**CHAPTER 2.1.17.**

**TRYpanosoma Evansi INFECTION[+] (including SURRA)**

**SUMMARY**

**Definition of the disease:** Trypanosoma evansi causes a *trypanosomosis* [disease] known as [*Trypanosomosis* ‘surra’. It affects a large number of wild and [species of] domesticated animals [species] in Africa, Asia, and Central and South America. The principal host species [affected] varies geographically, but camels, horses, buffalos and cattle are particularly affected, although other animals, including wildlife, are also susceptible. It is an arthropod-borne disease; several species of haematophagous flies, including *Tabanus* and *Stomoxes* [*Tabanus spp.* and *Musca spp.*], are implicated in transferring infection from host to host, acting as mechanical vectors. In Brazil, vampire bats are also [have been] implicated in a particular biological transmission.

**Description of the disease:** The general clinical signs of *T. evansi* infections: [disease in susceptible animals is manifested] pyrexia directly associated with parasitaemia together with a progressive anaemia, loss of condition and lassitude are not sufficiently pathognomonic for diagnosis. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes [often] observed in horses. Abortions have been reported in buffaloes and camels. Nervous signs are common in horses. [There are indications that the] The disease causes immuno-deiciencies that may be of high impact when interfering with other diseases or vaccination campaigns (foot and mouth disease and haemorrhagic septicaemia for example).

**Identification of the agent:** The general clinical signs of *T. evansi* infection are not sufficiently pathognomonic for diagnosis. Laboratory methods for detecting the parasite are required. In early infection or acute cases, when the [Examination of the host] blood is problematic as trypanosomes can be detected only when there is a high parasitaemia [high]. [Under these circumstances] examination of wet blood films, stained blood smears or lymph node materials might reveal the trypanosomes. In [other] more chronic cases, or more generally when the parasitaemia is low, [such as the carrier state,] the examination of thick blood smears, as well as methods of parasite concentration and the inoculation of laboratory rodents [animals] are required. In apparently healthy carriers [animals without clinical signs], parasites can rarely be observed and mouse inoculation gives the best results. Several primer pairs targeting the subgenus [*Trypanozoon*] or the species-specific [*T. evansi*] parasitic DNA sequences are available for diagnosis by polymerase chain reaction (PCR). PCR is more sensitive than parasitological examination, but it may give false-negative results when the parasitaemia is very low; in these cases, suspicion of potential carriers can only be confirmed by serological examination.

**Serological tests:** Infection gives rise to specific antibody responses and a variety of antibody detection tests have been introduced for laboratory and field use. Some have been partially validated, but await large-scale evaluation and standardisation. [Among those that are used regularly in the laboratory are immunoenzyme assays, card agglutination tests and latex agglutination tests.] The most relevant are immunofluorescence test (IFAT), enzyme linked immunosorbent assays (ELISA) and card agglutination test (CATT/T. evansi). For field use, only [both] CATT/T. evansi and latex [can be applied] [yet an individual test format]. [Fen side tests are currently unavailable.] [Assays for detection of circulating antibodies have high measures of validity.] Estimates of predictive values [of different serological tests] indicate that ELISA for detecting IgG [antibodies] are more likely to classify correctly uninfected animals, while the CATT is [and are] more likely to classify correctly truly infected animals. [An IgG ELISA would thus be suitable for verifying the] [that animals are] free status of animals [from infection] prior to movement or during quarantine. [In situations where there is overt disease,] CATT can be used to target individual animals for treatment with trypanocidal drugs. For
declaring a disease-free status, serial testing – CATT and ELISA followed by re-testing of suspect samples [by CATT] – is recommended, preferably completed by PCR. It must be stressed however, that there are considerable antigenic similarities among the different species of pathogenic trypanosomes, hence in areas where T. cruzi, T. equiperdum or tsetse-transmitted trypanosomoses occur, cross-reactions may occur with any serological test employed.

Requirements for vaccines [and diagnostic biologicals]: No vaccines are available for the disease.

### A. INTRODUCTION

Infection with Trypanosoma evansi causes a disease named surra in India and, amongst others, El Deba or El Gafar in Africa, Mal de Caderas or Murrin in Latin America. The clinical signs of surra [the disease caused by Trypanosoma evansi] are indicative but are not sufficiently pathognomonic, thus, diagnosis must be confirmed by laboratory methods. The disease in susceptible animals, including camels (dromedary and bactrian), horses, buffalo, cattle and pigs [sheep and goats] is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Such recurrent episodes lead to intermittent [a] fever (as high as 44°C in horses [Gill, 1977]) and parasitaemia [occur] during the course of the disease. Oedema, particularly of the lower parts of the body, rough coat in camels, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed. In advanced cases, parasites invade the central nervous system [CNS], which can lead to nervous signs (progressive paralyses of the hind quarters and, exceptionally, paraplegia), especially in horses, but also in other host species before complete recumbency and death. Abortions have been reported in buffaloes and camels (Gutierrez et al., 2005; Lohr et al., 1986[8–12]) and there are indications that the disease causes immunodeficiency (Dargantes et al., 2005b; Ongh et al., 1998[5–21]). In natural infection, sheep and goats rarely exhibit clinical signs.

There is considerable variation in the pathogenicity of different strains and the susceptibility of different host species [to disease]. The disease may manifest as an acute or chronic form [condition], and in the latter case may persist for several [many] months, possibly years. The disease is often rapidly fatal in camels, [buffalo] and horses, [cattle, llama and dog], but mild and subclinical infections may also be fatal [occur] in buffalo, cattle, llama and dog. However, these host species may also develop mild or subclinical infections. Wild animals such as deer, [and capybara and coat] can become infected and ill (including death), but they may also constitute a reservoir. Animals subjected to stress – malnutrition, pregnancy, work – are more susceptible to disease.

Biologically T. evansi is very similar to T. equiperdum, the causative agent of dourine (Claes et al., 2003[2]), and morphologically resembles the slender forms of the tsetse-transmitted trypanosomes, T. brucei, T. b. gambiense and T. b. rhodesiense. Most of the molecular characterisations indicate that various strains of T. evansi isolated from Asia, Africa and South America are very homogeneous and may have a single origin (Ventura et al., 2002), but other works suggest that T. evansi could have emerged from T. brucei in several instances (Jensen et al., 2008; Lai et al., 2008). Molecular characterisation using random amplified polymorphic DNA techniques and endonuclease fingerprinting showed that isolates of T. evansi and T. equiperdum formed a closely homogeneous group. The difficulties to differentiate T. equiperdum from the other Trypanozoon have been stressed (Claes et al., 2005; Zablotski et al., 2003), and the existence of T. equiperdum was even questioned. [One possibility of this finding is that T. equiperdum is not a genuine species per se and that the clinical outcome of disease is related primarily to the hosts’ immune response.]

Like all pathogenic trypanosomes, T. evansi is covered by a dense protein layer consisting of a single protein called the variable surface glycoprotein (VSG). This acts as a major immunogen and elicits the formation of specific antibodies. The parasites are able to evade the consequences of these immune reactions by switching the VSG, a phenomenon known as antigenic variation.

Clinical suspicion of surra can emerge from the field in case of fever and/or anaemia. Anaemia is a reliable indicator of trypanosome infection, but it is not in itself pathognomonic. On the other hand, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia (Dargantes et al., 2005a).

In enzootic areas, routine diagnoses can be made using parasitological techniques, while serological surveys can be carried out preferably by ELISA. CATT can be used to target individual animals for treatment with trypanocidal drugs.

For a definitive confirmation of the infection in suspected animals, mouse inoculation is the best test to apply.

For declaring disease-free status, at the individual level, serial testing by CATT and ELISA at 40-day intervals is recommended. However the conditions for importation of animals from infected to non-infected areas should be defined, including the status of the exporting farm, the status of exported animals, the application of a diagnostic protocol, and possibly the preventive administration of curative treatments.
In areas where *Trypanosoma evansi*, *T. equiperdum* or tsetse-transmitted trypanosomoses occur, cross-reactions may occur with any serological test employed. In such conditions, the exact status of an animal regarding trypanosomosis cannot be established.

The OIE has developed international standard monographs for trypanocidal drugs.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

The classical direct parasitological methods for the diagnosis of trypanosomosis, namely microscopic examination of blood or lymph node material, are not highly sensitive, but a number of techniques, including enrichment of the sample, rodent inoculation and DNA methods may increase the sensitivity. In regions where other *Trypanozoon spp.* occur in addition to *T. evansi*, specific identification by microscopy is not possible; molecular tools are then very useful for species-specific diagnosis. Specific DNA probes (19, 24) may enable identification of *trypanosome species* by non-radioactive DNA hybridisation. A species-specific polymerase chain reaction (PCR) based on *T. evansi* specific antigen (RoTat 1.2 VSG) has been developed, but has not been validated in the field (3).

**a)** Direct microscopic examination [methods]

- **Usual field methods**
  - **Blood sampling**
    
    *Trypanosoma evansi* is a parasite of the blood and tissues, [often inhabiting the deep blood vessels in cases of low parasitaemia. For this reason. As for other trypanosomes, it is recommended that blood for diagnosis be obtained from [both the peripheral ear or tail vein, even if jugular vein is most often preferred for practical reasons and deep blood vessels]. However it should be realised that less than 50% of infected animals may be identified by examination of [peripheral] blood.

    Peripheral blood is obtained by puncturing a small vein in the ear or tail. Deeper samples are taken from a larger vein by syringe. Cleanse an area of the ear margin or tip of the tail with alcohol and, when dry, puncture a vein with a suitable instrument (lancet, needle). Ensure that instruments are sterilised or use disposable instruments to avoid transmission of the [are used between individual animals, so that] infection [cannot be transmitted] by residual blood.

- **Wet blood films**

  Place a small drop of blood (2–3 µl) on to a clean glass slide and cover with a cover-slip to spread the blood as a monolayer of cells. Examine by light microscopy (200 ×) to detect any motile trypanosomes. Better observation can be obtained with dark-ground or phase-contrast microscopy (200–400 ×). The sensitivity of this method is low, approximately 10 trypanosomes per ml, which is frequent in early or acute infections only.

- **Stained thick smears**

  Place a large drop of blood (10 µl) on the centre of a microscope slide and spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. Air-dry for 1 hour or longer, while protecting the slide from insects. Place the slide in a horizontal position, stain the unfixed smear with Giemsa’s Stain (one drop of commercial Giemsa + 1 ml of phosphate-buffered saline, pH 7.2), for 25 minutes. After washing and drying, examine the smears by light microscopy at medium [high magnification (500–1000 ×)] with oil immersion. The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites, which are more visible owing to the haemolysis of the unfixed red cells. The disadvantage is that the trypanosomes may be damaged in the process, and the method is therefore not suited for species identification in case of mixed infections.

- **Stained thin smears**

  Place a small drop of blood (3–5 µl) at [20 mm from] one end of a clean microscope slide and draw out a thin film in the usual way. Air-dry briefly and fix in methyl alcohol for [2] minute and allow to dry. Stain the smears in Giemsa (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. Pour off, stain and wash the slide in tap water and dry. Nowadays, fast stains are most often used1, which allow to fix and stain within a few seconds. Slides are then washed in tap water and dried. [Unfixed smears can be stained by covering them with May–Grünwald stain for 2 minutes, then adding an equal volume of PBS, pH 7.2, and leaving the slides for a further 3 minutes. Pour off and add diluted Giemsa for 25 minutes. Pour off, wash the slides with tap

---

1 For example Diff-Quick® RAL555®
Examine at a high magnification (400–1000 ×) with oil immersion. This technique permits detailed morphological studies and identification of the Trypanosoma [trypansomal] species, but it is of a very low sensitivity (it can detect parasitaemia >500,000 trypanosomes/ml of blood), so it is used either for detection of all the haemoparasites or for morphological studies of trypanosomes. [Rapid staining techniques also exist (Field's stain, Diff-Quick®)].

v) Lymph node biopsies of oedema fluid

Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes. Select a suitable node by palpation and cleanse the site with alcohol. Puncture the node with a suitable gauge needle, and draw lymph node material into a syringe attached to the needle. Expel lymph on to a slide, cover with a cover-slip and examine as for the fresh blood preparations. Fixed thin or thick smears can also be stored for later examination. Similar examination can be done by collection of oedema fluid.

b) Concentration methods

In most hosts T. evansi can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the parasites. In these circumstances, concentration methods are necessary, as they increase the sensitivity of microscopic examination.

i) Haematocrit centrifugation technique (also known as Woo's technique, or HCT)

Collect blood (70 µl) into at least two heparinised capillary tubes (75 × 1.5 mm). Close the wet end with plasticine and centrifuge at 3000 g for 5[繁殖] minutes (generally 12,000 rpm in a haematocrit centrifuge machine). The capillary tube is examined and the value of the haematocrit is expressed as a percentage of packed red blood cells (RBCs) to total blood volume; this gives an indication on the anaemia of the animal. The capillary tube in then placed in a groove made with pieces of slide glued to a slide. Trypanosomes are large cells that concentrate at the junction between theuffy coat and the plasma, which is observed under the microscope (100–200 ×). Light conditions must be set to induce refringence of the cells to increase the visibility of the moving trypanosomes; this can be obtained when lowering the position of the light condensor or with intermediary positions of the turret light condenser. Specially designed reading chambers for HCT can be obtained at the OIE Reference Laboratory for Surra, at the Institute of Tropical Medicine (ITM) ². The fresher the sample, the better is the sensitivity as strong parasitic movements make trypanosomes more visible. This technique can detect around 50–200 trypanosomes/ml of blood (Desquesnes & Tresse, 1996). The Buffy coat can also be collected in a microtube and frozen; further on the sample can be prepared for polymerase chain reaction (PCR). [Place the capillary tube between two pieces of glass (25 × 10 × 1.2 mm) glued to a slide. Place a cover-slip on top at the level of the buffy coat junction where the trypanosomes will be concentrated. Flood the space around this part of the tube with water or immersion oil, and examine the buffy coat area under the microscope (×100–200). This technique can detect around 400 trypanosomes/ml. A simpler alternative is to examine the centrifuged capillary tube by placing a drop of immersion oil on the tube and ensuring that there is contact between the objective lens and the immersion oil.]

ii) Dark-ground/phase-contrast buffy coat method (also known as Murray's technique, or BCM)

This technique is very similar to the previous one. Collect blood into heparinised capillary tubes and centrifuge as above. Scratch the tube with a glass-cutting diamond and break it 0.5–1 mm below the buffy coat layer – the upper part thus contains a small top layer of RBCs, the buffy coat (white blood cells and platelets) and some plasma.

Partially expel the contents of this piece on to a slide; avoid expelling more than 5–8 µl of plasma, but make sure the buffy coat has been expelled (the small disk of the buffy coat should be visible to the naked eye), cover with a cover-slip, press to spread the buffy coat and examine by dark-ground, phase-contrast or similar microscopy to the previously described refringent conditions at a magnification of 200–500 ×. Trypanosomes are mostly present at the periphery of the thick buffy coat material.

Both the Woo and the Murray techniques allow anaemia to be estimated by measuring the packed cell volume and may be used in surveys of herds at risk. The value of the haematocrit can be used as an indicator (when <24% for example in cattle) to select a subset of samples to be submitted to the more expensive PCR analysis (Desquesnes et al., 1999).

[cover with a cover-slip and examine under dark-ground, phase-contrast or ordinary illumination.]

As an alternative to the electrically powered haematocrit centrifuge, hand-powered micro-centrifuges have been used successfully for detection of trypanosomosis in cattle and camels (9, 14).

iii) Haemolysis techniques

2 Laboratory of Parasite Diagnostic, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. pbuscher@itg.be, fclaes@itg.be
Sodium dodecyl sulphate (SDS) can be used as a reagent to haemolyse RBCs to facilitate detection of mobile trypanosomes in parasitised blood samples. As SDS is toxic, contact with skin, inhalation and ingestion should be avoided. SDS solution can be stored for several months at ambient temperature. Both the SDS solution and the blood samples should be used at a temperature above 15°C. At lower temperatures the trypanosomes may be destroyed.

Two general procedures, namely wet blood film clarification and haemolytic centrifugation, can be used.

### 1. Wet blood film clarification method

This method uses the partial lysis of RBCs to facilitate the detection of mobile trypanosomes. The method requires an SDS solution: 1% SDS dissolved in Tris/glucose/saline, pH 7.5, inoculating loops (10 µl), slides and cover slips (24 x 24 mm), and a drop of fresh meat or heparinised blood. Dissolve 100 mg of SDS in 100 ml of isotonic Tris/NaCl/glucose buffer, pH 7.5 (Tris base 14.0 g, NaCl 3.8 g, glucose 10.8 g; dissolve chemicals in 750 ml distilled H2O; add 90–100 ml 1 N HCl and adjust pH to 7.5 then make up to final volume of 1000 ml with distilled H2O). This buffer can be stored in small vials at ambient temperature for several months. The test does not give a significantly higher sensitivity than wet film technique. In addition, the SDS can cause problems when focusing the microscope and the movements of trypanosomes can be severely curtailed owing to the high viscosity of the SDS.

Put approximately 10 µl of blood on a slide. Add 10 µl of SDS solution using a dip inoculation loop and mix gently. Apply a cover-slip and examine the preparation without delay at low magnification (<100 or <200)

### 2. Haemolytic centrifugation technique

Nearly complete lysis of RBCs is required for this procedure. The materials needed include: SDS solution (0.1% SDS dissolved in Tris/glucose/saline, pH 7.5), conical centrifuge tubes, ordinary test tubes, large and fine plastic taping pipettes with attached bulb, slides, cover-slips (24 x 24 mm or 24 x 32 mm), and heparinised blood.

Using a pipette or syringe, transfer nine volumes (maximum 6.3 ml) of SDS solution into an ordinary test tube. Aspirate one volume (maximum 0.7 ml) of heparinised blood into a bulb pipette and expel it just above the surface of the SDS solution; mix quickly and thoroughly. Avoid foam formation, which may result in destruction of the trypanosomes. Wait for 10 minutes so as to achieve complete haemolysis.

Pour the haemolysed suspension into a conical centrifuge tube and spin at approximately 500 g for 10 minutes. With a clean bulb pipette, remove as much supernatant as possible without disturbing the sediment. Using a fine tapering bulb pipette, remove more supernatant, leaving 10–20 µl of undisturbed sediment at the bottom.

Very carefully collect the entire sediment and put on to a microscope slide. Apply a cover-slip and examine the preparation without delay at low magnification (<100 or <200).

### 3. Mini-anion exchange centrifugation technique

When a blood sample from animals infected with salivarian trypanosomes is passed through an appropriate DEAE-cellulose (diethylamino-ethylcellulose, such as Whatman DE 52) anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, can be [are] adsorbed onto the anion-exchanger (in pH and ionic strength conditions adapted to the host species), while the trypanosomes are eluted, retaining viability and infectivity (Lanham & Godfrey, 1970). This technique is mostly used for the purification of parasites from the blood (for example, for parasite antigen preparation), but miniature systems have been developed, especially for diagnosis in humans (Lundsen et al., 1981). A simplified field method for detection of low parasitaemia has been developed (Sachs, 1984). The sensitivity of this technique can be increased by approximately tenfold by the use ofuffy coat preparations rather than whole blood (Reid et al., 2001).

### Preparation of phosphate buffered saline glucose (PSG), pH 8

Na2HPO4 anhydrous (13.48 g); NaH2PO4•2H2O (0.78 g); NaCl (4.25 g); distilled water (1 litre). Solutions of different ionic strength are made by diluting the stock PBS, pH 8, and adding glucose to maintain a suitable concentration. For blood of mice, domestic and wild ruminants and dog, add four parts PBS to six parts distilled water and adjust the final glucose concentration to 1%. For blood of pigs and rabbits, add three parts PBS to seven parts distilled water and adjust the final glucose concentration to 1.5%. The PBS/glucose solution (PSG) must be sterile (however, PBS must be autoclaved before adding glucose).

### Equilibration of DEAE-cellulose

Suspend 500 g of DEAE-cellulose in 2 litres of distilled water. Mix for 20 minutes with a magnetic stirrer at low speed. Adjust the pH to 8 with phosphoric acid. Allow to settle for 30 minutes. Discard the supernatant fluid containing the finest granules. Repeat the procedure three times. Store the

---

3 All necessary materials and instructions can be obtained from the Institute of Tropical Medicine, Laboratory of Serology, Nationalestraat 155, B-2000 Antwerp, Belgium.
Packaging of equilibrated DEAE-cellulose

Place a 2 ml syringe without the plunger on a test-tube rack complete with a flexible pipe that can be capped with a clamp to act as a tap [see Note 20.4.2.1.1]. Put a disc of Whatman No. 41 filter paper at the bottom of the syringe and moisten by adding a few drops of PSG. Pour 2–2.5 ml of the slurry of equilibrated cellulose into the syringe and allow packing for 5 minutes before [i.e., pack] elution of the buffer. The height of the sediment should be approximately 3 cm. Wash and equilibrate the column with 2 ml of PSG without disturbing the surface.

Adsorption of blood eluate of the trypanosomes

Gently place 100–300 µl of heparinised blood (or preferably buffy coat) above the surface of the cellulose column, allow it penetrate the cellulose, but do not let the cellulose dry before pouring the eluting buffer. [Add ten drops of PSG and discard the first ten drops of the eluate] Progressively add 1.5 ml of PSG and start collecting the eluate into a finely tapered Pasteur pipette with a sealed end. The cellulose column should remain wet throughout the procedure. Put the filled pipette, protected by a conical plastic pipette tip, in a tube and centrifuge at 525 g (or up to 1000 g) for 10 minutes. Examine the bottom of the pipette under the microscope (100 × or 200 ×) using a special mounting device. Alternatively, the eluate could be collected into 50 ml plastic tubes, with conical bottoms, centrifuged at 1000 g and the sediment examined by dark-ground microscopy.

A derived method used in cattle, pig and goat is also referred to as the miniature anion exchange chromatography method (Gutierrez et al., 2004a; Reid et al., 2001; Sachs, 1984). In contrast, large amounts of blood or buffy coat can be handled on large columns for indirect fluorescence antibody test (IFAT), card agglutination test (CATT) or enzyme linked immunosorbent assay (ELISA) antigen preparations.

c) Animal inoculation

Laboratory animals may be used to reveal subclinical (non-patent) infections in domesticated animals. Trypanosoma evansi has a broad spectrum of infectivity for small rodents, and so rats and mice are often used. Rodent inoculation is not 100% sensitive (Monzon et al., 1990) but further improvement in its efficacy can be obtained by the use of buffy coat material. Such a procedure was able to detect as few as 1.25 T. evansi/ml blood (Reid et al., 2001; 25). This technique is suitable when highly sensitive detection is required.

Inoculate heparinised blood intraperitoneally into rats (1–2 ml) or mice (0.25–0.5 ml). Inoculate a minimum of two animals. Bleed animals from the tail after every 48 hours [three times a week] to detect parasitaemia. The incubation period before appearance of the parasites and their virulence depends on the strain of trypanosomes, their concentration in the inoculum, and the strain of laboratory animal used; however in most cases it is very short (5 ± 2 days), but can extend to 2 weeks in rare cases (Monzon et al., 1990). Sensitivity of this in-vivo culture system may [perhaps] be increased by use of immunosuppressed laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate, [α] X-ray irradiation, or splenectomy have been [are] used for this purpose. Such a procedure is only justified when the detection of a potentially infected host is of high importance (for example, importation into free area).

d) Recombinant DNA probes

Specific DNA probes have been used to detect trypanosomes in infected blood or tissue but are not routinely applied (28, 31). Although molecular methods have a potentially high analytical sensitivity there have been few convincing studies to critically evaluate the diagnostic sensitivity of these tests as compared with other techniques, such as serology.

d(e) Detection of trypanosomal DNA

Detection of minute amounts of trypanosomal DNA [using a PCR procedure] is a possible mean of identifying animals with active infections as the parasitic DNA does not remain for more than 24–48 hours in the blood of the host after the trypanosomes are killed (Desquesnes, 1997b) [and could have the sensitivity and specificity required (3, 20, 32)]. Experimental studies in buffaloes (10) showed the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation.

m DNA probes

Specific DNA probes have been used to detect trypanosome DNA in infected blood or tissue but are not routinely applied because their sensitivity is limited, they are time consuming and sometimes potentially toxic (Reid et al., 2001; Visesshakul & Panyim, 1990). PCR techniques are generally preferred and are routinely used in some laboratories.
Polymerase chain reaction (PCR)
Polymerase chain reaction (PCR), based on DNA sequences of various taxonomic levels is used. The gold standard, to date, for detection of the Trypanozoon subgenus are the NRP or TBR primers (Masiga et al., 1992; Moser et al., 1989). Other primers have been published and are being evaluated; some of them are specific for Trypanozoon (Desquesnes et al., 2011; Holland et al., 2001; Wuyts et al., 1994) and others for T. evansi + T. equiperdum (Artama et al., 1992; Claes et al., 2004; Panvim et al., 1993) (evaluation of the latter is very difficult because of the absence of collections of reference strains). To date, the most sensitive test is that of satellite DNA using TBR primers (Masiga et al., 1992); the sensitivity of the other primers is being compared under various conditions, including in laboratory rodents, but can only be validated with a sufficient batch of field samples from natural hosts. The use of TBR primers is recommended, at least in the first instance, and, if necessary, for example in areas and host species possibly infected with other trypanozoon such as T. brucei brucei, species confirmation can be obtained with more specific primers such as TEPAN (Panvim et al., 1993) or TE2249/2250 (Artama et al., 1992). Other primers specific for RoTat (Claes et al., 2004; Verloo et al., 2001) or non-RoTat strains (Nqaira et al., 2005), and other techniques such as the loop-mediated isothermal amplification (LAMP) (Thelwall et al., 2005) and Taqman (Taylor et al., 2008), are under development but need to be further evaluated and validated.

DNA preparation is an important step that determines the success and the sensitivity of the PCR. It can be done on plain blood (generally collected with anti-coagulant), or preferably, on the buffy coat to increase the sensitivity of the test (Desquesnes & Davila, 2002; Majwa et al., 1994). Several classical techniques are available, such as Chelex preparation (Solano et al., 1999), commercial kits and the phenol–chloroform preparation (Maciel et al., 2009). Blood conserved 1/1 in 70% alcohol, or on dry filter paper can also be used (Desquesnes, 2004; Holland et al., 2002; Omanwar et al., 1999).

The sensitivity of the PCR being dependent on the amount of DNA available, it is proportional to the parasitaemia. PCR is thus more sensitive in highly susceptible hosts (camels, horses, dogs, etc.) than in hosts of mild or low susceptibility (cattle, buffalo, pigs, etc.). Using a suitable DNA preparation and the most sensitive primers available (TBR), PCR allows as little as 1–5 trypanosomes/ml of blood to be detected (Panvim et al., 1993; Penchenier et al., 1996), or only 10 per ml in buffaloes with a quantitative real-time PCR (Konnai et al., 2009).

PCR offers the sensitivity and specificity required for detection of trypanosome infection (Masiga et al., 1992; Wuyts et al., 1994; Wuyts et al., 1995), but it gives false-negative results. Experimental studies in sheep have shown that PCR can remain negative for long during aparasitaemic periods (Bengaly et al., 2001), while in buffalo the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation (Holland et al., 2001). Nevertheless, PCR is the most sensitive technique for detection of the infection.

Antigen detection
Antigen detection is also a way to detect active infection. Several attempts to develop such tests have not yet reached a satisfactory level to be recommended for routine diagnosis (Desquesnes, 1996; Monzon, 2006; Morzaria et al., 1996).

Indirect methods
These methods involve tests that demonstrate the effects of the parasite on its host rather than directly detecting the parasite itself.

a) Haeematology
Anaemia is usually a reliable indicator of trypanosome infection, although it is not in itself pathognomonic. However, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia (4).

Anaemia can be estimated by measuring the packed cell volume and may be used in surveys of herds at risk. The technique is identical to that of haematocrit centrifugation. The capillary tube is examined and the results are expressed as a percentage of packed RBCs to total blood volume.

2. Serological tests
Historically, different methods have been used to detect nonspecific humoral antibodies to trypanosomal antigens but, more recently, there has been a tendency to concentrate on more easily standardized techniques such as ELISA (5, 7, 12, 22, 23, 24, 27) card agglutination tests (CATT) (4, 20, 24) and latex agglutination tests (LAT) (11, 16). Extensive evaluation of ELISA and CATT has been carried out in buffaloes in Indonesia and Vietnam (5, 11, 23). The collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (11). Other innovative modifications that might be developed in the future are the use of a colloidal-dye dipstick tests (13) that could enable the tests to be carried out under field conditions. It is vitally important that serological tests are validated and standardized if they are to be suitable for correctly identifying infected animals. This means that standard criteria for interpreting the tests might have to be developed for each animal species as well as each laboratory operating the procedure.
cases of surra infection. These methods are biochemical tests including flocculation, formol-gel, mercuric chloride precipitation and thymol turbidity tests, and are considered to be outdated although the formol-test may still have some use in the field because it is simple to perform. These tests all depend on an increase in serum globulins as a result of infection, but this increase is not specific for *T. evansi* infection. Mercuric chloride must not be used because of its toxicity. The formol-gel test is the test of choice in camels but has not been validated in other species. It is carried out by adding two drops of concentrated formalin solution (40% formaldehyde [w/v]) to 1 ml of serum. The test is positive if the serum coagulates immediately and turns white. In negative reactions, the serum remains unchanged or coagulation may take up to 30 minutes to appear.

Similarly, many different methods have been used to detect specific humoral antibodies to trypanosomal antigens, such as direct or indirect agglutination tests, complement fixation test (CFT), IFAT (Desquesnes, 1997a; Uilenberg, 1998) and the trypanolysis test. The IFAT is still useful for small-scale surveys. The trypanolysis test is used for individual confirmation of positivity because of its high specificity. The other tests are no longer used as, recently, they have been replaced by the more easily standardised techniques of ELISA (Davison et al., 1999; Franke et al., 1994; Rae et al., 1988; Reid & Copeman, 2002; Reid & Copeman, 2003; Tuntasuvan et al., 1996) and CATT (Bajyan Songa & Hamers, 1988; Njiru et al., 2004). Attempts to develop new techniques such as latex agglutination tests have not been successful so far (Gutierrez et al., 2004b; Holland et al., 2005; Morzaria et al., 1996).

Evaluations of ELISA and CATT have been carried out in camels, horses, cattle, buffaloes and pigs (Desquesnes et al., 2009; Diall et al., 1994; Holland et al., 2005; Payne et al., 1991; Reid & Copeman, 2003; Verloo et al., 2000). Tests should preferably be implemented with plasma or serum, but the collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (Holland et al., 2002; Hopkins et al., 1998). It is vitally important that serological tests be validated and standardised if they are to be suitable for correctly identifying infected animals; cross evaluation in different laboratories is thus required. Standard criteria for interpreting the tests might have to be developed for each animal species and standardised at least at a regional level (Desquesnes, 1997c). It is also necessary to take into consideration the various *Trypanosoma* species and strains (RoTat versus non-RoTat for example) present in a given area.

**a) Indirect immunofluorescent antibody test (IFAT)**

Although the technique is not adapted to large-scale surveys, it is still useful to screen a limited number of samples in laboratories that are carrying out the test for other purposes and/or that are not carrying out the ELISA. Cost of reagents is medium, around 0.5€/test, but the technique is time consuming.

**m Test procedure**

The antigen consists of dried blood smears containing from five to ten *T. evansi* per field at magnification 500×, collected from a highly parasitaemic mouse or rat (3–4 days post-infection). Smears are dried at room temperature for 1 hour and fixed with acetone (+ ethanol) for 5 minutes. When kept dry, the fixed smears may be stored at –20°C for several months. Better results are obtained using purified trypanosomes separated from the rat's buffy coat on a DEAE-cellulose column (Lanham & Godfrey, 1970) using a mixture of 80% cold acetone and 0.25% formalin in a normal saline solution.

On testing, the slides are first subdivided into several circles of 5 mm diameter with nail varnish using mounting media (Teflon-coated multisport slides may also be used), then washed in PBS, pH 7.2, at room temperature for 10 minutes.

After washing, a positive and a negative control serum and field sera to be tested (diluted 1/50 in PBS), are added and allowed to react at 37°C for 30 minutes in a humid chamber. The slides are washed three successive times in PBS for 5 minutes each. A rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate or other fluorescein-conjugated antisera specific to the animal species tested is then added at a suitable dilution and left at 37°C for 30 minutes in a humid chamber. The slides are rewashed in PBS, mounted with 50% glycerol in PBS with immunofluorescence mounting media, and examined by fluorescence microscopy. The glycerol solution should be stored at 4°C and renewed every 2 weeks.

The fluorescein conjugate should be stored at –20°C in small aliquots to avoid repeated freezing and thawing. The tube should be shielded from light in some way, for example by wrapping in aluminium foil. The conjugate is diluted in PBS, pH 7.2, or in PBS containing Evans blue 1/1000 (w/v) as a counterstain to facilitate discrimination between positive (green) and negative (red) fluorescence. In general, monospecific anti-IgG (gamma-chain) conjugates give the most specific results.

The IFAT *T. evansi* seroconversion can take 60–90 days (Jacquet et al., 1993). Compared with the CATT, IFAT is more sensitive, probably because it can detect aparasitaemic animals, but specificity is lower (Dia et
Various treatments of the antigen preparations have been applied to
by washing plate with 0.01
or reconstitute the frozen or freeze-
diluted in water so as to obtain a protein
thus obtained can be stored in small aliquots at –80
and brie-
Enzyme-linked immunosorbent assay (ELISA)
The principle of this technique is that specific antibodies to trypanosomes can be detected by enzyme-linked
anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen. The enzyme may be
peroxidase, alkaline phosphatase or any other suitable enzyme. The enzyme conjugate binds to the
antigen/antibody complex and then reacts with a suitable substrate to yield a characteristic colour change
either of the substrate itself or of an added indicator (the chromogen).

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat. The trypanosomes
are concentrated in theuffy coat by centrifugation and separated on a DEAE-cellulose column and washed
three times by centrifugation in cold PSG, pH 8 (PBS with 1% glucose). The final pellet is suspended in cold
PSG to a concentration of (2–5%), added with and anti-enzyme cocktail, submitted to five freeze–thawing
cycles, and ultrasonicated three times for 2 minutes on ice (and briefly ultrasonicated on ice for 30–120 seconds)
until disintegration of the organisms is complete. This preparation is centrifuged at 4°C and 14,000 g for
10(20) minutes. The supernatant is collected and the protein concentration estimated by UV readings at 260
and 280nm (WARBURG & CHRISTIAN, 1942). The soluble antigen (divided in water so as to obtain a protein
concentration of 1 mg/ml. The reagents thus obtained can be stored in small aliquots at –80°C for several
months. It can also be freeze-dried and stored at ~20°C. Coating of the ELISA plate is generally made with 5
µg/ml protein concentration in coating buffer [Various treatments of the antigen preparations have been applied to
improve the accuracy of antibody detection (32)].

Test procedure
i) Dilute the soluble antigen at 5 µg/ml in freshly prepared 0.01 M carbonate/bicarbonate buffer, pH 9.6.
Add 100 µl to each well of a 96-well microtitre plate and incubate overnight at 4°C or for 1 hour at 37°C.
For this step, Polysorp Nunc® immunoplates, which allow adsorption of the proteins under various
positions and thus allow access to all the epitopes, will be preferred to any other plate (for example
maxisorp Nunc immunoplates lead to a partial exposure of the epitopes owing to more specific
adsorption of the proteins) or reconstitute the frozen or freeze-dried antigen with freshly prepared 0.01 M
carbonate/bicarbonate buffer, pH 9.6. Add 100 µl to each well of a 96-well microtitre plate and incubate overnight at
4°C or for 1 hour at 37°C.
ii) Remove excess antigen and add 150 µl of blocking buffer (BB : 0.01 M PBS containing 0.1% Tween
20 and 5% skim milk powder for 1 hour at 37°C. The quality of the skim milk is very critical;
optimal skim milk concentration may vary from 0.5 to 7% depending on the skim milk origin. Bovine serum
albumin may also be used as blocking agent. by washing plate with 0.01 M PBS containing 0.05% Tween 20
(PBST). Add test serum dilutions in PBST (100 µl). Include control negative and positive sera. Dilutions must be
determined empirically, but are usually between 1:100 and 1:10,000.
iii) Add test serum dilutions in BB (100 µl), in duplicate or triplicate. Include control negative and positive
sera. Dilutions must be determined empirically, but are usually around 1:100–1:200. Incubate plates at
37°C for 30 minutes. Eject contents and wash five times with washing buffer (PBS-0.1% Tween 20).
iv) Add a specific peroxidase conjugated species-specific anti-globulin (100 µl) appropriately diluted in BB
(usually between 1:5000 and 1:20,000). If species-specific conjugates are not available, protein A or
protein G conjugates can be used. Incubate the plates at 37°C for 30 minutes, eject contents and wash
three times with washing buffer.
[iii] Incubate plates at 37°C for 30 minutes. Eject contents and wash three times with PBST.
iv) Add a specific peroxidase conjugated species-specific anti-globulin (100 µl) appropriately diluted in PBST (usually
between 1:1000 and 1:50,000).
v) Add the plates at 37°C for 30 minutes, eject contents and wash three times with PBST.
vi) For peroxidase conjugates a number of substrate/chromogen solutions can be used, consisting of
hydrogen peroxide with a chromogen, such as tetramethylbenzidine (TMB), 2,2'-azino-bis-(3-
ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ortho-diphenylenediamine (OPD). A suitable
substrate/chromogen solution for peroxidase conjugates is 30% hydrogen peroxide (0.167 ml and
35 mg) in citrate buffer (100 ml). pH 6.0. The citrate buffer is made up as follows: Solution A (36.85 ml):
0.1 M citric acid [21.01 g/litre]; Solution B (65.15 ml): (0.2 M, Na₂HPO₄ [35.59 g/litre]); and distilled
water (100 ml). Dissolve 10 mg TMB in 1 ml dimethyl sulphoxide and add to 99 ml of the citrate buffer.
A number of these combinations are available commercially in ready-to-use formulations that remain
stable at 4°C for up to 1 year. Add the substrate chromogen (100 µl) to the plates and incubate at room

---

4 For example: ref: 190-12865, Wako Pure Chemical Industries Ltd, Osaka, Japan.
vi) Read the plates or stop the reaction by adding 50 µl 1 M sulphuric acid. Read the absorbance of each well at 450 nm for TMB chromogen. Other chromogens may require the use of a different wavelength.

All tests should include three known high and medium positive control sera, three low and medium [a] negative control sera, and a buffer control. Results are expressed in relative percentage of positivity based on the optical densities of the control samples (Desquesnes, 1997c; Desquesnes et al. 2009).

A large variety of other test procedures exists, for example, using purified native antigen (Verloo et al. 1998) or more recently, using recombinant antigens (Tran et al. 2009). For closely related animal species, cross-reacting reagents may often be used (e.g. anti-bovine immunoglobulin for buffalos) and the use of monospecific anti-lgG conjugates is generally recommended. However, when specific conjugates are not available, nonspecific proteins able to fix on the Fc fragment of the immunoglobulins can be used, such as protein A (for detection of lgG) or protein G (for detection of IgM). Protein A conjugate has been validated for use in camels (Desquesnes et al. 2009).

There is a number of methods that can be used to determine a cut-off point to discriminate between positive and negative results. The simplest method is to base the cut-off on visual inspection of the test results from known positive and negative populations (Desquesnes, 1997c). These results are likely to show some overlap. The operator can choose the most appropriate point to modify the false-positive or false-negative results depending on the required application of the assay. An alternative is to base the cut-off on the mean +2 standard deviations (SD) or +3 SD values from a large sample of negative animals. Finally, if no suitable positive/negative samples are available, a cut-off can be based on the analysis of the data from animals in an endemic situation (Greiner et al. 1994). If a bimodal distribution separates infected from uninfected animals, then an appropriate value can be selected. The ELISA is likely to correctly identify uninfected animals (while the CATT would correctly qualify infected ones). A new ELISA/RoTat 1.2 based on the VSG from T. evansi RoTat 1.2 clone – a predominant antigen in T. evansi (Verloo et al. 2001) – was successfully used in the field in Vietnam (Holland et al., 2002; Verloo et al., 2000); protocols are available from the OIE Reference Laboratory at ITM (Antwerp) for use in equines, camellae and water buffaloes. Another test based on invariant surface glycoprotein has recently been developed at the ITM (Tran et al., 2009) and should proceed to inter-laboratory evaluation. [Equivocal results can be re-tested using CATT. The Institute of Tropical Medicine in Antwerp developed an ELISA using the VSG from a T. evansi RoTat 1.2 clone. It was shown (30) that this antigen is a predominant antigen in T. evansi and absent in T. brucei. This ELISA/RoTat 1.2 was successfully used in the field in Vietnam (11, 29). Protocols are available at ITM Antwerp for use in equines, camellae and water buffaloes.]

The VSGs may be too specific to be a universal test (see after RoTat versus non-RoTat parasites), while the ELISA using soluble antigens is not strain specific and this qualifies it as a universal test. Soluble antigens from whole lysate of T. evansi are able to detect immunoglobulins directed against T. evansi strains present in various host species and geographical areas (Laha & Sasm, 2008); they can also detect infections in heterologous systems owing to strong cross reactions with T. vivax, T. congolense and even T. cruzi. Trypanosoma evansi soluble antigen must then be considered as a universal reagent for detection of T. evansi, but consideration must be given to species specificity in multispecies areas. The cost of reagents is low, around 0.1€/test, and the technique is fast, allowing 500–1000 samples to be tested a day by experienced technicians.

\[\text{g[6]}\] Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. This finding was used as a basis for a test for the diagnosis of T. evansi, the card agglutination test – CATT/T. evansi [developed at the Laboratory of Serology, Institute of Tropical Medicine, Antwerp\textsuperscript{5}]. The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariant surface antigens take part in the agglutination reaction. The CATT is available in kit form from the OIE Reference Laboratory ITM. It consists of lyophilised stained parasites (‘antigen’), PBS, pH 7.4, plastic-coated cards, spatulas, positive and negative control sera and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 1 week [2days], but preferably should be used within 8 hours when kept at 37°C.

For screening, dilute test sera 1/4 or 1/8 in PBS on to circles inscribed on the plastic cards. Add 45 µl of the prepared antigen suspension (previously well shaken to homogenise the parasite suspension) onto circles inscribed on the plastic cards. Add 25 µl of each test serum. Mix and spread the reagents with a spatula and rotate the card for 5 minutes using the rotator provided in the kit (or at 70 rpm on a classical rotor agitator).

Compare the pattern of agglutination with the illustrations of different reactions provided in the kit.

Equivocal results can be re-tested using CATT. The Institute of Tropical Medicine in Antwerp developed an ELISA using the VSG from a T. evansi RoTat 1.2 clone. It was shown (30) that this antigen is a predominant antigen in T. evansi and absent in T. brucei. This ELISA/RoTat 1.2 was successfully used in the field in Vietnam (11, 29). Protocols are available at ITM Antwerp for use in equines, camellae and water buffaloes.

\[\text{g[6]}\] Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strands of salivarian trypanosomes from different areas. This finding was used as a basis for a test for the diagnosis of T. evansi, the card agglutination test – CATT/T. evansi [developed at the Laboratory of Serology, Institute of Tropical Medicine, Antwerp\textsuperscript{5}]. The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariant surface antigens take part in the agglutination reaction. The CATT is available in kit form from the OIE Reference Laboratory ITM. It consists of lyophilised stained parasites (‘antigen’), PBS, pH 7.4, plastic-coated cards, spatulas, positive and negative control sera and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 1 week [2days], but preferably should be used within 8 hours when kept at 37°C.

For screening, dilute test sera 1/4 or 1/8 in PBS on to circles inscribed on the plastic cards. Add 45 µl of the prepared antigen suspension (previously well shaken to homogenise the parasite suspension) onto circles inscribed on the plastic cards. Add 25 µl of each test serum. Mix and spread the reagents with a spatula and rotate the card for 5 minutes using the rotator provided in the kit (or at 70 rpm on a classical rotor agitator).

Compare the pattern of agglutination with the illustrations of different reactions provided in the kit. Blue granular deposits reveal a positive reaction visible to the naked eye. The cost of reagents is medium, around 0.5€/test, around 200 tests can be carried out a day by one technician.

5 CATT/T. evansi kits are available at the Laboratory of Parasite Diagnostic, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. (pbuscher@itg.be ; fclaes@itg.be)
As the CATT principally detects Ig M (agglutinating pentavalent immunoglobulins the half life of which is short), it is suitable for detection of early infections or late infections with recent circulation of parasites in the blood, and can detect active infections with a high positive predictive value. The CATT is more likely to classify correctly truly infected animals, it can be used to target individual animals for treatment with trypanocidal drugs.

An alternative test format (LATEX/T.evansi) using latex beads coated with native RoTat 1.2 VSG is currently under evaluation.

d) Latex agglutination tests

A kit is available from ITM, Antwerp. It comprises a lyophilised latex suspension coated with T.evansi RoTat 1.2 variable antigens, PBS, positive and negative controls, test cards, plastic spatulas and a rotator.

Reconstitute the antigen-coated latex particles using distilled, deionised water. Mix gently. Add 20 µl of latex suspension onto each black spot on the test cards.

Dilute test sera with PBS (1/2 to 1/4) and add 20 µl to the latex suspension. Include appropriate controls. Mix the reagents carefully with a plastic spatula.

Rotate test cards at 70 rotations/minutes for 5 minutes and view cards under a good light source at the end of the incubation. Positive sera will cause agglutination of the latex particles.

d) Immune trypanolysis test

Immune trypanolysis test detects specific 'trypanolytic' antibodies directed against a given parasitic strain able to induce trypanolysis in the presence of complement. It is performed with T. evansi variable antigen type RoTat 1.2 and may therefore be positive with hosts that produce trypanolytic immunoglobulins directed against RoTat 1.2 VAT (Van Meirvenne et al., 1995). Sera are tested at a 1/4 dilution. Live trypanosomes are incubated for 60 minutes with test serum in the presence of guinea-pig serum as the source of complement. When variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes occurs. The sample is considered positive for the presence of anti-RoTat 1.2 antibodies when 50% or more of the trypanosomes are lysed. This test required the growth of trypanosomes in rodents and is thus costly. At present, it is mostly used to confirm samples suspected to be positive using other tests. It can be carried out at the ITM, Antwerp, on request. The cost of the test is very high (250€/test).

C. REQUIREMENTS FOR VACCINES [AND DIAGNOSTIC BIOLOGICALS]

No vaccines are available for this disease.

REFERENCES


Chapter 2.1.17. - Trypanosoma evansi infection (surra)


Chapter 2.1.17. - Trypanosoma evansi infection (surra)


Chapter 2.1.17. - Trypanosoma evansi infection (surra)


Chapter 2.1.17. - Trypanosoma evansi infection (surra)
