteristics and sequences of the main primers used to diagnose trypanosomes by mono-specific PCR

Specificity	Name and sequence of the primers	Locali-sation	Size of the products (bp)	T° hybrid	REF
T. vivax	TVW 1 : CTGAGTGCTCCATGTGCCAC	ADN satellite	150	55	Masiga et al., 1992 Int J Parasitol
	TVW 2 : CCACCAGAACACCAACCTGA				
T.brucei s.l.	TBR1: 5' CGAATGAATATTAAACAATGCGCAG 3'	ADN satellite	173	55	Moser et al., 1989 Parasitology
	TBR2: 5' AGAACCATTTATTAGCTTTGTTGC 3'				
T.congo savane	TCS1: 5' CGAGCGAGAACGGGCAC 3'	ADN satellite	321	55	modifiés de Moser et al 1989 Parasitology
	TCS2: 5' GGGACAAACAAATCCCGC 3'				
T.congo forest	TCF1: 5' GGACACGCCAGAAGGTACTT 3'	ADN satellite	350	55	Masiga et al., 1992 Int. J. Parasitol.
	TCF2: 5' GTTCTCGCACCAAATCCAAC 3'				
T. congo 'Kilifi'	TCK1 : 5' GTGCCCAAATTTGAAGTGAT 3'	ADN satellite	294	55	Masiga et al., 1992 Int. J. Parasitol.
	TCK2 : 5' ACTCAAAATCGTGCACCTCG 3'				
T. simiae	TSM1: 5'CCGGTCAAAAACGCATT3'	ADN satellite	437	55	Masiga et al., 1992 Int. J. Parasitol.
	TSM2: 5'AGTCGCCCGGAGTCGAT3'				
T. (N.) Tsavo	TST1: 5' GTCCTGCCACCGAGTATGC 3'	ADN satellite	450	55-60	Majiwa et al., 1993 Parasitology (ILO892/3)
	TST2: 5' CGAGCATGCAGGATGGCCG 3'				
T. congo godfreyi	DGG1: 5' CTGAGGCTGAACAGCGACTC 3'	ADN satellite	149	60	Masiga et al., 1996 Vet. Parasitol.
	DGG2: 5' GGCGTATTGGCATAGCGTAC 3'				
T. evansi	EVA1: 5' ACATATCAACAACGACAAAG 3'	minicercles	139	58	These Masiga 1994 Njiru Vet Par 2004
	EVA2: 5' CCCTAGTATCTCCAATGAAT 3'				
	RoTat1.2F: 5' GCGGGGTGTTTAAAGCAATA 3'	ADN génomique (VSG)	205	59	Claes et al., KBD 2004
	RoTat1.2R: 5' ATTAGTGCTGCGTGTGTTCG 3'				
	TEPAN1: 5' AGTCACATGCATTGGTGGCA 3'	séquence répétée	122	60	Panyim et al, 1993 Pruvot VetPar 2010
	TEPAN2: 5' GAGAAGGCGTTACCCAATCA 3'				
	ESAG6/7F: 5' ACATTCCAGCAGGAGTTGGAG 3'	ADN génomique (R Tsferrin)	237	55	Braem, 1999 these Holland Vet P 2001
	ESAG6/7R: 5' CACGTGAATCCTCAATTTTGT 3'				
T.brucei gambiense	TRBPA1: 5' GCGCCGACGATACCAATGG 3'	Séquence microsat	149 - 203	60	Herder OCEAC 1997 Truc TRSTMH 2002
	TRBPA2: 5' AACGGATTTCAGCGTTGCAG 3'				
T.brucei gambiense	Tgs-GP F: 5' GCTGCTGTGTTCGGAGAGC 3'	Tbg specific glycoprotein	308bp	63	Radwanska et al 2002a
	TgsGP R: 5' GCCATCGTGCTTGCCGCTC 3'				
T.brucei rhodesiense	Tbr F: 5' ATAGTGACAAGATGCGTACTCAACGC 3'	SRA	284	68	Radwanska et al 2002b
	Tbr R: 5' AATGTGTTCGAGTACTTCGGTCACGCT 3'				
T. lewisi	LEW1S: 5' ACCACCACACGCTCTCTTCT 3'	ITS1	220	64	Desquesnes et al. IGE, 2011
	LEW1R: 5' TGTATGTGCGTGCTTGTTCA 3'				
T. theileri	TthCATL1: 5' CGTCTCTGGCTCCGGTCAAAC 3'	CATL (Cathepsin L-like)	273bp	65	Rodrigues et al, 2010 Parasitol. Int.
	DTO155: 5' TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA 3'				

When using satellite DNA primers, it must be mentioned that a “pattern” of PCR products is obtained, due to the higly repetitive chain sequence, giving roughtly: (1) the product expected and (2) the double and (3) triple size products, such as for example for *T. vivax* (TVW primers, product size expected 150bp): products obtained are: 150bp, 300bp; 450bp…

3.2.3 Multi-specific PCR

The development of diagnostic methods using multi-specific PCR has been made possible by the amplification of internal transcribed spacer 1 of ribosomal DNA (ITS1 rDNA) of trypanosomes. ITS1 is highly conserved within a species, whereas its sequence, and in most cases its length, differ from one species to another but are conserved within a species. These characteristics of ITS1 also make it an excellent candidate for universal barcoding of species in protists. Furthermore, as ITS1 is flanked on both sides by highly conserved sequences within genes 18S and 5.8S, it is easy to define primers in these parts that can be used to obtain a PCR product varying in length according to the species. It was thus demonstrated that one could distinguish and even detect simultaneously the DNA of (in decreasing order of ITS1 length): *T. congolense* forest, savannah and Kenya coast, *Trypanozoon* and *T. vivax*. Nevertheless, when an amplification product differs slightly from the expected size, sequencing must be done for identification purposes, as with any amplification product when the epidemiological context is unclear. Indeed, TRYP1 primers, for example, can amplify trypanosomes, but also *leishmania leptomonas* and even *Babesia*; the interpretation of the results is only clear in well-defined epizootiological contexts. Classical PCRs have thus been developed with two sets of primers TRYP1R & S and TRYP4R & S, and a nested PCR has also been developed. Table 3 shows the primers and size products of the main multi-specific PCR. The various protocols (with addition of 5% DMSO) and the expected results are detailed in the references mentioned.

3.2.4 LAMP

PCR methods for amplifying DNA at a constant temperature (LAMP: loop-mediated isothermal amplification) have been described, but they are still too expensive to be used in veterinary medicine and the superiority of their performance has yet to be demonstrated with field samples, or in competition with the ‘gold standards’, which are still monospecific PCRs by amplification of satellite DNA.

The main theoretical advantage of LAMP is to eliminate the need for a laboratory and to allow a direct visual reading of the amplification results. However, the latter is often debatable and gel migration is ultimately required, which negates the whole advantage of the method, which, moreover, frequently returns false-positive results. For these reasons, LAMP has not been widely adopted for the diagnosis of trypanosomes.

Table 3: Characteristics and sequences of the main primers to diagnose trypanosomes by multi-specific PCR

Specificity	Name and sequence of the primers	Locali-sation	Size of the products (bp)	T° hybrid	REF
T.brucei, T. vivax, T. congolense	TRYP1S : CGTCCCTGCCATTTGTACACAC	ITS1	Tb 520, Tv 310,	55	Desquesnes et al
Savannah & forest, T. lewisi	TRYP1R : GGAAGCCAAGTCATCCATCG	rDNA	Tcs-Tcf 680-750, Tl. 623		KBD 2002
T.brucei, T. vivax, T. congolense	TRYP4S: 5' AAGTTCACCGATATTG 3'	ITS1	Tb 487, T.v 242, Tcs 697	45	Desquesnes + DEA B.
savannah, forest & kilifi	TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA	, Tcf 727, Tck 627		DESCAMPS 2003
NESTED: T.brucei,	R1-TRYP18.2C: 5' GCAAATTGCCCAATGTCG 3'	ITS1		51	Desquesnes
T. vivax, T. congolense	R1-TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA			+ DEA B.
savannah, forest	R2-IRFCC: 5' CCTGCAGCTGGATCAT 3'	ITS1	Tb 392, Tv 147, Tcs 602,	47	DESCAMPS
& Kilifi	R2-TRYP5RCG: 5' ATCGCGACACGTTGTG 3'	rDNA	Tcf 632) Tck 532		2003
NESTED: T.brucei,	TRYP18.2C: 5' GCAAATTGCCCAATGTCG 3'	ITS1		51	Desquesnes
T. vivax, T. congolense	TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA			+ DEA B.
savannah, forest	IRFCC: 5' CCTGCAGCTGGATCAT 3'	ITS1	Tth 310, Tb 426, Tv 181,	47	DESCAMPS
Kilifi & T. theileri	TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA	Tcs 636, Tcf 666, Tck 566		2003

4. Serological tests

Several antibody detection techniques have been developed for the diagnosis of animal trypanosomosis, with varying degrees of sensitivity and specificity. The method of choice for an individual examination is indirect immunofluorescence (IIF). The most suitable method for population studies is ELISA, which remains the gold standard among these techniques. The identification of major antigens of trypanosomes and their production as recombinant molecules or synthetic peptides could lead to the development and validation of new tests based on the use of these molecules, which would make it possible to reach a level of standardisation inaccessible to techniques using total parasite extracts. Nevertheless, to date no recombinant antigen has proved sufficiently effective to compete with the classical ELISA.

IIF and ELISA techniques for the detection of antibodies have been adapted to analyse blood samples collected on filter paper. Blood in a heparinised capillary microtube is poured onto a filter paper (Whatman® No. 4). The samples are air-dried in the dark and then placed in a plastic bag with a desiccating substrate. The bag is sealed and should be kept cool if the samples are not refrigerated or frozen.

4.1 Indirect immunofluorescence test

For the preparation of slides, the parasites are grown in mice or rats and blood is collected at peak parasitaemia. Originally, blood smears were prepared, but when the method for separating trypanosomes and blood cells on a DE52 column became available, the parasite separation step was added to the protocol so that only the parasites were fixed on the slides. These original methods have been replaced by a technique for preparing trypanosome antigens that involves fixing live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in physiological saline.

Protocol

i) Prepare thin smears from heavily parasitaemic blood or, better still, from a trypanosome suspension. Air dry and fix in acetone for 5 min;

ii) Draw circles 5 mm in diameter on glass slides using nail varnish;

iii) Using a pipette, place the test serum diluted 1/40 in each circle ensuring that the area in each circle is completely covered;

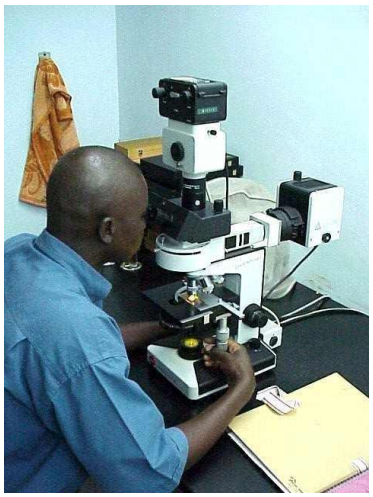
iv) Incubate the antigen/test serum preparation at 37°C for 30 min in a humid chamber;

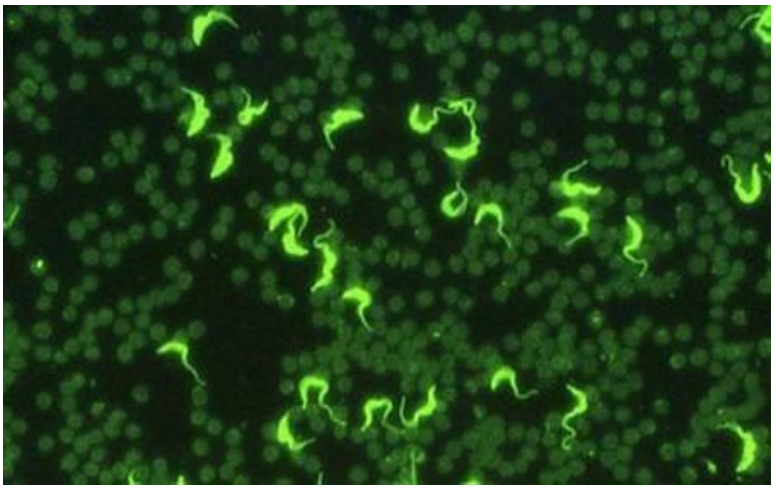
v) Wash the preparation three times in PBS for 5 min each time at 4°C, with gentle agitation. Air-dry the slides;

vi) Apply the 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.





If the examination shows marked fluorescence the result is not in doubt, but when it is moderate, the interpretation will vary according to the observer and the context, rendering the examination subjective and therefore not very reliable in cases close to the limit of positivity.

4.2 IgG Antibody detection enzyme-linked immunosorbent assay

Although detection of IgM is theoretically possible, due to the inconsistancy and variability of the results obtained for IgM detection (inconsistant presence of IgM in the serum due to immun-complexe formation and to its short half-life), only IgG detection is routinely carried out by ELISA for detection or follow-up of trypanosomoses.

The ELISA method has been developed for use in large-scale surveys of bovine trypanosomosis. Plates coated with soluble antigens of various species of trypanosomes are used in routine examinations; the method is also applied in other host populations, including buffaloes, horses, sheep, goats, pigs, dogs, elephants, etc. Plates pre-coated with *T. congolense* and *T. vivax* antigen present the advantage of using standardised denatured antigen that can be stored for long periods at room temperature.

The standard antigen for antibody detection tests is derived from trypanosomes present in the blood. Antigens are prepared as a soluble fraction of trypanosomes purified by DEAE anion-exchange chromatography of parasites from whole blood of infected rats, with lysis using several freeze–thaw cycles and centrifugation to eliminate particles.

4.2.1 Protocol for the preparation of soluble antigens

a) Cultivation of parasites and their harvesting from blood

Rats are used in preference to mice in order to obtain sufficient volumes of blood. The parasites are cultivated in rats immunosuppressed by IP injection of cyclophosphamide (200 mg/Kg) 2 to 3 days prior to injection of parasites, in order to generate very high levels of parasitaemia. At peak parasitaemia (in the ascending phase of parasitaemia, when it has reached 10 million parasites /ml), the animals are anaesthetised before being bled by direct cardiac puncture or by opening the thorax and excising a lung after the addition of several drops of citrate buffer. The blood is collected using a pipette and citrate is regularly added to prevent coagulation. After collection, the blood (more than 10 ml are usually collected from an adult rat) is diluted 1:4 in phosphate buffered saline glucose (PSG) (1 volume of blood + 3 volumes of PSG), then centrifuged at 5000 rpm for 10 minutes at 4°C to collect the buffy coat, which will be placed in a previously prepared DE52 column. With 10 ml of blood collected from a rat, the volume of buffy coat harvested and placed in the DE52 column will be approximately 4-6 ml. The quantity of antigens prepared from a given rat will on average be sufficient to perform 10 000 to 20 000 ELISA tests in the conditions described below.

Handling of rat

Intraperitoneal injection
(M. Desquesnes)

b) Preparation of buffers

Stock solution of phosphate saline: PS pH 8

Na_2HPO_4 (anhydrous)	13.48 g	NaH_2PO_4, $2H_2O$	0.78 g
NaCl	4.25 g	H_2O sqf	1000 ml

Solutions of different ionic strengths are prepared by diluting PS, pH 8, in distilled water and adding glucose. The PSG solution must be sterile (the PS must be autoclaved before adding glucose to avoid the glucose becoming caramelised).

- For blood from mice, rats, ruminants and dogs, add 4 parts of PS to 6 parts of distilled water and adjust the final concentration of glucose to 1%, giving an ionic strength of 0.145.
- For blood from pigs and rabbits, add 3 parts of PS to 7 parts of distilled water and adjust the final concentration of glucose to 1.5%.
- For the separation of *T. lewisi* it is preferable to use 6 parts of PS to 4 parts of water to increase the ionic strength (0.217), then adjust the glucose concentration to 1%. Nevertheless, the results obtained in this species are rather inconsistent.

Preparation of the phosphate buffered saline glucose (PSG pH 8):

Buffer solution	Volume **PS**	Volume H2O	% w/vol glucose	Ionic strength
PSG: 6:4	6	4	1%	0.217
PSG 5:5	5	5	1%	0.181
PSG 4:6	**4**	**6**	**1%**	**0.145**
PSG 3:7	3	7	1.5%	0.109
PSG 2:8	2	8	2.5%	0.072
PSG 1:9	1	9	5%	0.036

To prepare 500 ml of PSG 4:6, prepare 200 ml of PS + 300 ml of distilled water, and add 5 g of glucose.

Depending on the host species, composition of the buffer may be adjusted as indicated in the next table:

Host species	PS	H20	Glucose	Ionic strength
Rat/mouse	6	4	1%	0,217
goat	4	6	1%	
sheep	4	6	1%	
cattle	4	6	1%	
Guinea pig	4	6	1%	
pig	3	7	1 .5%	
rabbit	3	7	1.5%	
human	5	5	1%	0,181

c) Preparation of the cellulose

To estimate the required volume of DE52, one gram of dry DE52 will give approximately 1.5 ml of cellulose suspension. Never use metallic objects (stirrer, spatula, etc.) when stirring or manipulating DE52.

To estimate the required volume of DE52, approximately six times more DE52 will be needed than blood or buffy coat loaded onto the column. For example, for a rat, if 3 ml of buffy coat has been collected, an average of 18 ml of DE52 (i.e. 12 g of DE52 will need to be prepared; taking into account losses and dead volumes, 15 g of DE52 should be prepared.

Place it in 100 ml of distilled water for a first rinsing.

Mix for 20 minutes with a plastic-coated magnetic stirrer (no metal in contact with DE52) at low speed (do not break the cellulose) and then adjust to pH 8 using phosphoric acid.

Allow to settle for 30 minutes. Discard the supernatant fluid containing fine granules of cellulose as they could block the column.

Repeat the procedure twice (rinse, adjust the pH and discard the supernatant).

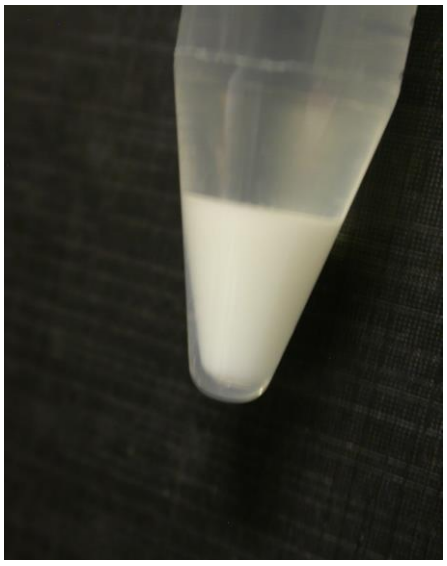
After final settling, remove the supernatant and add a volume of water equivalent to the remaining DE52 volume; then store the cellulose in the refrigerator until use.

d) Preparation of the column and equilibration of the DE-cellulose

Place a 50-ml syringe upright on a test-tube rack, preferably with a central nozzle, connected to a rubber tube that can be closed with a clamp to act as a tap (when a large volume of blood is to be separated a flat-bottom porcelain funnel can be used). Cut two discs of Whatman No. 41 filter paper to exactly the same size as the interior of the syringe (or funnel). Place one of the discs at the bottom of the syringe (it must be able to drop down and lie flat at the bottom of the syringe without being pushed, otherwise it will lose its shape). Moisten the filter paper with a few drops of PSG; add a sufficient volume of buffer to allow it to flow and fill the rubber tube, and then close the clamp. Check there is no air in the pipe. Shake the DE52 and gently pour the 50 ml into the syringe without disturbing the filter paper. Allow to settle for 5 minutes before elution of the buffer. Place the second disc of filter paper on top of the column. Check that it always remains in the buffer and do not allow too much buffer to escape. The top of the column must NEVER be dry. Wash the column with 50 ml of PSG then close it until the sample is added.

e) Separation of parasites on the DE-cellulose column

Gently place the 3 ml of blood or buffy coat on the filter paper on the top of the column; open the column and allow the sample to penetrate the cellulose. When the whole of the sample has penetrated the cellulose, close the column to avoid it drying and gently pour the PSG on top taking care not to disturb column of cellulose (pour the buffer very gently onto the filter). Open the column again. Gradually add 20 ml of PSG and start to collect fractions as soon as the parasites appear at the bottom of the column. Check the arrival of the parasites by regularly examining a drop of eluate between a slide and cover-slip with a dark-field microscope (magnification X400 to X500). The fractions are collected on ice in Falcon tubes. When the trypanosomes start separating, the liquid collected in the bottom of the column becomes opalescent (see figure below: centre). When the liquid has become limpid again check the collection is complete by examining a drop under the microscope. Centrifuge the collected liquid at 1000 ***g*** (or approximately 5000 rpm) for 10 minutes to allow the parasites to settle, in the form of a pellet (photo on the right); discard the supernatant. Wash twice with PBS.



Glass funnel Syringe (opalescent buffer) Pellet of parasites after centrifugation (M. Desquesnes)

The parasites are then suspended in distilled water; the volume of the buffer should be 20 to 30 times greater than the volume of the parasite pellet. Adding an anti-enzyme cocktail will avoid the lysis of proteins on thawing. For this purpose, use Complete Protease inhibitor cocktail (Roche, ref. 11697498001 [20 tablets] or ref.11836145001 [3 X 20 tablets]), by placing 1 tablet in 2 ml of distilled water, then adding 40 µl of this cocktail per ml of parasite lysate. Freeze, preferably at -80°C or in liquid nitrogen.

e-bis) Separation of trypanosomes by differential centrifugation

An alternative to this procedure is differential centrifugation of the blood; this enables moderate quantities of parasites to be separated and provides a limited degree of purification since blood cells or blood cell debris are still present; nevertheless, this method can be useful for experimental preparations.

At the peak of parasitaemia, blood is collected in the same way as before, diluted 1:6 in 1% PSG 1then centrifuged at low speed (200 ***g*** or about 1200 rpm X 5 minutes), causing the sedimentation of blood cells but not that of trypanosomes, which have slighter higher sedimentation parameters. The method is effective for large trypanosomes but slightly less so for small trypanosomes, such as some types of *T. congolense* which tend to have fairly similar sedimentation parameters to those of white blood cells. After a first centrifugation (5 minutes at 1200 rpm), the plasma containing the parasites is harvested. The pellet of cells can then be resuspended in PSG and a second centrifugation performed at low speed, to harvest again the parasites which will be in the supernatant. The supernatants from the 2 centrifugations at low speed are then combined and centrifuged at a higher speed (800 ***g*** or about 6000 rpm X 10 minutes) to allow the parasites to settle and to discard the plasma- rich supernatant. The pellet can be gently resuspended in PSG and washed again, and the supernatant discarded. This step must be performed twice consecutively to ensure that the parasites are washed thoroughly. At this point, if the aim is to prepare soluble antigen, the parasites are then suspended in distilled water, in a volume of buffer 20 to 30 times greater than the volume of the parasite pellet. An anti-enzyme cocktail is then added prior to freezing and the rupture of the parasites.

A similar protocol has been proposed (Davita Pillay), which consists of initially diluting the blood 1:8 in PSG with 0.5% glucose, then centrifuging at 200 ***g*** X 5 minutes and harvesting the supernatant, which is then centrifuged at 300 ***g*** X 5 minutes. The second supernatant is harvested and centrifuged at 1800 ***g*** for 10 minutes. The third supernatant is discarded and the pellet resuspended until used. This method might be more suitable for *T. congolense*.

f) Preparation of soluble antigens

The parasite lysate is thawed in a water bath at 20°C. Carefully monitor the thawing process and as soon as the ice has disappeared plunge the tube back into the liquid nitrogen to avoid heating the lysate.

Perform 5 cycles of freezing in liquid nitrogen / thawing in a water bath at 37°C (about 6 X 5 min). Leave in the cryotube or place in an appropriate flask for the sonicator (carefully clean the probe) and apply 3 cycles of sonication on ice (power 60 W) for 1 minute (output

power 7). Then perform 1 cycle of freezing in liquid nitrogen / thawing in a water bath at 20°C. After thawing, centrifuge the lysate at 8000 rpm for 15 min at 4°C and recover the supernatant, which comprises the soluble antigen; discard the pellet containing cell debris and insoluble particles.

Water bath & liquid nitrogen bath for freeze/thaw cycles

Sonicator

f) Quantitation of proteins

A UV reader can be used.

As the optical density of the lysate is normally too high for a direct reading, dilutions are made at 1:5, 1:10 and/or 1:20, and the dilution buffer used (PBS or distilled water) serves as the blank.

The ODs of soluble antigens are measured at wavelengths of 260 and 280 nm.

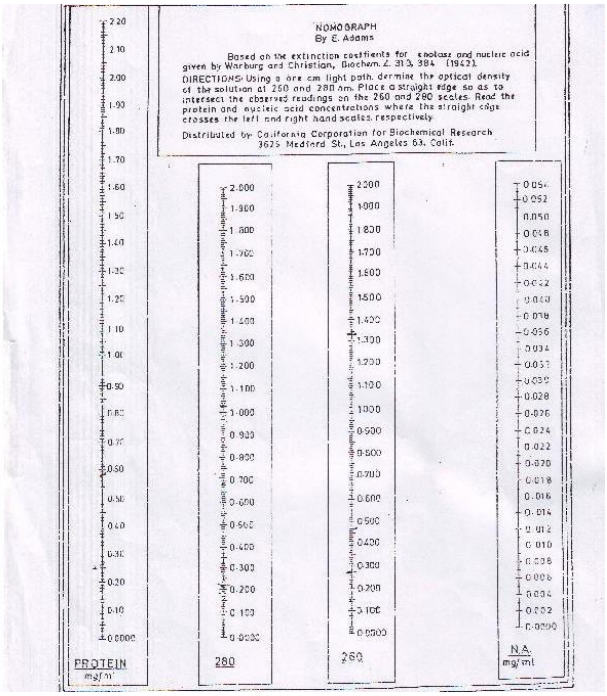
To determine the protein concentration of the measured dilution, a nomograph based on the Warburg-Christian method is used. After correction (X10 or X20) depending on the measured dilution, determine the protein concentration of the soluble antigen, which should normally be between 1 and 10 mg/ml. The antigen is aliquoted into cryotubes, labelled as follows: Ag Sol *species*, date, protein concentration in mg/ml. Store the aliquoted antigen (at approximately 2-6 mg/ml) at –80°C, in aliquots of 100 µl.

Example of measurement of the protein concentration of antigens

OD	260 nm	280 nm	protein concentration	final
Dilution 1:10	1.953	1.387	0.71	7.1 mg/ml
Dilution 1:50	0.387	0.259	0.13	6.5 mg/ml

The mean concentration of this antigen is therefore considered to be 6.8 mg/ml.

A more modern, fast and effective solution is the NanoDrop, a device that uses microvolumes and is based on the same principles of spectrophotometry.



Nomograph for measuring DNA and protein concentrations NanoDrop

Bradford technique

The Bradford method is a colorimetric assay based on the modification of the absorbance of Coomassie blue, linked with a complexification of the links of Coomassie blue with the basic amino acids (arginine, histidine, lysine) and the hydrophobic residues of amino acids of the proteins present in the trypanosome lysate. A change in the colour of the stain (from reddish brown to blue) occurs in the presence of proteins in the sample being tested and is proportional to the concentration of proteins. The maximum absorption spectrum is estimated at 595 nm. The Bradford method is less sensitive to interference by various agents present in the protein samples. However, it is affected by detergents, is modified by the pH and also gives a positive result to hydrosoluble polyphenols of high molecular weight (tannins).

To determine the protein concentration of a sample, prepare a solution of bovine serum albumin (BSA) at 5 mg/ml, which will be used to prepare the range of calibration by serial dilutions at 1:2. Add 10 µl of each dilution or sample and 90 µl of Bradford solution (Sigma) into the microtitre plate wells; after an incubation period of 5 minutes at room temperature, absorbance at 595 nm is measured using a spectrophotometer. Determine the calibration curve and the corresponding linear regression line and equation. The OD value of the

sample should be within the OD values of the linear regression line. Simply introduce the OD of the sample in the linear regression line equation to obtain the protein concentration of the sample.

Bicinchoninic acid technique

The proteins reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{1+}) in an alkaline medium. Bicinchoninic acid is a highly specific chromogenic reagent for Cu^{1+} that forms a purple complex having peak optical absorbance at 562 nm (can be read between 540 and 590 nm). Absorbance is in proportion to the concentration of proteins. A calibration range is prepared with serial dilutions at 1:2 with a standard protein (e.g. bovine serum albumin [BSA]). Mix 50 µl of the dilution or the sample to be assayed and 150 µl of copper sulphate bicinchoninic acid solution. After incubation for 20 minutes at 37°C, reading is done with a spectrophotometer set at a wavelength of 550 nm. Determine the calibration curve and the corresponding linear regression line and equation. The OD value of the sample should be within the OD values of the linear regression line. Simply introduce the OD of the sample in the linear regression line equation to obtain the protein concentration of the sample.

4.2.2 ELISA assays

The soluble antigens of 3 or 4 species of trypanosomes are regularly used in ELISA assays: *T. vivax*, *T. congolense* savannah and *T. brucei brucei* or *T. evansi* (the latter two give quite similar results). The parasites are produced as described above, using laboratory rats. The soluble antigens are kept either at -80°C for long-term storage or at -20°C in the case of working aliquots for use in the medium or short term. The classical steps of an ELISA assay are: sensitisation (coating), blocking, serum incubation, washing, conjugate incubation, washing, substrate-chromogen incubation, reading.

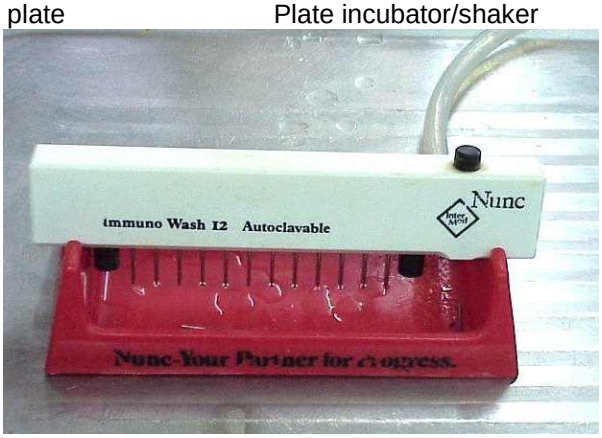
The equipment required consists of sets of pipettes and multichannel pipettes, plate incubator-shaker, manual or automatic plate washer, UV spectrophotometer connected to a computer (ELISA reader). Optical densities are transferred and entered into an Excel file, where the data are automatically reorganised and the final result is provided.

The Excel file is programmed to facilitate the presentation and interpretation of the data and performs several simple tests to check that the plate for validity.

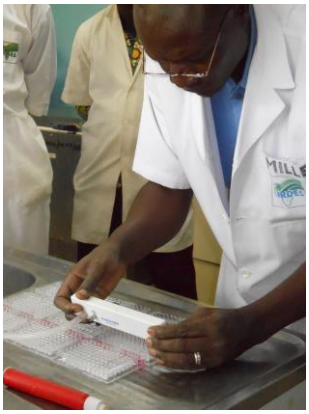
Preparation of the ELISA plate

Plate incubator/shaker

Automatic washer

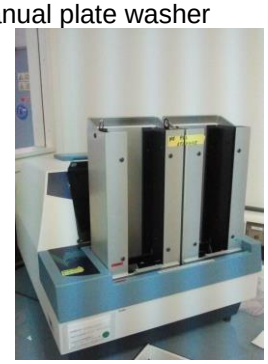


Manual plate washer



Using a manual washer

ELISA UV plate reader



ELISA multiplate UV-reader

Plate with substrate-chromogen

Preparation of buffers:

PBS X 1: Na_2HPO_4 1.44g + KH_2PO_4 0.24g + NaCl 8g + KCl 0.2g + water QSP 1 litre

Or, with anhydrous products:

PBS X 1: Na_2HPO_4 1.21g + KH_2PO_4 0.20g + NaCl 8g + KCl 0.2g + water QSP 1 litre

pH 7.4 to be adjusted with H_3PO_4 or NaOH.

Carbonate buffer: Na_2CO_3 1.58g + $NaHCO_3$ 2.93g + water QSP 1 litre, adjusted to pH 9.6 with HCl.

Washing buffer (WB): PBS + 0.1% Tween 20 (1ml / litre)

Blocking buffer (BB): 100 ml de WB + 5 to 7 g powdered milk (or 0.2 g of casein)
Substrate buffer-chromogen (for ABTS): citric acid ($C_6H_8O_7$) 9.6g per litre of water. Adjust to pH 4.
H_2O_2 1.875%: H_2O_2 30% 500µl + 7.5ml of water, store in a cool (4°C) dark place; ABTS 20mg/ml: ABTS 135mg + 6.25 ml of water, store in a cool (4°C) dark place.

Implementing the ELISA protocol:

Sensitisation: Nunc Immunosorp flat bottom 96-well plates are used, with a carbonate coating buffer. Dilute the trypanosome soluble antigen to a final concentration of 5 µg/ml. Place 100 µl per well, overnight at 4°C or for 2 hours at 37°C on a shaker at 300 rpm.
Blocking: Empty the plates by inversion and place 150 µl of blocking buffer (BB) per well; place on a shaker at 300 rpm for 30 minutes at 37°C;
Serum dilution: in the case of serum or plasma, perform an initial dilution at 1/10 in a round-bottom polypropylene plate (U); this operation can be done during blocking, with BB (or with WB + 0.2% BSA).
Transfer: after blocking, empty the plates by inversion, rinse once with PBS then add 90 µl of BB. Transfer rapidly, using an 8-channel pipette, 10 µl of diluted serum to 2 horizontally neighbouring wells (final dilution 1/100); place on a shaker at 300 rpm for 30 minutes at 37°C;
Washing: Empty the plates by inversion and fill the wells with washing buffer (WB); empty and wash 4 times; empty the wells and tap the plates vigorously on absorbent paper towels;
Conjugate: Place 100 µl of conjugate diluted in the BB (see dilutions according to the conjugate in the appendix); place on a shaker (300 rpm) for 30 minutes at 37°C;
Washing: Empty the plates by inversion and fill the wells with washing buffer (WB); empty and wash 4 times; empty the wells and tap the plates vigorously on absorbent paper towels;
Substrate-chromogen: add to each well 100 µl of substrate-chromogen: either Kblue or TMB, or a mixture of citric acid 25 ml + 125 µl ABTS + 100 µl H_2O_2. Keep in the dark for 30 minutes at laboratory temperature.
Reading: at the appropriate wavelength for the chromogen (Kblue and TMB: 620 nm; ABTS: 405 nm).

Using sera spotted onto filter paper

For storage at room temperature and especially for dispatching by post, a practical solution is to spot the serum onto filter paper; place one or two 100-µl spots of serum on Whatman No. 1 paper, well apart. Then, using a pencil, draw a circle around each of the two moistened zones (this generally results in circles of about 2.5 cm in diameter) and write the reference number on the sample. When the filter paper is full, allow it to dry and then sandwich it

between 2 sheets of A4 paper, stapled to prevent any movement of the filter paper between the A4 sheets and to avoid contamination between the samples. When the samples are thoroughly dry, these 'sandwiches' can be inserted in a hermetically sealed plastic bag for shipping. The antibodies degradation on filter paper can be fast and the test must take place within one month after the deposition of the serum on filter paper. In the case of camelids, which have a particular antibody structure, the use of frozen serum should be preferred.

On receipt, the Whatman No. 1 filter paper is cut into small pieces (2-4 mm^2) with scissors and placed in a microtube 1.5 ml with 500 µl of PBS, triturated with a pipette, then put on a rotating shaker (shaker-incubator) at 300 rpm for 2 hours at 25°C. The result is a serum diluted 1/5 that gives virtually the same result in ELISA as a fresh serum.

4.2.3 ELISA standardisation

The reading of each ELISA microplate is referenced against three positive reference sera (strong, medium and weak) and three negative reference sera (strong, medium and weak), which are necessary in order to define control values within the context of quality control. The value of each sample tested by ELISA is expressed as a percentage of the mean of the positive reference standards and of the negative reference standards (RPP: relative percentage of positivity), according to the following relationship:

$$\text{RPP sample} = \frac{\text{mOD sample} - \text{mOD negative controls}}{\text{mOD positive controls} - \text{mOD negative controls}}$$

The results are thus quantifiable. A positivity threshold is generally determined on the basis of the results obtained in a population of uninfected animals (mean plus 2 or 3 standard deviations), and its plausibility is checked with samples of known status or, better still, obtained under experimental conditions. Ideally, the reference samples placed on the plate should be representative of infected populations, positive, and non-infected populations, negative (their RPP values should be close to the mean values for each population). A detailed protocol is given in the appendix.

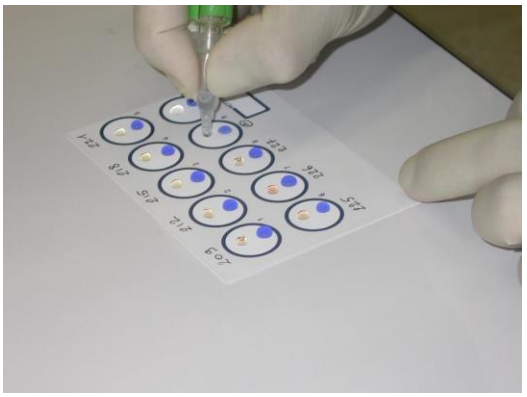
This test has a high sensitivity and specificity at genus level; however, species specificity is generally low. IIF and ELISA detect an immune response to current and past infections and consequently can only give rise to a presumptive diagnosis of infection. Nevertheless, when the sample has been collected more than 6 months after treatment, seropositivity is highly suggestive of active infection. Immunodiagnosis requires expensive and sophisticated equipment and appropriate experience, which are not always available. It is carried out in specialised laboratories and there is a considerable delay between sample collection and

obtaining the results. Nevertheless, ELISA lends itself well to a high degree of automation and standardisation. The collection of samples and their shipping by post has been made easy by the use of filter paper. All these factors make ELISA a very useful test for large-scale surveillance of the distribution of tsetse-transmitted trypanosomoses.

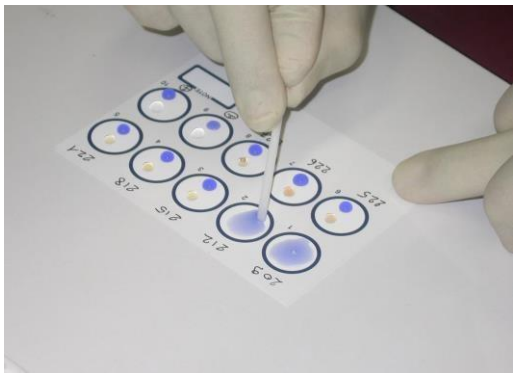
4.3 Detection of IgM antibodies

The CATT (Card Agglutination Test for Trypanosomes)/*T. evansi* is an agglutination test that can detect recent infections with *T. evansi*. It consists of mixing fixed parasites and serum (or blood) containing immunoglobulins M (IgM). IgM are pentavalent and can fix several parasites to form a network of agglutinates visible to the naked eye after incubation and rotary agitation of the mix for several minutes.

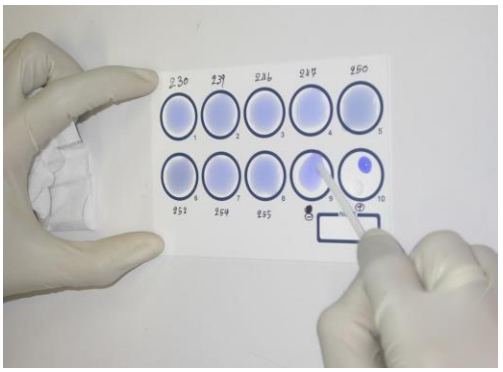
Reagents: buffer, antigen and control sera



Placing of antigen and then diluted serum

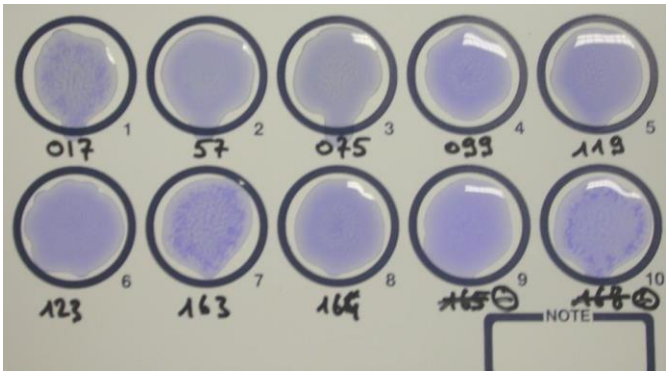


Mixing of antigen and serum



Mixing of reference samples

Rotary shaker at 70 rpm



Reading the results

Although the test was developed with *T. evansi*, it can be crossed with other trypanosome species and thus also detect IgM antibodies directed against *T. vivax* and, to a lesser extent, those directed against *T. congolense*. As IgM antibodies are consumed during IgM / parasite immune reactions, their availability in the blood fluctuates, which means that the test can give false-negative reactions (when the IgM antibodies have been consumed in eliminating a large population of parasites). Conversely, non-specific agglutinations are sometimes observed and regularly give 2% to 5% or more false positives. This proportion can vary from one population to another and from one host species to another. The test remains quite useful for detecting infected animals in recent outbreaks, where it has a high positive predictive value. It has good sensitivity in susceptible host species, such as horses and camels, but it is low, or very low, in host species with low susceptibility to *T. evansi*, including cattle and buffaloes. As this is a commercially available test, the protocol is supplied with the test kit. For the record, it is included in the appendix.

4.4 Immune trypanolysis test

The immune trypanolysis test (ITT) detects specific antibodies directed against variable surface antigens of live trypanosomes; it is based on the ability of complement to bind to antigen–antibody complexes and provoke the lysis of trypanosomes by successive cleavage of their components. The test requires the availability of live trypanosomes and guinea-pig complement. The test is difficult to set up, costly and time-consuming and requires the sacrifice of several animals. Despite these drawbacks, the ITT has a major advantage: it is a functional test that shows the presence of protective antibodies, and can therefore reveal infections that are undetectable by parasitology. Developed for use in humans with *T. b. gambiense*, it uses live trypanosomes expressing 3 types of antigenic variants (LiTat 1.3, LiTat 1.5, and LiTat 1.6). The test is positive when the percentage lysis of trypanosomes is greater than 50% for at least one of the three variants. The test can be performed on serum, plasma or blood on filter paper (25 µl of blood on Whatman No. 4 filter paper). An ITT developed with *T. evansi* RoTat 1.2 is carried out at the OIE Reference Laboratory for Surra, at ITM in Antwerp. The method is not available for the other species of trypanosome (*T. vivax*,
T. congolense and *T. brucei*) as one would first need to identify strains presenting universal variants. In view of its drawbacks, this method is not recommended for routine diagnosis in animals; it might however prove useful as a sensitive screening test for animals from a surra-infected area prior to export to uninfected areas.

5. Recommendations on using the tests

The tests and methods described in this compilation are, like most biological tests, limited in terms of sensitivity and specificity. Furthermore, test performance and parameters will vary considerably between species and from one geographical location to another. For example, there is no parasitological or serological test that can distinguish *T. evansi* from other *Trypanozoon* (*T. brucei* ssp.), and, to date, even molecular tests have difficulty in distinguishing *T. evansi* from other *Trypanozoon* (*T. brucei* ssp.), since there is no single PCR test capable of characterising *T. evansi*. Consequently, the diagnosis of “surra” is most often the result of a combination of epizootiological information and laboratory results and observations. For these reasons, this paragraph recommends a combination of tests and measures adapted to a variety of contexts and to the most important host species. The guidelines and advice given here can be useful but they will need to be adapted to any new situation to reach a diagnosis that is as sensitive and accurate as possible and is adapted to the study objectives. Any situation where animals from an infected zone are to be imported into a disease-free zone will require very strict controls using the most sensitive methods.

5.1 Recommended diagnostic methods

5.1.1 Microscopic examination

Microscopic observation (x400–1000 in oil immersion) of a Giemsa-stained thin blood smear from the host or from a laboratory rodent acting as an amplifier, allows identification of the subgenera *Trypanozoon*, *Duttonella*, *Nannomonas*, *Herpetomonas*, *Schizotrypanum* or *Megatrypanum*, based on morphological and morphometric criteria of the parasites.

When fresh samples are available, HCT or BCM tests can be used to increase sensitivity; motility criteria can also be useful in identifying the parasites.

When high sensitivity is required (e.g. suspect animals in a free zone or animals from an infected zone for export to a free zone), the HCT method and inoculation of laboratory rodents are recommended.

5.1.2 PCR

For better sensitivity, DNA should be prepared from buffy coat obtained by 8000 *g* centrifugation of 0.5 ml of blood on anticoagulant, failing which it can be prepared with blood,

preferably using the more sensitive phenol chloroform method, or else with a commercial kit (Chelex, Qiamp, etc.). PCR examinations are performed as described above and, according to the case, with various sets of primers, or even with all the available sets of primers if it is not known which species might possibly be present in the sample. PCR is very sensitive but does have its limits: when parasitaemia is below 1-20 trypanosomes per ml, PCR examinations are usually negative. Furthermore, contamination can sometimes lead to false positives. Strict controls and precautions are therefore required.

5.1.3 ELISA

The ELISA methods are poorly species-specific and cross-reactions between subgenera are variable and sometimes quite strong; several tests can therefore be applied depending on the context: ELISA *T. congolense* (savannah), ELISA *T. vivax*, ELISA *T. brucei* (low sensitivity in cattle), ELISA *T. evansi*, etc. The scores from the various tests can even be compared to deduce which species are present, but these methods are tricky to interpret. The serum or plasma samples are tested using genus-specific conjugates, which limits their use in genera for which specific conjugates are not commercially available (camels, elephants, deer, etc.). In these cases one can use a conjugate of protein A (validated in camels) or protein G (evaluated in elephants in Asia). In these particular cases and/or when positive and negative reference samples do not exist, only optical density can be considered. In such cases only a suspected diagnosis can be made.

ELISA methods for trypanosomes have been widely evaluated, used and validated in infected geographical sectors (Africa, Latin America and Asia). In other sectors, in the absence of need, no preliminary evaluation has been carried out. Caution is needed when applying these methods. For example, when ELISA *T. evansi* was used in France for the first time, it produced high rates of false-positive results in sheep (a phenomenon that was not observed in cattle or horses). VSG ELISA was also applied in this batch of sera and revealed a similar rate of false-positive samples, but not necessarily the same samples. The source of these serological cross-reactions has never been identified. In such cases, recourse to the immune trypanolysis test could present advantages, which would need to be evaluated, but its high cost is in most cases a major obstacle.

5.1.4 CATT/*T. evansi*

Serum or plasma samples are diluted at 1:4 and tested as described by the manufacturer. Positive samples are those presenting results ≥1+, doubtful samples being considered

negative. The test is not species-specific, and it has been demonstrated that it cross reacts, notably with *T. vivax*. The test regularly has good positive predictive value in horses and camels. Unfortunately, it clearly presents false-positive results, notably in horses, and false-negative results, especially in cattle (demonstration performed in Thailand under experimental infection conditions). It is therefore of limited diagnostic value, and an animal cannot be declared “infected” solely on the basis of a positive CATT test, other tests being needed to confirm these results.

5.2 Recommended methods in equids and camelids for surra

International trade and movement of animals are particularly important in the case of sports horses and camels, strict controls are therefore required, especially for surra. The following principles can be applied (fuller recommendations based on these tests and statuses are given in paragraph 4.4):

- An equid or camelid is negative to surra if it is negative to the following tests: ELISA-*T. evansi* (anti-horse IgG whole molecule in equids and protein A conjugate in camels), CATT/*T. evansi*, PCR-TBR and microscopic examination of blood or buffy coat.
- An equid or camelid is considered infected by *Trypanozoon* spp. if it is positive on microscopic examination or after cultivation in rodents, and it is considered suspected if positive to the PCR-TBR test (or other primers allowing detection of *T. evansi*); in the latter case it must be re-sampled and tested again 1 week later; if it tests positive again, its status as suspected is confirmed.

- An equid or camelid is considered seropositive to surra if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*. Equids should, in this case, undergo a complement fixation test for dourine (CFT-dourine); if it is positive for CFT-dourine it is also considered as seropositive to dourine; if it is negative to CFT-dourine, it is considered as seropositive to surra only.

Please note that the analysis report will indicate only that an animal is “negative to tests for surra” and not that it is uninfected or disease-free: absence of proof of infection is not proof of absence of infection.

5.3 Recommended methods in other species for all trypanosomoses

An animal is negative to tests for African trypanosomosis if it is negative to the following tests:
- ELISA-*T. evansi*, ELISA *T. vivax*, ELISA *T. congolense* (savannah) and ELISA *T. brucei* (for conjugates, see below);
- CATT/*T. evansi*;
- PCR with the following primers: TBR, TVW, TCS, TCF, TCK, and TRYP1 or nested ITS1; in the case of positivity with only the last 2 sets of primers (which amplify ITS1 of ribosomal DNA), all the other specific primers should be used (detection of *T. simiae, T. godfreyi*, *T. theileri* etc., see PCR paragraph);
- Microscopic examination preferably of buffy coat but otherwise of blood.

An animal is considered infected by an African trypanosome if it is positive to one of the PCR tests or to microscopic examination and if it was not possible to identify *T. theileri* (cattle). In such cases, attempts must be made to isolate the parasite by intraperitoneal inoculation of infected blood into rodents.

An animal is considered seropositive to African trypanosomosis if it is positive to at least one of the serological tests (ELISAs or CATT).
The conjugates should be used according to the host, as follows:
- Cattle, water buffalo: anti-bovine IgG whole molecule;
- Pigs: anti-pig conjugate;
- Pigs and hog deer (*Cervus porcinus*): Protein G conjugate;
- Sheep and goats: anti-goat and anti-sheep conjugates;
- Camels, Asian elephant: Protein A conjugate;
- Rats: anti-rat IgG whole molecule;
- Dogs: anti-dog conjugates.

Conjugates have yet to be defined for other host species.
Please note that the analysis report of a negative test is indeed “test negative”, and not “animal uninfected” or “animal disease-free”.

5.4 Proposed recommendations for international trade of equids or camelids

The high level of trade and the risk of introducing a parasite transmitted mechanically and therefore potentially cosmopolitan means that special measures need to be taken with equids and camelids being exported from an infected zone to a free zone.

The following proposals have also been submitted to the OIE Biological Standards Commission to supplement the chapter on surra in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*:

For international trade of equids and camelids from an infected zone to a free zone:
Two 4-week quarantine periods must be applied, the first in the **farm of origin** and the second in the **farm of destination** for international trade in equids and camelids **from an infected zone to a free zone**.

To be **authorised for sale, an animal** must be from a "**non-infected farm**" situated in a "**suspicion-free zone**" and be **"negative to all tests for surra"** on 2 occasions at an interval of 3 to 4 weeks in each of the quarantine periods.
A farm is situated in a "**suspicion-free zone**" if, within a 30-km radius of the farm, no cases of surra have been reported within the past 3 years.
Shall be considered a "**non-infected farm**", a farm situated in a "suspicion-free zone" that within the past 3 years has only introduced animals from non-infected farms (and therefore situated in suspicion-free zones) having been subjected to the 2 quarantine periods and having produced negative results to tests for surra.
To obtain the status of "**non-infected farm**", a farm must be situated in a suspicion-free zone, and all mammals on the farm must have tested negative on two occasions at an interval of 3 months.
To maintain this status, all mammals on the farm must produce negative results to tests every 10–12 months.

6. Laboratory notebooks

To enable quality to be monitored in a laboratory, it is essential to be able to trace all the operations carried out (traceability). For this purpose, a laboratory notebook is assigned to each technician or, more commonly, to each work station. Laboratory notebooks are permanently bound to avoid any pages being added or removed. Entries are to be made in indelible ink.

1) Date all events. Enter the name of the technician if there are several users.

2) Enter the referenced protocol being followed (contained in the compilation of authorised protocols).

3) Enter precise details of any operations carried out alongside or outside the referenced protocol.

4) Note down any change in reagent or stock and the references of the products used. Make a careful note of any deviations from the protocol (incubation time, concentration, new product, power cut, temperature deviation, stock outage, etc.). At this point, a link with the stock management book will make it easier to monitor stocks and place orders in time to avoid shortages.

5) Unusual observations, salient facts, events in parallel with the protocol. Provide figures wherever possible.

6) Indicate clearly and precisely where the samples or data were stored at the end of the protocol. Attach any photos (PCR) with comments and conclusions, even if they are only partial; this will help to trace the chains of logic leading up to the subsequent steps.

7) If necessary, and/or to save time, a check-list can be drawn up and the points completed ticked off as the protocol progresses.

8) At the end of the procedure, the work must be validated by the head of the laboratory.

7. Reference samples and strains

A reference laboratory for trypanosomes must be equipped with a collection of reference biological material, including reference parasite strains, DNA and sera.

7.1 Reference strains

Mammal trypanosome strains must be stored alive at the reference laboratory to have standard biological material available at all times and to be able to share this material with other laboratories, produce live parasites on request, and characterise strains of special interest by cultivating them on mammals, either livestock or rodents.

A pool of **reference parasite strains**, kept alive, will be defined during the twinning programme and will include international standard strains, local standard strains and strains characterised in various ways for specific properties, such as chemoresistance, pathogenicity, source of the host, geographical origin, etc.

A **list of reference strains** will be established and these strains will be cultivated in rats in order to prepare and distribute cryopreserved stabilates of these reference parasite strains to reference laboratories. This pool of reference strains must include a minimum of one to three strains of each species: *T. vivax, T. congolense* savannah*, T. congolense* forest*, T. congolense* Kenya coast*, T. brucei brucei, T. evansi, T. lewisi* (for identification in rat colonies) and if possible: *T. simiae*, *T. godfreyi*, *T. tsavo* and even *T. theileri* and *T. musculi* (for identification in mouse colonies).

Preparation of cryopreserved stabilates: live trypanosomes are preferably preserved in liquid nitrogen but in the short term can also be kept in a freezer at -80°C. The procedure is as follows: parasitized blood, preferably collected during the ascending phase of high parasitaemia, is mixed with a cryostabilising buffer comprised of PSG 1% (buffer obtained by the addition of 1 g of glucose per 100 ml of PBS) with 15% of glycerol (85 ml of PSG 1% + 15 ml of glycerol). The blood must be collected with an anticoagulant (EDTA, heparin or citrate), placed on ice as quickly as possible and the parasitaemia checked, and then the cryostabiliser added drop by drop is mixed with the blood in equal quantities. The stabilised blood is placed in cryotubes labelled as indicated below, then placed in a freezer box. The freezer box can either be purchased or made by placing an approximately 1-cm-thick layer of plasticine around a glass or plastic freezing container and then taped together with Elastoplast. The freezing container must be maintained at -20°C before use. When the cryopreserved stabilates are ready, remove the box from the freezer at -20°C, place the cryotubes inside and then leave the box overnight in a freezer at -80°C; the next day, plunge the tubes into liquid nitrogen. In the absence of a freezer at -80°C, the cryotubes can be

suspended in gaseous phase liquid nitrogen overnight, before plunging them into liquid nitrogen.

When the cryopreserved stabilates are to be used, remove them from the nitrogen and thaw at room temperature or in the palm of the hand; as soon as the cryopreserved sample is liquid again, place it on crushed ice until use. For cultivation in rodents, it is generally recommended to start with a single passage through a mouse (0.1 ml intraperitoneally) before transfecting rats for larger scale production.

Nomenclature for referencing cryopreserved stabilates: to be able to identify and distinguish an isolate, a strain (cultivated in rodent or *in vitro*), or even a clone, the referencing of parasites is important. Even if the species or subspecies in field samples have not yet been fully identified, they will be after one or more passages through rodents. It is therefore normal practice to indicate the individual species of trypanosome only after characterisation and cultivation in rodent. When cryopreserving biological material that has not yet been fully characterised, it is advisable simply to indicate optionally (in parentheses) the species and the name of the isolate. There are three different types of cryopreserved samples:

- **Field isolates** are blood samples collected from the host of origin and directly cryostabilised; the reference of the animal of origin is assigned to these isolates, which will subsequently be characterised (by PCR) and possibly cultivated in the form of single parasite strains, or sometimes multiple strains in the case of mixed infections, but the latter situation is rarely encountered as parasite strains are frequently excluded during passages in rodents. It may also happen that a trypanosome is identified on detection, in which case a species code can be given, but the species will subsequently need to be confirmed. These isolates are referenced in the following way, using codes: (putative species), species of the host of origin, host's reference number, place where isolation was performed, the date cryospreserved sample was prepared; all this information must be contained in a single line (see example below).

- P**arasite strains** are isolates that have been cultivated one or more times in laboratory rodents; they are assumed to have been precisely identified by PCR. These strains are referenced in the following way, using codes. The first line indicates: *Trypanosoma* species, species of host of origin, host's reference number, place where isolation was performed (town or province), year of isolation, and a second line indicates the number of passages in mouse (M#), the number of passages in rat (R#), the date the cryopreserved sample was prepared. The information on the second line is potentially variable, whereas the information on the first line will remain unchanged throughout the various passages in rodents.

- **parasite clones** are strains obtained by culture from a single specimen and cultivated one or more times in laboratory rodents; as several clones may be derived from the same strain, they are given an identifying reference code. The first line indicates: reference of the clone, *Trypanosoma* species, species of host of origin, host's reference number, place where isolation was performed (town or province), year of isolation, and the second line indicates the number of passages in mouse (M#), the number of passages in rat (R#), the date the cryopreserved sample was prepared.

The following codes should be used:

Trypanosoma species		Host species	
T. vivax:	TV	Beef cattle	BC
T. congolense savannah	TCS	Dairy cattle	DC
T. congolense forest:	TCF	*Bos taurus*	BT
T. congolense Kenya coast:	TCK	*Bos indicus*	Bi
T. simiae:	TSM	Water buffalo (*Bubalus bubalis*)	BB (or B)
T. brucei brucei:	TBB	Sheep (ovine)	OV (or S)
T. brucei gambiense:	TBG	Goat (caprine)	CP (or G)
T. brucei rhosesiense:	TBR	Horse	HH (or H)
T. evansi:	TE	Donkey (Mule DH	DD (or A)
T. equiperdum:	TEQ	Pig (*Sus scrofa*)	SS (or P)
T. lewisi:	TL	Dog (canine)	CN (or D)
T. lewisi-like:	TLL	Asian elephant	EM (or E)
T. musculi:	TM	*Rattus norvegicus*	RN (or R)
T. theileri:	TT	*Mus musculi*	MM (or M)
Trypanosoma species	Tsp	Other: initials of the name of the species	

The breed is not given; this information should be supplied in the accompanying data sheets.

Examples:

- the cryopreserved sample of a field isolate, collected at Gaoua from a *baoulé* named 'F32' on 25 November 2012, identified microscopically as *T. vivax* and placed directly in liquid nitrogen, will be referenced as:

(TV)BT-F32 Gaoua-25/11/12

- the cryopreserved sample of a strain of *T. evansi*, found at Nan, in Thailand, in a beef cow named 'P12', on 10 October 2010, initially isolated in mouse and cultivated twice in rat and placed in liquid nitrogen on 16 April 2012, will be referenced as:

Line 1 (invariable): TE-BC-P12-Nan2010
Line 2 (variable): M1R2-16/04/12

- the cryopreserved sample of a *T. congolense* savannah clone named IL1180, isolated in a lion in the Serengeti National Park in 1971, and passaged 5 times in mice and 3 times in rats, placed in liquid nitrogen on 20 December 2008, will be referenced as:

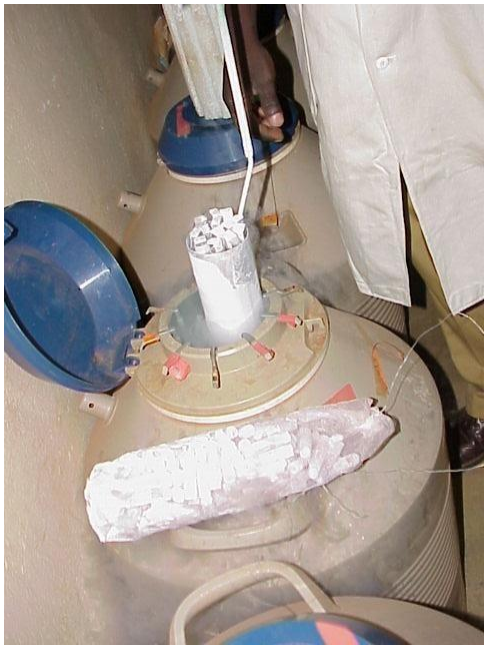
Line 1 (invariable): IL1180-TCS
Line 2 (variable): M5R3-20/12/08

7.2 DNA reference samples

DNA reference samples will be prepared from these parasite reference strains; they will be used as positive controls in the PCR assays, based on a range of known concentrations, and will be made available internationally to enable laboratories to put in place equivalent, referenced diagnostic systems.

To evaluate the sensitivity of the PCR reaction, the concentration of DNA in the positive control should not be too high, so as to detect any loss of sensitivity of the reaction. The semi-quantitative nature of the PCR will thus be used for quality control purposes. If necessary, 2 positive reference samples can be used for the PCR, one in the form of diluted purified DNA and the other in the form of a naturally positive field sample (blood from an animal) prepared using Chelex, for example. However, this double control comes at a cost, which must be added that of the 2 positive and negative controls. The use of 2 types of positive control, one strong and one weak, makes it easier to detect any loss of sensitivity, even though the overall performance of the PCR is maintained.

During the twinning programme, CIRDES is responsible for reconstituting the bank of cryopreserved reference samples and to produce, in sufficient quantities for storage in reference laboratories and for distribution to partner laboratories, aliquots of DNA from the following species (one to three strains per species): *T. vivax*, *T. congolense* savannah, *T. congolense* forest, *T. congolense* Kenya coast, *T. brucei brucei*, *T. evansi*, *T. lewisi* (for identification in rat colonies) and if possible: *T. simiae*, *T. godfreyi*, *T. tsavo* and even *T. theileri* and *T. musculi* (for identification in mouse colonies).



Strains being preserved in liquid nitrogen

Stocks of reference sera stored at -80°C

7.3 Reference sera or plasma

Positive reference sera generated in sufficient quantities (20-40 ml), preferably during **experimental infections** carried out with reference parasite strains, are used as positive strains in the ELISA tests and are made available internationally to enable laboratories to put in place equivalent diagnostic systems. In the case of trypanosomoses, **numerous species** may be involved as they potentially have very many host species (cattle, sheep, goats, horses, donkeys, dogs, pigs, etc.).

Likewise **negative reference sera**, generated in sufficient quantities (20-40 ml) and preferably in the same animals as before, just before their experimental infection, will be prepared, stored and used in conjunction with the positive reference sera.

These positive and negative reference sera or plasma must be obtained from at least 3 different individuals, and be aliquoted and preserved under optimal conditions, either in a freezer at -20°C in the case of aliquots in current use or in a low-temperature freezer (-80°C) in the case of long-term storage. When there are sufficient quantities for distribution to the three laboratories, these samples will be shipped on dry ice to serve as a common reference. Failing this, a limited quantity of these samples can be sent, which can then be used to calibrate the tests in the other two laboratories. Better still, if the technology is available, these samples can be lyophilised for easier storage and distribution without any risk of rapid deterioration.

If a sufficient number of experimental infections to generate reference sera cannot be carried out, **field samples** can be used if they have been duly characterised (the positive reference samples must be positive to several tests and the negative reference samples must be negative to all the tests).

In this case, the procedure is to study the distribution of responses of individuals in the population, calculate the mean and then select 3 positive reference samples and 3 negative reference samples representative of the target population, namely that present an ELISA reaction (1) equal to the mean, (2) 10% below the mean and (3) 10% above the mean. These three samples placed in duplicate (i.e. in 6 different wells) must give the expected response in at least 5 of the 6 cases or else the ELISA plate must be rejected. Only the head of the laboratory can validate the plates in terms of the results of the controls.

8. Internal quality control and biosafety

Internal quality control consists of using a set of fundamental parameters to evaluate the repeatability and reproducibility of serological and other laboratory tests. In the present context of globalised trade and the emergence or re-emergence of animal diseases and especially zoonoses, this is based on ISO standards, using two approaches:

1. Certification, which consists of evaluating compliance with the requirements of the management system, and
2. Accreditation, which consists of evaluating compliance with the requirements of the system, but more particularly the technical and managerial competence of the laboratory.

At CIRAD in Montpellier, the procedures applied are described in a document entitled "Procedure for handling requests and samples for diagnosis of animal trypanosomosis within the framework of the OIE Reference Laboratory for Animal Trypanosomoses of African Origin", and the diagnostic methods applied are detailed in the present compilation. Quality management is notably based on CIRAD's accreditation for serology (/ASTRE unit), under standard NF in ISO/CEI 17025 and the Cofrac rules of application (Reference No. 1-2207), the general implementation of these same operating rules, the use of P2 and P3 laboratories, and the sharing of equipment controlled within the framework of the accreditation (calibration, metrology, etc.).

At CIRDES, the laboratory is ISO 17025 accredited for its *Glossina palpalis* genotyping activity. For the laboratory to obtain this status, the genotyping method underwent a validation stage followed by a pre-evaluation. A flexible scope quality management system was put in place, with a manual or policy on quality, procedures and operating methods, internal audits, a training plan, a metrology control plan and a document management system. The final evaluation was carried out under the control of an accreditation body licensed by ISO.

Steps are currently being taken to obtain certification of a method of trypanosomosis diagnosis by serology and PCR.

Based on the experience gained as a result of the accreditation of the CIRDES genotyping laboratory, the flexible scope management system already in place will be

extended to include matters relating to certification of its activity on serological and molecular diagnosis of trypanosomosis. The diagnostic team at the CIRDES laboratory is very much involved in the quality management system and will capitalise on its experience to rapidly achieve the intended objective.

Biosafety covers all measures designed to prevent and address the hazards associated with the handling and use of biological materials (microorganisms, genetically modified organisms), in research laboratories, hospitals and industry. First of all, the level of risk must be calculated, so that the appropriate safety rules can then be applied. There needs to be a classification of levels of risk. For laboratories working with organisms that pose a health risk, the different levels of risk are classified into risk groups. This classification enables laboratories to determine their appropriate level of containment.

There are four risk groups:

- **Risk Group 1**

Biological agents in Risk Group 1 are unlikely to infect healthy humans or healthy animals. They therefore represent a low risk for the individual and a low risk for the community. They include *Trypanosoma theileri*, for example, which is frequently observed in the blood of cattle.

- **Risk Group 2**

Pathogens that can cause human or animal disease but are unlikely to be a serious hazard to laboratory personnel, the community or the environment. Exposure to these pathogens rarely causes infection leading to serious disease. Effective treatment and preventive measures are available and the risk of spread is limited. To summarise, the risk is moderate for someone working with the organism and the risk is low for the community. These include, for example, *Trypanosoma brucei brucei* and *T. brucei gambiense*; by extension, other animal species, such as *T. congolense,*
T. vivax and *T. lewisi*, are treated in the same way.

- **Risk Group 3**

These pathogens usually cause serious human disease but it does not ordinarily

spread by casual contact from one individual to another. They rarely cause untreatable diseases. Effective preventive and therapeutic measures usually exist. The risk is therefore high for the individual and low for the community. These comprise *Trypanosoma brucei rhodesiense* and *T. cruzi.*

- **Risk Group 4**

Pathogens that usually produe very serious, often untreatable, human disease that is readily transmitted from one individual to another by direct or indirect contact. The risk to the individual is high and the risk to the community is high (e.g. Ebola).

The biosafety rules applied to the diagnosis of trypanosomosis in the CIRDES laboratory relate principally to preventing contamination with the products known or suspected to be carcinogenic used during PCR tests. These rules include the requirement for all laboratory personnel to wear long-sleeved white gowns and gloves. Furthermore, the prevention of respiratory problems from the use of aerosols or exposure to laboratory chemicals is ensured by daily elimination of ambient air using an industrial vacuum pump as soon as the laboratory opens.

Within the framework of quality assurance and competence assessment programmes, CIRDES and CIRAD follow the general criteria for competence of testing and calibration laboratories, and conduct blind analyses of samples, defined below as inter-laboratory standardisation.

9. Inter-laboratory standardisation

A reference laboratory is by definition accredited to determine the status of a sample with regard to the various tests it is qualified to perform. It disseminates diagnostic methods to other laboratories, along with the relevant protocols and possibly also reference samples. Within a network of reference laboratories, the status of samples determined in one laboratory should be the same in the other laboratory or laboratories, though occasional exceptions may occur, for example if samples become altered during transport or if a sample is close to the limit of positivity. Statistical comparisons must be carried out, based on a series of control samples; this will ensure good agreement between the laboratories (reproducibility).

To check the proficiency of a network of reference laboratories, the procedure is to distribute, within this network, batches of samples of known status for testing, and to compare the results obtained at the various sites. These exchanges of biological material are conducted in a blinded fashion between the laboratories and should be scheduled on a regular basis to check the continued reliability of the results.

As indicated in the OIE *Terrestrial Manual*, (Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases), a check sample panel needs to have undergone repeated testing by more than one analyst conducting multiple runs of the test on different days. Sufficient values should be generated to assure the unequivocal status of the test material, including homogeneity.

Note on exempt biological materials for shipping purposes. The following animal samples are considered to be exempt and not subject to the regulations governing fresh materials: biological materials that do not contain infectious substances (i.e. purified DNA or RNA), or that contain inactivated substances (e.g. after treatment at 65°C during 3 h) or that correspond to dried serum or blood on absorbent material.

In the case of diagnostic tests for trypanosomosis, methods applied to fresh samples are unsuitable for inter-laboratory comparisons; only methods applied to refrigerated or dried samples are suitable for comparative evaluations within the laboratory network. To conduct these comparisons, control samples in the form of dry biological material will be preferred as this simplifies shipping. The following will be exchanged between laboratories (i) stained smear slides, (ii) sera on filter paper, (iii) purified DNA samples in microtubes and/or on filter paper, (iv) blood samples on filter paper (v) and lyophilised sera.

Qualitative comparisons will be carried out for readings of smears and PCR; quantitative comparisons will be carried out for ELISA and CATT tests.

Twenty blood smears, of which at least 10 and no more than 15 are to present at least 2 species of trypanosome, will be prepared in the 3 laboratories and circulated in a blinded fashion for examination, with results being expressed qualitatively (species observed) and quantitatively (frequency of observation of parasites on the smear). A deviation of 10% in the quantification of parasites will be allowed.

Series of 30 control samples, between 10 and 20 of them positive, will be prepared in each of the 3 laboratories for each species of trypanosome available locally. At CIRDES and at CIRAD, 3 series of control samples will be prepared for *T. vivax*, *T. brucei brucei* and *T. congolense* savannah. In Thailand, 1 series of 30 control samples will be prepared for *T. evansi*. Volumes of 100 µl of serum, blood or DNA will be placed on Whatman 4 filter paper and circulated within the network of laboratories for testing by PCR (blood and DNA) and ELISA and CATT (serum and blood).

The statistical analysis will tolerate deviations of 10% from one laboratory to another for qualitative results (positive/negative PCR, CATT and ELISA) and will require agreement >90% for the semi-quantitative tests (CATT & ELISA).

10. Packaging and shipment of samples

All shipments of samples should be by the fastest and most direct method. All shipments should comply with IATA regulations concerning the shipment of biological materials, the import must comply with national or supranational (i.e. European for France) regulations of the importing country, export must comply with national or supranational regulations of the country of export. Legislation is evolving and it must be followed.

In all cases, the use of lyophilised sera, plasma, blood, buffy coat, DNA or antigens will be preferred to all other forms of conservation in view of the advantages of lyophilisation (transport not requiring refrigeration and hazard free).

10.1 Sample types and information required

Samples required for the different diagnostic techniques

Type of test	**Sample type**	**Volume to be shipped**
Parasitological	Blood on anticoagulant (EDTA or heparin)	200 µl
Serological: plasma	Blood on EDTA or heparin, centrifugation 2500 rpm 5-10 min to recover plasma	100 µl (if plasma or sera)
serum	Blood in dry tube, wait for retraction of clots at room temperature or centrifuge after several hours to recover the serum	500 µl if whole blood
	Serum/plasma on Whatman filter paper	
PCR: blood or buffy coat	Blood on anticoagulant (EDTA or heparin), whole blood or buffy coat	200 µl
	Blood on FTA or Whatman filter paper	
DNA	DNA already extracted	20 µl at 50 ng/µl

Required information, which must be contained in the document accompanying the samples:

- Identification of the animal:

  - animal's name, tag or chip number,
  - host species, sex, age, breed if available,
  - owner, farm, geographical location (GPS if for a survey)
- Case history, history of the animal (presence in trypanosome endemic zone? contact with an infected animal?), clinical examination data
- Contact details of the sender: surname, first name, position (veterinarian, owner), postal address, email address and phone number
- Type of samples, volume and requested test
- Clearly mark the tube (identification of the animal, type of sample, sampling date)

10.2 Health and safety regulations

The international regulations for the transport of infectious substances by any mode of transport are based upon the Recommendations made by the Committee of Experts on the Transport of Dangerous Goods (UNCETDG), a committee of the United Nations Economic and Social Council.
There are specific regulations for each mode of transport (air, rail, maritime, road, post). For example, packaging and transport of samples by air are subject to the regulations issued by IATA (International Air Transport Association).
Packaging and transport requirements will depend on **classification of samples, considered as dangerous substances**.

Reminder of the classification of dangerous substances:

The substances can be classified into three categories: A (=UN 2900), B (=UN 3373) or exempt

- **Category A:** UN 2814 (*INFECTIOUS SUBSTANCE, AFFECTING HUMANS*) or **UN 2900** (*INFECTIOUS SUBSTANCE, AFFECTING ANIMALS only*): An infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals; example: substance containing foot and mouth disease virus or African swine fever virus;
- **Category B or UN 3373**: an infectious substance which does not meet the criteria for inclusion in Category A; this category includes **blood or serum samples from animals suspected of or infected with trypanosomosis, as well as strains of live trypanosomes**;
- **Exemptions**: Substances that do not contain infectious substances or that are unlikely

to cause disease in humans or animals are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class. Substances containing microorganisms which are non-pathogenic to humans or animals are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class. Substances in a form that any present pathogens have been neutralized or inactivated such that they no longer pose a health risk are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class. Examples: purified DNA, sera heated for at least 3 h at 65°C, serum for a CATT test on a healthy dog having lived in France, to enable it to enter into a foreign country dried blood spots on absorbent paper. These samples must therefore be maked with the words “Exempt animal specimen”

The packaging of substances, the maximum quantities to be transported and the documentation requirements depend on the category.

- If Category A: requirements include approved triple packaging, a maximum quantity of 50 g or 50 ml for a passenger aircraft, 4 kg or 4 litres for a cargo aircraft, specific labels on the outer packaging and specific import documents.
- If Category B: requirements include approved triple packaging, a maximum quantity of 4 kg or 4 litres, specific labels on the outer packaging and specific import documents.
- Exempt substances: triple packaging but not subject to a given standard.

Basic triple packaging system: 1) a watertight, leak-proof receptacle containing the specimen, 2) a second, watertight, leak-proof receptacle with absorbent material, 3) outer packaging.

Hand carriage (i.e. cabin or hold baggage) of Category A and B samples and transport of these materials in diplomatic pouches are strictly prohibited; such samples must be transported by a specialist firm (depending on the country: DHL, SDV, Air France Cargo, World Courier, etc.). The country of import may carry out checks on the documents and the external packaging, for example from the Veterinary services of the Border Inspection Post (PIF) in France.

Exempt animal specimens can be transported as cabin or hold baggage, but must comply with the regulations on triple packaging.

Details of the requirements for packaging, labelling and documentation are available on the WHO or Pasteur Institute websites:

https://www.pasteur.fr/fr/sante-publique/CNR/envoi-de-materiel-biologique/cadre-reglementaire

http://www.who.int/ihr/publications/who_hse_ihr_20100801/fr/index.html

Pay attention, the documents are regulary updated : they must be consulted by the head of laboratory. The English version of the WHO document is online before the French version.

Refrigerated transport

In general, samples should be transported chilled: around 4°C for blood prior to parasitological tests, preparation of serum or plasma or extraction of DNA.
For long, international transport of plasma or sera: shipping in dry ice is advisable to ensure freezing. Dry ice is considered “dangerous goods” (notably due to the risk of asphyxiation from the release of CO_2) and requires special transport by a specialist shipper (depending on the country: DHL, SDV, Air France Cargo, World Courier, etc.). The agreement of the shipper must be sought in advance (for example, DHL does not transport samples on dry ice from Burkina Faso to France).

Importation into the European Union, in the case of CIRAD

Any sample of animal blood or serum originating from a country outside the continental European Union is opened and handled in a containment level 3 (EU level P3) laboratory, at CIRAD.

The shipment of samples must be arranged in advance: this will include obtaining quotations, preparing the documents and arranging the collection and delivery dates with the carrier.

For the import of samples to the Cirad Montpellier laboratory, the following shipping documents are required:

- the import authorization provided by the Departmental Directorate for the Protection of Populations (which summarizes the type of samples, quantity, packaging, consignor, and consignee) or A Health Support Document for the cultivation of pathogens;
- The number of the air waybill supplied by the carrier, as well as the arrival date and flight number;
- A declaration for the customs, indicating “no commercial value” and a nominal value of € 25;
- A separate label on the package, bearing the words “Exclusivement destinés à usage technique ou pharmaceutique et produits non destinés à l'alimentation humaine ou animale” (“Products destined exclusively for technical or pharmaceutical use and not intended for human or animal consumption”) and other labels specific to Category B, including “dry ice”, if appropriate;
- Delivery address: Cirad-Bios UMR17 TA A-17/G c/o CLASQUIN ROISSY**,** Fret 6,

Bâtiment 3210, 20 Rue du Pavé, BP 15339, Tremblay en France, 95705 Roissy Charles de Gaulle cedex, France

10.3 Rules on intellectual property

Specimens exclusively used for the diagnosis of diseases are not subject to specific regulations on Access and Benefit Sharing (ABS).

However, if the samples are **intended for research** (development of a new diagnostic test, genetic diversity of the parasite, immune response of the host, etc.), they represent a biological resource. Whether trypanosomosis strains, DNA, RNA or sera, the parties (consignor and consignee) are required to sign documents that **meet the international Nagoya Protocol on ABS**. The Nagoya Protocol has been signed in 2010, and it has entered into force since October 12, 2014. At a minimum, an MTA (Material Transfer Agreement), which specifies the framework of the ABS, must be signed.

Information is available on the website of the Convention on Biological Diversity https://www.cbd.int/ and https://absch.cbd.int/search/national-records/NFP which specifies the status of each state and provides the contact details of the national focal points. According to the Rio Convention 1992 (updated in Nagoya, 2010), genetic resources (animal, plant and microbial) constitute a world heritage of humanity but are under the sovereignty of the states that shelter them. These states therefore have the right to legislate on access to these resources, on their use and on the sharing of the benefits that could arise from their uses.

➔ **The consignor and the recipient of the samples must therefore verify the regulations in force in their countries, prepare and sign the necessary documents** to trace the biological material, to clarify the intellectual property clauses and the conditions of Access and Benefits Sharing in the event of publication, patent filing or transfer of material to a third party ...

11. Bibliography

1. ISO/IEC International Standard 17025 (2005). General requirements for the competence of testing and calibration laboratories. International Organisation for Standardisation (ISO)/International Electrotechnical Commission (ISO/IEC), ISO Central Secretariat, 1 rue de Varembé, Case Postale 56, CH - 1211, Geneva 20, Switzerland.
2. International Organisation for Standardisation (ISO) (1997). Proficiency testing by interlaboratory comparisons. Part 1: Development and operation of proficiency testing schemes. Part 2: Selection and use of proficiency testing schemes by laboratory accreditation bodies. ISO/International Electrotechnical Commission (ISO/IEC), Guide 43. ISO/IEC, Geneva, 19 pp.
3. ISO International Standards 9000:2005, 9001:2000; 9004:2000 (2000–2005). Quality management and quality assurance. International Organization for Standardization (ISO), ISO Central Secretariat, 1 rue de Varembé, Case Postale 56, CH - 1211, Geneva 20, Switzerland.
4. Bajyana Songa, E., Hamers, R., 1988. A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of Trypanosoma evansi. Annales de la Société Belge de Médecine Tropicale 68, 233-240.
5. Bengaly, Z., Sidibé, I., Boly, H., Sawadogo, L., Desquesnes, M., 2002a. Comparative pathogenicity of three genetically distinct *Trypanosoma congolense* - types in inbred Balb/c mice. Veterinary Parasitology 105, 111-118.
6. Bengaly, Z., Sidibe, I., Ganaba, R., Desquesnes, M., Boly, H., Sawadogo, L., 2002b. Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. Vet Par 108, 1-19.
7. Delafosse, A., Bengaly, Z., Duvallet, G., 1995. [Absence of interaction between *Trypanosoma theileri* infections with the diagnosis of animal trypanosomiasis by detection of circulating antigens]. Rev Elev Med Vet Pays Trop 48, 18-20.
8. Desquesnes, M., 1996. Evaluation of three antigen detection tests (monoclonal trapping ELISA) for African trypanosomes, with an isolate of *T. vivax* from French Guyana. Annals of the New York Academy of Sciences 791, 172-184.
9. Desquesnes, M., 1997a. Evaluation of a simple PCR technique for the diagnosis of *Trypanosoma vivax* in the serum of cattle in comparison to parasitological techniques and antigen-enzyme linked immunosorbent assay (Ag-ELISA). ACTA Tropica 65, 139-148.

10. Desquesnes, M., 1997b. Standardisation internationale et régionale des épreuves immuno-enzymatiques: méthode, intérêts et limites. Rev Sci Tech Off Int Epiz 16, 809-823.
11. Desquesnes, M., 2004a. Les trypanosomoses des ruminants: Diagnostic différentiel des trypanosomoses des ruminants. Fiche technique, Santé animale, CIRDES, BP454 Bobo-Dioulasso, Burkina Faso 4, 1-8.
12. Desquesnes, M., 2004b. Livestock trypanosomoses and their vectors in Latin America. CIRAD-EMVT publication, OIE, Paris, ISBN 92-9044-634-X, 174 pages. http://www.oie.int/doc/ged/D9818.PDF
13. Desquesnes, M., Bengaly, Z., Dia, M.L., 2003. Evaluation de la persistance des anticorps détectés par Elisa-indirect *Trypanosoma vivax* après traitement trypanocide chez des bovins naturellement infectés. Rev Elev Med Vet Pays Trop 56, 141-144.
14. Desquesnes, M., Bengaly, Z., Millogo, L., Meme, Y., Sakande, H., 2001. 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.
15. Desquesnes, M., Bossard, G., Thevenon, S., Patrel, D., Ravel, S., Pavlovic, D., Herder, S., Patout, O., Lepetitcolin, E., Hollzmuller, P., Berthier, D., Jacquiet, P., Cuny, G., 2009. Development and application of an antibody-ELISA to follow up a *Trypanosoma evansi* outbreak in a dromedary camel herd in France. Vet Parasitol 162, 214-220.
16. Desquesnes, M., Bosseno, M.F., Breniere, S.F., 2007a. Detection of Chagas infections using *Trypanosoma evansi* crude antigen demonstrates high cross-reactions with *Trypanosoma cruzi*. Infection Genetic and Evolution 7, 457-462.
17. Desquesnes, M., Dávila, A.M.R., 2002. Applications of PCR-based tools for detection and identification of animal trypanosomes; a review and perspectives. Vet par 109, 213-231.
18. Desquesnes, M., Itard, J., Cuny, G., Solano, P., Authie, E., 2010. Trypanosomoses: diagnosis, In: Lefèvre, P.-C., Blancou, J., Chermette, R., Uilenberg, G. (Eds.) Infectious and parasitic diseases of livestock. Lavoisier, Paris, France, pp. 1911-1926.
19. Desquesnes, M., Kamyingkird, K., Kengradomkij, C., Pruvot, M., Sarataphan, N., Jittapalapong, S., 2008. Standardisation of an ELISA for *Trypanosoma evansi* and its application to dairy cattle in Thailand. Proceedings of the 15th Congress of the Federation of Asia Veterinary Association (FAVA), OIE joint symposium on emerging diseases; 27-30 October 2008, Bangkok, Thailand, 269-270.
20. Desquesnes, M., Kamyingkird, K., Yangtara, S., Milocco, C., Ravel, S., Wang, M.-H., Lun, Z.-R., Morand, S., Jittapalapong, S., 2011. Specific primers for PCR amplification of the ITS1 (ribosomal DNA) of *Trypanosoma lewisi*. Infect Genet Evol 11, 1361-1367.

21. Desquesnes, M., Michel, J.F., De La Rocque, S., Solano, P., Millogo, L., Bengaly, Z., Sidibe, I., 1999. Enquête parasitologique et sérologique (ELISA-indirecte) sur les trypanosomoses des bovins dans la zone de Sidéradougou, Burkina Faso. Revue Elev Med Vet Pays Trop 52, 3-4.
22. Desquesnes, M., Patout, O., Brugidou, R., Faye, B., Cuny, G., 2007b. Un foyer de trypanosomose observé pour la première fois en France. Bulletin des GTV 39, 8-10.
23. Desquesnes, M., Ravel, S., Cuny, G., 2002. PCR identification of *Trypanosoma lewisi*, a common parasite of laboratory rats. Kinetoplastid Biol Dis 1, 2.
24. 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.
25. Desquesnes, M., Tresse, L., 1996. Evaluation de la sensibilité du test de WOO pour la détection de *Trypanosoma vivax*. Revue d'Elevage et de Médecine Vétérinaire des Pays Tropicaux 49, 315-321.
26. Greiner, M., Shivarama, B.T.P., R.J., Kakaire, D., Shares, G., Bohning, D., Zessin, K., Mehlitz, D., 1997. Impact of biological factors on the interpretation of bovine trypanosomosis serology. Prev Vet Med 30, 61-73.
27. Gutierrez, C., Corbera, J.A., Morales, M., Buscher, P., 2004. Performance of serological tests for *Trypanosoma evansi* in experimentally inoculated goats. Ann N Y Acad Sci 1026, 152-153.
28. Gutierrez, C., Desquesnes, M., Touratier, L., Buscher, P., 2010. *Trypanosoma evansi*: recent outbreaks in Europe. Vet Parasitol 174, 26-29.
29. Herbert, W.J., Lumsden, W.H., 1976. *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. Exp Parasitol 40, 427-431.
30. Hirumi, H., Hirumi, K., Peregrine, S., 1993. Axenic culture of Trypanosoma congolense: Application to the detection of sensitivity levels of bloodstream trypamastigotes to diminazene aceturate, homidium chloride, isometamidium chloride and quinapyramine sulphate. J Protozool Res 3, 52-63.
31. Hopkins, J.S., Chitambo, H., Machila, N., Luckins, A.G., Rae, P.F., van de Bossche, P., Eisler, M., 1998. Adaptation and validation of antibody-ELISA using dried blood spots on filter paper for epidemiological surveys of tsetse-transmitted trypanosomosis in cattle. Preventive Veterinary Medicine 37, 91-99.
32. Howie, S., Guy, M., Fleming, L., Bailey, W., Noyes, H., Faye, J.A., Pepin, J., Greenwood, B., Whittle, H., Molyneux, D., Corrah, T., 2006. A Gambian infant with fever and an unexpected blood film. PLoS Med 3, e355.
33. Ilboudo, H., Jamonneau, V., Camara, M., Camara, O., Dama, E., Leno, M., Ouendeno, F., Courtin, F., Sakande, H., Sanon, R., Kabore, J., Coulibaly, B., N'Dri, L., Diarra, A., N'Goran, E., Bucheton, B., 2011. Diversity of response to *Trypanosoma brucei*

gambiense infections in the Forecariah mangrove focus (Guinea): perspectives for a better control of sleeping sickness. Microbes Infect 13, 943-952.

34. Jamonneau, V., Bucheton, B., Kabore, J., Ilboudo, H., Camara, O., Courtin, F., Solano, P., Kaba, D., Kambire, R., Lingue, K., Camara, M., Baelmans, R., Lejon, V., Buscher, P., 2010. Revisiting the immune trypanolysis test to optimise epidemiological surveillance and control of sleeping sickness in West Africa. PLoS Negl Trop Dis 4, e917.
35. Jittapalapong, S., Inpankaew, T., Sarataphan, N., Herbreteau, V., Hugot, J.P., Morand, S., Stich, R.W., 2008. Molecular detection of divergent trypanosomes among rodents of Thailand. Infect Genet Evol 8, 445-449.
36. Katakura, K., Lubinga, C., Chitambo, H., Tada, Y., 1997. Detection of *Trypanosoma congolense* and *T. brucei* subspecies in cattle in Zambia by polymerase chain reaction from blood collected on filter paper. Parasitol Res 83, 241-245.
37. Katende, J.M., Musokoe, A.J., Nantulya, V.M., Gooderis, B.M., 1987. A new method for fixation and preservation of trypanosomal antigens or use in the indirect immunofluorescence antibody test for diagnosis of bovins trypanosomiasis. Trop Med Parasitol 38, 41-44.
38. Komoin-Oka, C., Truc, P., Bengaly, Z., Formenty, P., Duvallet, G., Lauginie, F., Raath, J.P., N'Depo, A.E., Leforban, Y., 1994. [Prevalence of Trypanosoma infections in different species of wild animals in the Comoe national park on the Ivory Coast: preliminary results of the comparison of 3 diagnostic methods]. Rev Elev Med Vet Pays Trop 47, 189-194.
39. Kratzer, R.D., Ondiek, F.O., 1989. The buffy coat double centrifugation technique, an improved method for the diagnosis of African trypanosomiasis. in: 20th meeting of the International Scientific Committee for Trypanosomosis Research and Control (ISTCRC), Mombasa, Kenya, 10-14 April 1989.
40. Lefrançois, T., Solano, P., Bauer, B., Kaboré, I., Touré, S.M., Cuny, G., Duvallet, G., 1999. Polymerase chain reaction characterization of trypanosomes in *Glossina morsitans submorsitans* and *G. tachninoides* collected on the game ranch of Nazinga, Burkina Faso. Acta Tropica 72, 65-77.
41. Luckins, A.G., 1977. Detection of antibodies in trypanosome-infected cattle by means of a microplate enzyme-linked immunosorbent assay. Trop Anim Hlth Prod 9, 53-62.
42. Lumdsen, W., Kimber, C., Dukes, P., Haller, L., Stanghellini, A., Duvallet, G., 1981. Field diagnosis of sleeping sickness in Ivory Coast 1. Comparison of the miniature anion-exchange centrifugation technique with other protozoological methods. Trans Roy Soc Trop Med Hyg 75, 242-250.

43. 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. International Journal of Parasitology 22, 909-918.
44. Murray, M., Murray, P.K., McIntyre, W.I.M., 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. Trans Royal Soc Trop Med Hyg 71, 325-326.
45. Nantulya, V.M., Bajyana Songa, E., Hamers, R., 1989. Detection of circulating trypanosomal antigens in Trypanosoma evansi-infected animals using a T. brucei group-specific monoclonal antibody. Trop Med Parasitol 40, 263-266.
46. 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.
47. Njiru, Z.K., Constantine, C.C., Masiga, D.K., Reid, S.A., Thompson, R.C. and Gibson, W.C., 2006. Characterization of *Trypanosoma evansi* type B. Infect Genet Evol 6(4): 292-300. DOI: 10.1016/j.meegid.2005.08.002.
48. Osorio, A.L., Madruga, C.R., Desquesnes, M., Soares, C.O., Ribeiro, L.R., Costa, S.C., 2008. *Trypanosoma (Duttonella) vivax*: its biology, epidemiology, pathogenesis, and introduction in the New World - a review. Mem Inst Oswaldo Cruz 103, 1-13.
49. Paris, J., Murray, M., McOdimba, F., 1982. A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. Acta Trop 39, 307-316.
50. 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.
51. Radwanska, M., Chamekh, M., Vanhamme, L., Claes, F., Magez, S., Magnus, E., de Baetselier, P., Buscher, P. and 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**(6), 684-690.
52. Rae, P.F., Luckins, A.G., 1984. Detection of circulating trypanosomal antigens by enzyme immunoassay. Ann Trop Med Parasitol 78, 587-596.
53. Rebeski, D.E., Winger, E.M., Rogovic, B., Robinson, M.M., Crowther, J.R., Dwinger, R.H., 1999. Improved methods for the diagnosis of African trypanosomosis. Memorias do Instituto Oswaldo Cruz 2, 249-253.
54. Toure, S., Kebe, B., Seye, M., Sa, N., 1976. Biométrie, morphologie et virulence de *Trypanosoma* (*Nannomonas*) *congolense* à travers 640 passages sur souris en 10 ans. Revue d'élevage et de médecine vétérinaire des pays tropicaux, 29 (1): 17-22. 29, 17-22.

55. Truc, P., Jamonneau, V., N'Guessan, P., N'Dri, L., Diallo, P.B., Cuny, G., 1998. *Trypanosoma brucei* ssp. and *T. congolense*: mixed human infection in Cote d'Ivoire. Trans R Soc Trop Med Hyg 92, 537-538.
56. Tuntasuvan, D., Chompoochan, T., Vongpakorn, M., Mohkaew, K., 1996. Detection of *Trypanosoma evansi* antibodies in pigs using an enzyme linked immunosorbent assay. J. Thai Vet. Med. Assoc. 47, 45-53.
57. Tuntasuvan, D., Luckins, A., 1998. Status of Surra in livestock in Thailand. Journal of Protozoological Research 8, 162-170.
58. Tuntasuvan, D., Mimapan, S., Sarataphan, N., Trongwongsa, L., Intraraksa, R., Chanprasert, B., 1998. Cerebral trypanosomosis in hog deer (*Cervus porcinus*) Proceedings of the 24th Annual Conference of the Thai Veterinary Medical Association (TVMA) and the 4th Conference of the Veterinary Practitioner Association of Thailand, 5-9 August 1998, Bangkok (Thailand), 56-64.
59. Van Meirvenne, N., Magnus, E., Buscher, P., 1995. Evaluation of variant specific trypanolysis tests for serodiagnosis of human infections with *Trypanosoma brucei gambiense*. Acta Trop 60, 189-199.
60. Woo, P.T.K., 1970. The heamatocrit centrifuge technique for diagnosis of African trypanosomiasis. Acta Tropica 27, 384-386.
61. Wright, P.F., Nilsson, E., Van, R., 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.
62. Wuyts, N., Chokesajjawatee, N., Panyim, S., 1994. A simplified and highly sensitive detection of *Trypanosoma evansi* by DNA amplification. Southeast Asian J Trop Med Public Health 25, 266-271.
63. Zweygarth, E., Sabwa, C., Rottcher, D., 1986. An enzyme-linked immunosorbent assay for the detection of antibodies to *Trypanosoma (T.) brucei evansi* in camels (*Camelus dromedarius*) using peroxidase-conjugated protein A. Trop Med Parasitol 37, 105-106.

12. Appendices: Test protocol datasheets

12.1 Identification of *Glossina*

Although the identification of *Glossina* species is not, strictly speaking, an integral part of trypanosomosis diagnosis, it is an essential precondition.

General morphology

Elongated flies: 6-16 mm; colour: blackish brown, never metallic; at rest, the wings are folded one over the other on the back; the biting mouthparts project forward horizontally.

Morphology of the head

Compound eyes: one pair; three (3) ocelli in a triangle between the compound eyes

2 antennae, each with 3 segments: the 3rd segment is the largest and has an arista at its base. The arista bears a fringe of silky hairs on its dorsal side.

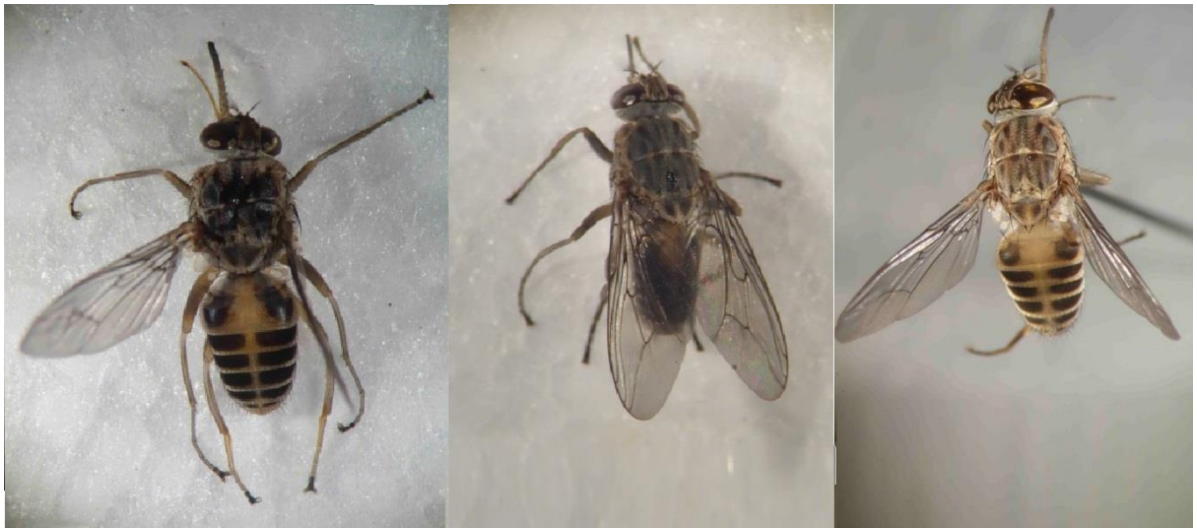
The ptilinal suture surrounds the first antennal segments.

Mouthparts:

- maxillary palps are as long as the proboscis and act as a protective sheath; they lift out of the way during biting.

proboscis:

- labium = rigid lower lip
- hypopharynx = tube for pumping saliva
- labrum = upper lip



Glossina morsitans *Glossina plapalis* *Glossina tachinoides*

(M. Desquesnes)

Morphology of the thorax

- Three (3) segments with hairs on the side.
- One (1) pair of wings, crossed at rest above the abdomen

  discal cell shaped like a hatchet: characteristic of the genus *Glossina.

- Halteres: balancing organs, the remnants of a second pair of wings.
- Three (3) pairs of legs: coxa (fixed to the thorax), trochanter, femur, tibia, tarsus (5 segments, the last having a pair of claws and a pair of pulvilli. Colour→systematic).
- Two (2) pairs of respiratory spiracles on the sides.

Morphology of the abdomen

- Eight (8) segments, seven (7) of which are visible dorsally
- Each segment comprises: –rigid dorsal tergite
  –elastic ventral sternite
  –one (1) pair of respiratory spiracles
- 'Abdominal pattern' colouring →systematic

G. morsitans *G palpalis* *G. tachinoides*
(M. Desquesnes)

Tarsal extremity black (*Gm*); All tarsal segments black (*Gp* and *Gt*)
(M. Desquesnes)

- 8th segment: Genitalia = external reproductive apparatus: shape and dimension are characteristic of individual species and sub species.

*Male genitalia:

- Hypopygium: convex callus, with only the basal part (= epandrium) visible at rest; articulated with the 7th tergite and rotates about 180° at the start of copulation.
- Hectors: middle of the 5th sternite; help to hold on to the female during mating.
- Upper claspers = cerci, at the end of the epandrium
  may or may not be linked by a connective membrane]
  ]
  →→systematic
  ending in a tooth that is more or less sharp]
- Phallic apparatus on the inside of the epandrium; highly complex anatomy:
  phallotheca
  claspers = lower claspers → systematic in *palpalis* group
  penis: systematic, based on the distal aedeagus (harpes).

* Female genitalia: no protruding organs

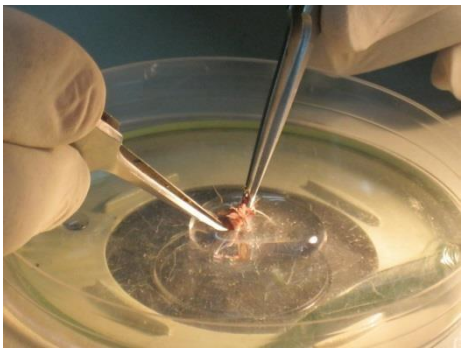
- 2 triangular dorsal plates
- 1 small medio-dorsal plate
- 2 anal plates that are convex and pointed on the inside
- 1 sternal plate shaped like a nipple
- 6 hairy plates around the vulva and the anus

(*number, shape, hairiness are used in systematics*)

12.2 Detection of trypanosome infection in *Glossina*

I- Dissection of *Glossina* and preparation of organs between slide and cover-slip

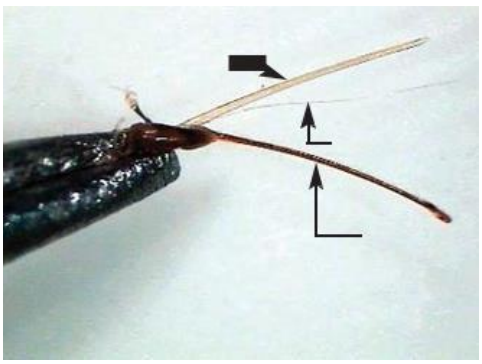
Dissection is always carried out using a stereoscopic microscope (magnification X60), with the insect placed in a petri dish, in a large drop of saline solution to maintain the fly's organs in a hydrated and isotonic environment.



In the teneral fly, a slight pressure on the thorax will release the ptilinum, an organ in the form of an inflatable pouch. Dissection is not necessary in this case as the fly has not yet taken its first blood meal.

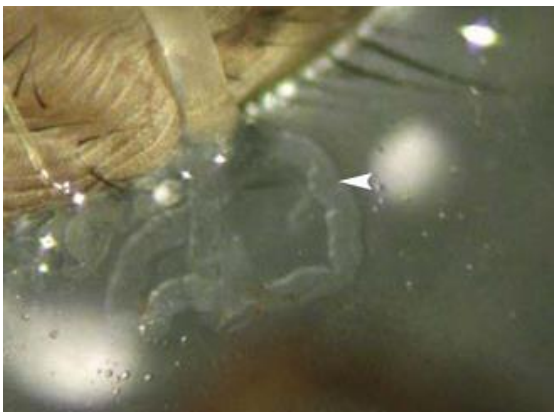
Dissection must be carried out in the following order: first the proboscis, then the salivary glands and finally the midgut. As the midgut often contains a large number of trypanosomes, this order reduces the risk of contaminating the other organs. Nevertheless, the instruments used for dissection must be sterilised in a mild bleach solution between each organ and each fly.

I-1 First, the proboscis is firmly held on the thecal bulb and removed; the labrum, hypopharynx and labium are separated using a purse-string needle or directly using the tip of the fine forceps and placed between the slide and cover-slip in a drop of saline solution.

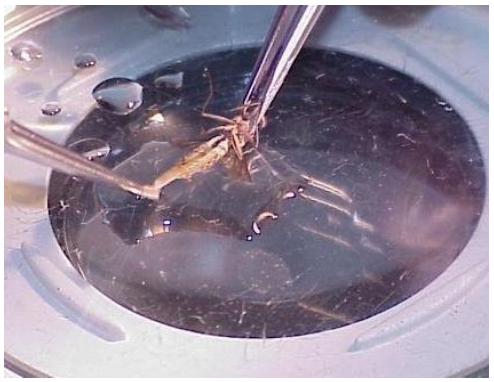


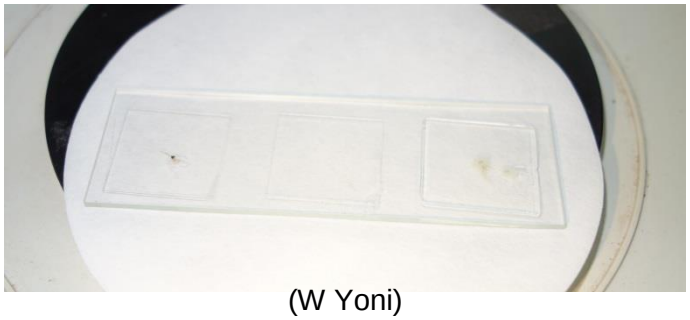
I-2 The salivary glands, resembling two long, thin translucent tubes, are retrieved from the lateral and anterior portions of the abdomen, and then placed between slide and cover-slip in a drop of saline solution.





I-3 The midgut is then extracted from the abdomen and the Malpighian tubules and fat bodies are removed; the midgut is then arranged in a 'U' shape between slide and cover-slip in a drop of saline solution.





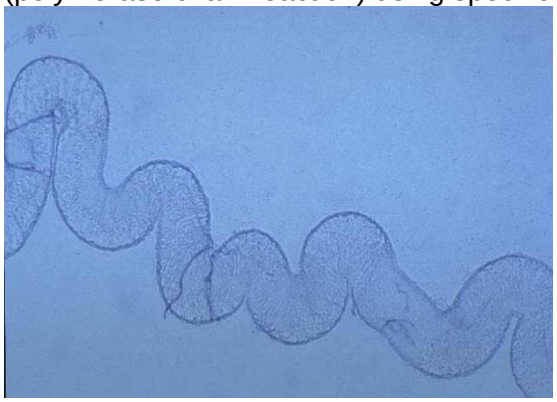
(W Yoni)

II- Microscopic examination (magnification X400) to search for trypanosomes

The specificity of the microscopic examination is low because the infection of an organ may go unnoticed or be temporary. Consequently, this diagnosis is only indicative when the development cycle is fully implemented:

* *Trypanosoma vivax* is only found in mouthparts;
* *Trypanosoma congolense* is only found in intestine and mouthparts;

* *Trypanosoma brucei* has a more complex cycle and may be found in intestine, salivary glands and mouthparts.
It is recommended that the trypanosome species are laboratory-confirmed by PCR (polymerase chain reaction) using specific trypanosome primers.



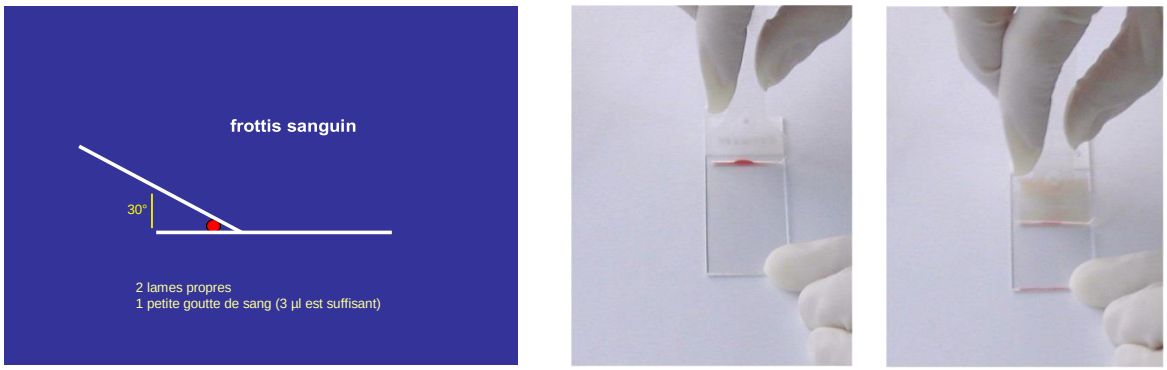
Trypanosomes in the salivary glands

A rosette of trypanosomes in the proboscis

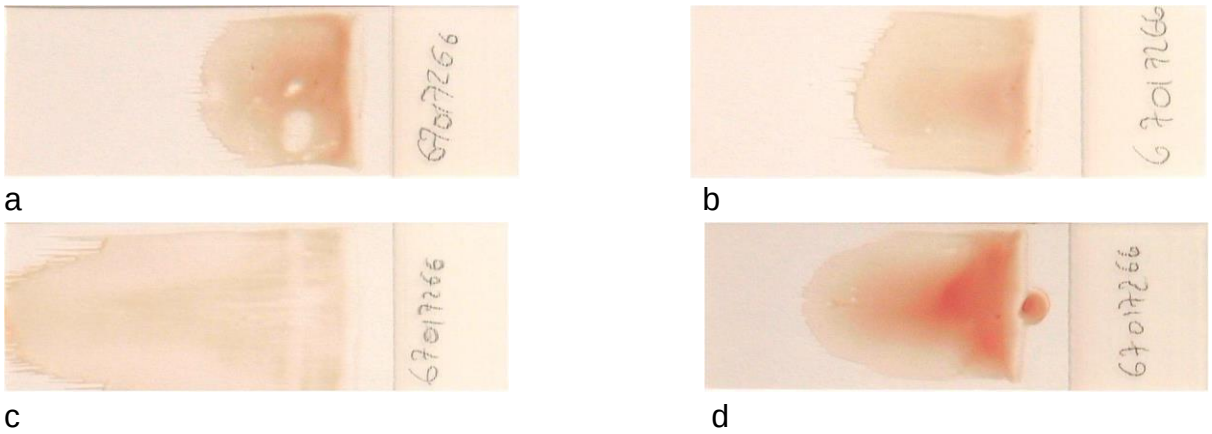
(D. Cuisance)

12.3 Preparation of Giemsa-stained blood smears

1. Spread a small drop of blood (3-4 µl, which can be placed using a capillary tube, the cap of an EDTA tube or the tip of a pipette) on a glass slide, using another slide, placed at an angle of approximately 30° to produce an even film of blood by capillary traction. Clean slides are crucial to the success of this examination (degrease the slides thoroughly with ethanol and dry them before making the smear). Clean the spreader slide immediately after use without allowing the blood to dry, otherwise the next smear will be streaky and unreadable.



Capillary traction of blood using a spreader slide. The larger quantity of blood in the centre of the slide gives smear a bullet shape. If the blood is too fluid (acute anaemic state), the result will be as shown below in (c); in this case, repeat the smear with a smaller quantity of blood (only 1-2 µl). If the slide has not been properly degreased, the smear will appear vacuolised (a); in this case, repeat the smear using a slide that has been degreased with ethanol. If too much blood has been used, the cells will be superimposed and the smear may be difficult or even impossible to read (d).



(a) Greasy slide, “vacuolisation” of the smear; (b) correct (c) excess blood and absence of “head” of the smear; (d) excess blood and inadequately spread; superimposed cells.

2. Once the smear is ready and has dried, fix the smear by immersing the slide in methanol for 30 seconds (for a single preparation, the methanol can be dropped on the smear rather than immersing the slide in the beaker of methanol).

3. The buffer stock solutions are as follows:

Solution A: KH_2PO_4 (Merck Art. 4873) 9.08 g/l (= 1/15 molar)
Solution B: Na_2HPO_4 .2 H_2O (Merck Art. 6580) 11.87 g/l (= 1/15 molar)

4. Mix 8.75 ml of solution A with 41.25 ml of solution B. Shake and allow to stand for 10 minutes, then check for pH 7.2–7.4. Adjust if necessary (HCl / NaOH). The resulting buffer solution will keep for one day only.

5. Filter the Giemsa solution and add 5 ml of Giemsa to 45 ml of the buffer solution (dilution at 1/10). Mix and allow to stand for 10 minutes

6. Immerse the slides in the stain or pour the stain onto the microscope slide and leave to stain for 30 min

7. Rinse thoroughly with tap water, then drain and allow to dry completely before observation (if the slide is not totally dry, the mixture of immersion oil and water will render the slide unreadable). If the staining is too strong, reduce the stain incubation time or increase the dilution to 1/20.

The stained smear is then dried thoroughly before being examined at X500 or X1000 magnification in oil immersion and direct lighting (with the light condenser raised).
It is important to dry the slide thoroughly, otherwise the mixture of water and immersion oil will render the slide unreadable due to the light interference created by the water and the air trapped under the oil. A well-prepared slide will show a light rim around the head of the smear; this zone is particularly rich in white cells (and possibly trypanosomes), due to the strong capillary forces linked to their large size. This “concentration” phenomenon is similar to that on which the haematocrit centrifugation method is based.
Approximately, 50–100 fields of the stained slide are examined at a magnification of X500 and then X1000 with immersion oil before a sample can be considered negative. Even after a trypanosome has been observed, about 20 more fields must be examined to determine if more than one species is present. Particular attention should be given to exploring the head of the smear as it has the highest concentration of white cells, trypanosomes and any parasitized RBCs (due to their increased size and therefore their adherence to the spreader slide).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes. Usually, both a thin and a thick smear are made from the same sample. Thick blood films contain more blood than thin blood smears and consequently have a higher diagnostic sensitivity. Thin smears, on the other hand, allow *Trypanosoma* species identification.

12.4 DNA preparation for PCR

Blood samples:

- Blood samples on anticoagulant: it is recommended that blood samples are concentrated by high speed differential centrifugation (>8000 rpm) followed by collection of the buffy coat, as described for concentration methods prior to microscopic examination. The buffy coats can be collected either from capillary tubes (approximately 10-15 µl of buffy coat samples placed on 30 µl of distilled water) or from tubes or microtubes (approximately 100-150 µl of pure buffy coat) and can then be prepared in various ways to optimise the sensitivity of the PCR tests.

- Blood spotted on filter paper: blood spotted on filter paper (e.g. confetti) can be eluted as follows:

1. Add 1 ml of 5% Chelex-100 to a 2 cm^2 disc of Whatman filter paper spotted with blood in a 1.5 ml tube.

2. Incubate the tubes for 1 h at 56°C and for 30 min at 95°C in the PCR cycler.

3. Centrifuge the tubes for 5 min at 14 000 rpm to sediment the Chelex and use the supernatant for the PCR.

4. Keep the Chelex-treated samples at –20°C

A solution of 5% Chelex-100 in sterile H_2O is prepared, and kept at +4°C.
Chelex-100 (Chelating Ion Exchange Resin, Biorad ref 143-2832, 100 g) is a chelating resin with a high affinity for polyvalent metal ions. It prevents DNA degradation by chelating the metal ions that can act as catalysts in this degradation.

REF: Biotechniques 1991;10(4):506-513.

Sensitivity studies have been carried out to compare different methods of preparing infected blood samples with series of dilutions of parasites. Recent studies with a parasite of the subgenus *Trypanozoon* have shown that the classical phenol–chloroform method remains the best in terms of both the sensibility of the tests and the quality, integrity and duration of preservation of the extracted DNA (Pruvot et al 2013). However, in view of the time-

consuming nature of the method and the danger of handling toxic products (phenol and chloroform), other, faster and in some cases economical methods have proved effective, such as the use of Chelex (Walsh et al., 1991; Penchenier et al., 1996). There are also numerous commercial kits for preparing or harvesting DNA, but the cost is generally prohibitive, especially for routine diagnosis in large batches of samples. However, their diagnostic superiority has rarely been demonstrated.

Preparation with phenol–chloroform: Place 500 µl of denaturing solution (guanidine thiocyanate) in a 1.5-ml microtube and add 100 µl of buffy coat (or, if not available, blood), vortex mix at high speed for 5 minutes. Add 150 µl of chloroform and 150 µl of phenol, vortex mix at high speed for 5 minutes, then centrifuge at 13 000 rpm (15493 *g*) for 5 minutes. Collect the supernatant and place it in a new tube. Again add 150 µl of chloroform and 150 µl of phenol, vortex mix at high speed for 5 minutes, then centrifuge at 13 000 rpm for 5 minutes. Collect 400 µl of supernatant, transfer it to a new 1.5-ml tube and add 1 ml absolute ethanol and leave to precipitate overnight at -20°C. Centrifuge cold for 10 minutes at 13 000 rpm and discard the supernatant. Wash the pellet twice with 75% ethanol and centrifuge at 13 000 rpm for 5 minutes. Eliminate the supernatant. Allow the pellet to dry in the air. Re-suspend in 50 µl of Tris-EDTA (TE) buffer. This method enables the sample to be concentrated from an initial volume of 100 µl to a final volume of DNA in 50 µl.

Preparation with Chelex: blood or buffy coat samples are stored frozen. The sample is thawed and an equivalent volume of 5% Chelex-100 (suspension of 5% Chelex-100 in distilled water) is added. It is kept for 1 h at 56°C, then vortex mixed and kept for 30 minutes at 95°C, then vortex mixed and centrifuged for 2 minutes at 5000 rpm; the supernatant is pipetted to avoid debris particles and resin beads blocking the tip and/or interfering with the PCR.

DNA extraction with PBS-saponin: Take 2 to 4 confettis, place them in a 1.5 ml Eppendorff tube, add 1 ml of 0.05% PBS-saponin, shake, incubate for 4 h at 4°C or overnight. Centrifuge for 1 min at 15000 *g*, discard the supernatant, add 1 ml of PBS; shake, then incubate for 90 min at 4°C. Centrifuge for 1 min at 15000 *g*, discard the supernatant, add 100 µl of 10% Chelex then incubate for 10 min at 95° while mixing continuously. Centrifuge for 5 min at 15000 *g* then harvest the supernatant without Chelex beads in a clean 1.5 ml tube. Keep at -20°C in readiness for PCR.

Genomic DNA extraction with DNeasy Blood and Tissue Kit (Qiagen ref. 69504 and 69506) from a pellet of trypanosomes

1. Allow the pellet to thaw (2 x 108 parasites on average) then resuspend the parasites in 200 µl PBS (not supplied with the kit),
   If the pellet is not dry: centrifuge at 1800 rpm for 5' then remove the supernatant before resuspending the parasites in 200 µl PBS.

2. Add 20 µl proteinase K then add 200 µl buffer AL, mix thoroughly by vortexing and incubate for 10 min at 56°C. The sample and buffer AL must be well mixed immediately in order to obtain a homogenous solution,

3. Add 200 µl ethanol (96-100%) (not supplied) and mix thoroughly by vortexing to obtain a homogenous solution,

4. Transfer all the contents of the tube to a DNeasy column already placed in a 2 ml tube (supplied). Centrifuge for 1 min at 8000 rpm at room temperature. Discard the eluate and the 2 ml tube.

5. Place the DNeasy column on a new 2 ml tube (supplied), add 500 µl buffer AW1 and centrifuge for 1 min at 8000 rpm. Discard the eluate and the 2 ml tube.

6. Place the DNeasy column on a new 2 ml tube, add 500 µl buffer AW2 and centrifuge for 3 min at maximum speed (14 000 rpm) to dry the membrane. Discard the eluate and the 2 ml tube.

7. Place the DNeasy column on a new 2 ml tube and centrifuge for 1 min at maximum speed (14 000 rpm) to eliminate any trace of residual ethanol,

8. Place the DNeasy column on a 1.5 ml tube and add 200 µl buffer AE directly on the membrane. Incubate for 1 min at room temperature then elute by centrifuging for 1 min at 8000 rpm.
   Elution can also be achieved using 100 µl buffer AE, which increases DNA concentration but reduces the yield.

9. Repeat the elution by transferring the DNeasy column to a new 1.5 ml tube and adding 200 µl buffer AE directly on the membrane. Incubate for 1 min at room temperature then elute by centrifuging for 1 min at 8000 rpm.

Important: do not elute more than 200 µl in a 1.5 ml tube, to avoid the column coming into contact with the eluate.
The indicated centrifugation speeds are intended for a fixed-rotor centrifuge for 1.5-2 ml tubes.

Tsetse organ samples

Equipment: sterilised 0.5 ml Eppendorf tubes, distilled water, 100 ul pipette, yellow tips, stand

Storage and preparation

Proboscis and salivary glands: place in 30 ul distilled water; midgut: place in 50 ul distilled water

Label tubes; Keep samples cold in an icebox (field) until they can be frozen (laboratory)
NB: The stated volumes of distilled water must be strictly respected to ensure the sensitivity of DNA examinations of the parasites.
At the laboratory, the probosces are triturated with a yellow-tipped pipette. For the salivary glands and midgut, the samples are homogenised by pipetting several times before adding 30 µl of 5% Chelex for salivary glands or 50 µl of 5% Chelex for midgut.
Then, the same volume (30 µl) of 5% Chelex is added and the Chelex programme applied, i.e. 1 h at 56° then 30 min at 95°.
Vortex the samples and then centrifuge them for 2 min. Pipette the supernatant for PCR to avoid any impurities that could inhibit the PCR.

12.5 Separation of trypanosomes on a DE52 column

Equipment

Whatman DE 52 cellulose (VWR 0904201) Na_2HPO_4 (anhydrous)
NaH_2PO_4, 2 H_2O NaCl H_3PO_4 Glucose
50-ml syringe Whatman No. 41 filter paper 50-ml Falcon tubes

Preparation of solutions

PBS (2 litres)

Na_2HPO_4 (anhydrous)-- 26.96 g
NaH_2PO_4, 2 H_2O--------- 1.56 g
NaCl---------- 8.5 g

Dissolve in sterile water.
Adjust to pH 8 with 5% phosphoric acid (H_3PO_4).
QSP at 2 litres.
Filter at 0.22 µm, autoclave and if necessary readjust the pH.
Keep at 4°C.

Cellulose

For a rat, prepare two columns of 40 ml cellulose. Weigh 60 g for two columns.
Add 200 ml PBS pH 8 and stir for approximately 10 min with a magnetic stirrer.
Allow to settle and then discard the supernatant.
Add 200 ml PBS pH 8 and stir for approximately 10 min with a magnetic stirrer.
Allow to settle and then discard a large part of the supernatant; keep approximately half cellulose and half buffer (supernatant).
Autoclave.

PSG 6:4 ratio recommended for *T. congolense* in rat.

For 1 litre
600 ml PBS pH 8
400 ml sterile H_2O
10 g glucose (1% final)
1 litre of PSG 6:4 is required for 2 columns.
Keep at 4°C.

Preparation of columns

Equipment: Whatman No. 41 filter paper discs cut to the same diameter as that of the syringe; 50-ml syringes; 50-ml Falcon tubes; device for adjusting the flow of liquid rubber hosepipe and clamp); ice bucket; retort stand.

For the autoclaved cellulose, check the pH (pH 8 required).
Position the filter paper disc at the bottom of the syringe and check that it is flat.
Add cellulose to the syringe until a volume of 50 ml of resin has been reached. Allow to settle for 15 min.

Position the second filter paper disc on top of the resin (to avoid disturbing the liquids when dropping the buffer).
Rinse the column with 100 ml PBS pH 8 and check the pH. The pH must reach 8.

CAUTION

Equilibrate the column with 150 ml cold PSG.
Add the freshly drawn rat blood (i.e. 5 ml per column), harvested in 50 ml tubes placed in ice.
The elution is done with PSG.
Allow approximately 15 ml to pass and then start checking the eluate containing the trypanosomes (drop placed between slide and cover slip).
Stop the flow from the column when no more trypanosomes are visible under the microscope.

Collection of the pellet

The collection tubes are centrifuged at 3000 rpm for 10 min at 4°C. A pellet should be visible at the bottom of the tube.
The supernatant is discarded using a pipette.

Storage of the pellet

Store the trypanosome pellet at -80°C.

12.6 Preparation of soluble antigens for ELISA

An anti-enzyme cocktail is added to the eluate obtained during the separation of trypanosomes on a DEAE cellulose column. Then, 7 cycles of freezing (-20 or -80°C, or, better, by immersion in liquid nitrogen) / thawing (slowly at 4°C, or at 37°C) are performed to obtain a total lysate. A sonication phase is recommended (where possible) to promote rupture of the membranes and disintegration of the internal structures of the parasites. This followed by high-speed centrifugation, if available with a low-temperature centrifuge (8000 rpm for 10 min) or simply with a haematocrit centrifuge equipped with an Eppendorf rotor (10-12 000 rpm) to eliminate insoluble particles. The supernatant containing the soluble proteins of the parasite is collected.

The protein concentration is determined using a spectrophotometer by:

- measuring the optical density (OD) at 260 nm and at 280 nm (the sample initially sued may to be diluted if the protein concentration is too high);
- deducing the concentration using a scale known as the Adams nomograph;

Or by any other protein concentration measurement method (Bradford technique, nanodrop, etc.).

The antigen thus obtained is then divided into small volumes (i.e. aliquoted) in cryotubes and frozen at -80°C or in liquid nitrogen until use in serological tests. An aliquot in regular use can be stored at –20°C but for better conservation it is recommended to keep stocks of antigen at lower temperatures.

These antigens can be used in indirect ELISA.

12.7 ELISA protocols

Indirect ELISA protocol for *Trypanosoma* species

1. Solutions

Coating buffer

	Quantity	Supplier	Ref.
Na_2CO_3	1.58 g	Sigma	S2127
$NaHCO_3$	2.93 g	Sigma	S6014

H_2O qsp 1 litre adjusted to pH 9.6

PBS

	Quantity	Supplier	Ref.
Na_2HPO_4	1.21 g	Sigma	71643
KH_2PO_4	0.2 g	Sigma	P8281
NaCl	8 g	Sigma	S7653
KCl	0.2 g	Sigma	P9541

H_2O qsp 1 litre adjusted to pH 9.6

Washing buffer (WB): PBS at 0.1% Tween 20

PBS + 1 ml Tween 20 (Sigma ref. P7949) per litre of buffer

Blocking buffer (BB)

5 g skimmed milk powder for 100 ml WB

Chromogen

Kblue substrate (Neogen, Ref. 03-014)

2. Equipment

Nunc polysorp flat-bottom well plate (Ref. 055137, Dutscher) incubator at 37°C with shaker speed up to 300 rpm; P200 multichannel pipette; P10, P200 and P1000 single channel pipettes; ELISA plate reader with a 620-nm filter.

3. Method

Coating buffer with the following dilutions:

Sub-species	Final concentration	Antigen volume	Concentration of antigen after lysis	Volume for a 96-well plate
T. evansi	5µg/ml	100 µl	0.5 mg/ml	10 ml
T. vivax	5µg/ml	50 µl	1 mg/ml	10 ml
T. brucei	5µg/ml	40 µl	1.3 mg/ml	10 ml
T. congolense	5µg/ml	50 µl	1 mg/ml	10 ml

In a 96-well Nunc plate, add 100 µl/well of the solution at 5 µg/ml of trypanosome antigen. Incubate for 2 h at 37°C on a shaker at 300 rpm or overnight at 4°C. Empty the plate by inversion.

Blocking

Add 150 µl of blocking buffer (BB) to each well.

Incubate for 30 min at 37°C on a shaker at 300 rpm.

Empty the plate by inversion – fill the wells with washing buffer (WB) – empty.

Dilutions of samples: (final dilution: 1/100)

- First dilution (1/50) *to be done during the blocking step*: in a Greiner plate (U), place 3 µl of the serum to be tested in 147 µl BB.
- Transfer of samples (dilution at ½): place 50 µl BB in the wells of the ELISA plate, and then 50 µl of the sera diluted at 1/50 (see above).

Please note: the first two columns (A1-H1, B2-H2) are used for controls.

Incubate for 30 minutes at 37°C on a shaker at 300 rpm.

Washing

Empty the plate by inversion – fill wells with washing buffer (WB) – empty. Repeat 4 times.

Secondary antibody

Add 100 µl of secondary antibody (corresponding to the animal species being tested) diluted in BB.

Species	Supplier	Ref.	Dilution
Anti-horse HRP	Sigma	A6917	1/20 000
Anti-bovine HRP	Sigma	A5295	1/10 000
Anti-sheep HRP	Sigma	A9452	1/ 4000
Anti-dog HRP	Sigma	A9042	1/10 000

Dilution of protein G HRP (Sigma ref. P8170) 1:2000 in BB

Dilution of protein A HRP (Sigma ref. P8651) 1:10 000 in BB

Incubate for 30 min at 37°C on a shaker at 300 rpm.

Washing

Empty the plate by inversion – fill wells with washing buffer (WB) – empty. Repeat 4 times.

Chromogen

Add 100 µl KBlue substrate per well.

Incubate for 30 min in the dark.

Read the plate at 620 nm.

Plate layout, to be completed before each reaction:

	1	2	3	4	5	6	7	8	9	10	11	12
A	vide	vide										
B	blanc	blanc										
C	T1+	T1+										
D	T1-	T1-										
E	T2+	T2+										
F	T2-	T2-										
G	T3+	T3+										
H	T3-	T3-										

12.8 PCR protocols

Primer sequences, targets, expected product size, hybridisation temperatures to be applied and published references

Table 1: Mono-specific PCR

Specificity	Name and sequence of the primers	Locali-sation	Size of the products (bp)	T° hybrid	REF
T. vivax	TVW 1 : CTGAGTGCTCCATGTGCCAC	ADN satellite	150	55	Masiga et al., 1992 Int J Parasitol
	TVW 2 : CCACCAGAACACCAACCTGA				
T.brucei s.l.	TBR1: 5' CGAATGAATATTAAACAATGCGCAG 3'	ADN satellite	173	55	Moser et al., 1989 Parasitology
	TBR2: 5' AGAACCATTTATTAGCTTTGTTGC 3'				
T.congo savane	TCS1: 5' CGAGCGAGAACGGGCAC 3'	ADN satellite	321	55	modifiés de Moser et al 1989 Parasitology
	TCS2: 5' GGGACAAACAAATCCCGC 3'				
T.congo forest	TCF1: 5' GGACACGCCAGAAGGTACTT 3'	ADN satellite	350	55	Masiga et al., 1992 Int. J. Parasitol.
	TCF2: 5' GTTCTCGCACCAAATCCAAC 3'				
T. congo 'Kilifi'	TCK1 : 5' GTGCCCAAATTTGAAGTGAT 3'	ADN satellite	294	55	Masiga et al., 1992 Int. J. Parasitol.
	TCK2 : 5' ACTCAAAATCGTGCACCTCG 3'				
T. simiae	TSM1: 5'CCGGTCAAAAACGCATT3'	ADN satellite	437	55	Masiga et al., 1992 Int. J. Parasitol.
	TSM2: 5'AGTCGCCCGGAGTCGAT3'				
T. (N.) Tsavo	TST1: 5' GTCCTGCCACCGAGTATGC 3'	ADN satellite	450	55-60	Majiwa et al., 1993 Parasitology (ILO892/3)
	TST2: 5' CGAGCATGCAGGATGGCCG 3'				
T. congo godfreyi	DGG1: 5' CTGAGGCTGAACAGCGACTC 3'	ADN satellite	149	60	Masiga et al., 1996 Vet. Parasitol.
	DGG2: 5' GGCGTATTGGCATAGCGTAC 3'				
T. evansi	EVA1: 5' ACATATCAACAACGACAAAG 3'	minicercles	139	58	These Masiga 1994 Njiru Vet Par 2004
	EVA2: 5' CCCTAGTATCTCCAATGAAT 3'				
	RoTat1.2F: 5' GCGGGGTGTTTAAAGCAATA 3'	ADN génomique (VSG)	205	59	Claes et al., KBD 2004
	RoTat1.2R: 5' ATTAGTGCTGCGTGTGTTCG 3'				
	TEPAN1: 5' AGTCACATGCATTGGTGGCA 3'	séquence répétée	122	60	Panyim et al, 1993 Pruvot VetPar 2010
	TEPAN2: 5' GAGAAGGCGTTACCCAATCA 3'				
	ESAG6/7F: 5' ACATTCCAGCAGGAGTTGGAG 3'	ADN génomique (R Tsferrin)	237	55	Braem, 1999 these Holland Vet P 2001
	ESAG6/7R: 5' CACGTGAATCCTCAATTTTGT 3'				
T.brucei gambiense	TRBPA1: 5' GCGCCGACGATACCAATGG 3'	Séquence microsat	149 - 203	60	Herder OCEAC 1997 Truc TRSTMH 2002
	TRBPA2: 5' AACGGATTTCAGCGTTGCAG 3'				
T.brucei gambiense	Tgs-GP F: 5' GCTGCTGTGTTCGGAGAGC 3'	Tbg specific glycoprotein	308bp	63	Radwanska et al 2002a
	TgsGP R: 5' GCCATCGTGCTTGCCGCTC 3'				
T.brucei rhodesiense	Tbr F: 5' ATAGTGACAAGATGCGTACTCAACGC 3'	SRA	284	68	Radwanska et al 2002b
	Tbr R: 5' AATGTGTTCGAGTACTTCGGTCACGCT 3'				
T. lewisi	LEW1S: 5' ACCACCACACGCTCTCTTCT 3'	ITS1	220	64	Desquesnes et al. IGE, 2011
	LEW1R: 5' TGTATGTGCGTGCTTGTTCA 3'				
T. theileri	TthCATL1: 5' CGTCTCTGGCTCCGGTCAAAC 3'	CATL (Cathepsin L-like)	273bp	65	Rodrigues et al, 2010 Parasitol. Int.
	DTO155: 5' TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA 3'				

Table 2: Multi-specific PCR

Specificity	Name and sequence of the primers	Locali-sation	Size of the products (bp)	T° hybrid	REF
T.brucei, T. vivax, T. congolense	TRYP1S : CGTCCCTGCCATTTGTACACAC	ITS1	Tb 520, Tv 310,	55	Desquesnes et al
Savannah & forest, T. lewisi	TRYP1R : GGAAGCCAAGTCATCCATCG	rDNA	Tcs-Tcf 680-750, Tl. 623		KBD 2002
T.brucei, T. vivax, T. congolense	TRYP4S: 5' AAGTTCACCGATATTG 3'	ITS1	Tb 487, T.v 242, Tcs 697	45	Desquesnes + DEA B.
savannah, forest & kilifi	TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA	, Tcf 727, Tck 627		DESCAMPS 2003
NESTED: T.brucei,	R1-TRYP18.2C: 5' GCAAATTGCCCAATGTCG 3'	ITS1		51	Desquesnes
T. vivax, T. congolense	R1-TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA			+ DEA B.
savannah, forest	R2-IRFCC: 5' CCTGCAGCTGGATCAT 3'	ITS1	Tb 392, Tv 147, Tcs 602,	47	DESCAMPS
& Kilifi	R2-TRYP5RCG: 5' ATCGCGACACGTTGTG 3'	rDNA	Tcf 632) Tck 532		2003
NESTED: T.brucei,	TRYP18.2C: 5' GCAAATTGCCCAATGTCG 3'	ITS1		51	Desquesnes
T. vivax, T. congolense	TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA			+ DEA B.
savannah, forest	IRFCC: 5' CCTGCAGCTGGATCAT 3'	ITS1	Tth 310, Tb 426, Tv 181,	47	DESCAMPS
Kilifi & T. theileri	TRYP4R: 5' GCTGCGTTCTTCAACGAA 3'	rDNA	Tcs 636, Tcf 666, Tck 566		2003

Laboratory worksheet to be completed when performing a PCR

(in blue, data to be adjusted according to the protocole used)

nom de l'opérateur: ……		**date : ……**				**nom des amorces ……**
				nombre de		
	volumes d'échantillon (µl)	**1**	**8**	**réaction à faire**		
	volume du master-mixt / tube (µl)	**9**	préparer l'équivalent pour			
	volumes finaux (µl)	**10**	**9**	**réactions**		
CONCENTRATIONS FINALES		vol indiv	working sol		**volumes**	
1	X 10mM Tris; **0** mMMgcl2; 50mMKcl	**1,00**	**10**	**X**	**9,00**	tampon 10X
200	µM dNTP with stock sol dNTP at	**0,80**	**2500**	**µMole**	**7,20**	dNTP
1,5	mM MgCl2 avec du Mgcl2 à …. mM	**0,30**	**50**	**mM**	**2,70**	Mgcl2
1	µM **Primer 1**	**0,50**	**20**	**µM**	**4,50**	**primer 1**
1	µM **Primer 2**	**0,50**	**20**	**µM**	**4,50**	**primer 2**
0,5	unit TaqPol per 11 µl of MM	**0,10**	**5**	**UI/µl**	**0,90**	TaqPol
0	% DMSO final	**0,00**	**net**		**0,00**	DMSO
l	eau distilée	**5,80**			**52,20**	eau distillée
	Vérification du volume final	**9,00**	**MASTER MIX**		**81**	

nombre de cycles: 30	**t°C**	**temps**		
dénaturation initiale	**94**	**60**	volume échantillons	**1**
dénaturation	**94**	**30**	volume master mix	**9**
hybridation	**60**	**60**	nécessaire	**72**
extension	**72**	**30**	volume total dispo	**81**
extension finale	**72**	**120**	reste (extra)	**9**

gel d'agarose à 2%	**120 volts**	**1 H**	
BAS		**HAUT**	
1		1	
2		2	
3		3	
4		4	
5		5	
6		6	
7		7	
8		8	
9		9	
10		10	
11		11	
12		12	
13		13	
14		14	
15		15	
16		16	
17		17	
18		18	
19		19	
20		20	
21		21	
22		22	
23		23	
24		24	
25		25	
26		26	

commentaires :

This Compendium has been compiled to serve as a reference tool for the diagnostic methods for trypanosomoses used at the OIE Reference Laboratory for Animal Trypanosomoses of African Origin, CIRAD, Montpellier, France, and its partner laboratory, CIRDES, Bobo-Dioulasso, Burkina Faso. It was generated in the framework of the CIRAD-CIRDES Twinning project of the OIE for the Reference laboratory on animal trypanosomes of African origin (2012-2017).

The Compendium describes the standard parasitological, serological and molecular biology methods recommended by the OIE for the diagnosis of African trypanosomoses, as published in Chapter 2.4.17 'TRYPANOSOMOSIS (tsetse-transmitted)' of the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (hereafter, the *Terrestrial Manual*). It describes the main characteristics of these methods, enabling users to correctly interpret the results of the tests. It describes all the protocols in detail so that other regional or national laboratories can use these methods to carry out tests independently of OIE Reference Laboratories. However, it is recommended that permanent links are maintained with these Reference Laboratories for quality control purposes. The Compendium includes a description of the quality control methods needed to ensure the continued validity of the tests carried out.