CHAPTER 3.6.3.

# DOURINE IN HORSES (*TRYPANOSOMA EQUIPERDUM* INFECTION)


## SUMMARY

*Dourine is a chronic or acute contagious disease of breeding equids that is transmitted directly from animal to animal during coitus. The causal organism is* Trypanosoma (Trypanozoon) equiperdum. Trypanosoma equiperdum *is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids. It is present in the genital secretions of both infected males and females. The incubation period, severity, and duration of the disease vary considerably; it is often fatal, however spontaneous recoveries do occur as do latent carriers and subclinical infections. Donkeys and mules are more resistant than horses and may remain inapparent carriers. Infection is not always transmitted by an infected animal during every copulation. Although adaptation to other hosts is not always possible, dogs, rabbits, rats and mice can be infected experimentally and be used to isolate and maintain strains of the parasite indefinitely.* Trypanosoma equiperdum *strains are best stored in liquid nitrogen.*

*The clinical signs are marked by periodic exacerbation and relapse, ending in death, sometimes after paraplegia, or, possibly, recovery. Moderate fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial and lip paralysis, ocular lesions, anaemia, and emaciation may all be observed. Oedematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are still considered as pathognomonic, although they are also occasionally found in equids infected with* T. evansi.

***Detection of the agent:*** *Definitive diagnosis depends on the recognition of clinical signs, and identification of the parasite and evidence of sexual transmission. As this is rarely possible, diagnosis is usually based on clinical signs, serological or molecular evidence and epizootiological context.*

***Serological tests:*** *Humoral antibodies are present in infected animals with or without clinical signs. The complement fixation test (CFT) is used to confirm infection in clinical cases or in latent carriers. Non-infected animals, especially donkeys, often yield unclear results. The indirect fluorescent antibody test can be used to confirm infection or resolve inconclusive CFT results. Enzyme-linked immunosorbent and immunochromatographic assays are also used.*

***Molecular tests:*** *Genetic markers that allow unequivocal differentiation of* T. equiperdum *from* T. evansi *within the subgenus* Trypanozoon *are missing.*

***Requirements for vaccines:*** *There are no vaccines available for this parasite. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted mating because infection may be transmitted through contaminated fomites.*


## A. INTRODUCTION

Dourine is a chronic or acute contagious disease of equids that is primarily transmitted directly from animal to animal during coitus. Other routes of transmission, e.g. vertical or congenital, may be possible but have not been documented. The causal organism is *Trypanosoma equiperdum* (Doflein, 1901).

*Trypanosoma equiperdum* is a flagellated extracellular protozoan parasite belonging to the order Kinetoplastida, the family Trypanosomatidae and the subgenus *Trypanozoon*. Other species within this subgenus are *T. brucei* causing nagana (Chapter 3.4.14 *Nagana: infections with salivarian trypanosomoses* [*excluding* Trypanosoma evansi *and* T. equiperdum]) and *T. evansi* causing surra (Chapter 3.1.21 *Surra in all species* [Trypanosoma evansi *infection*]).

Both species are able to cause some similar clinical signs to dourine in chronic infections such as ventral oedema, emaciations, anaemia and neurological signs (Büscher *et al*., 2019). However, dourine initially often presents with genital lesions that may progress to neurological and chronic disease over a period of weeks to month. Morphologically and genetically these three species are very similar.

Dourine is also known under other names: mal de coït, syphilis du cheval, el dourin, morbo coitale maligno, Beschälseuche, slapsiekte, sluchnaya bolyezni, and covering disease (Barner, 1963; Hoare, 1972). So far, no human case has been reported.

Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in an infected Algerian horse (Rouget, 1896). *Trypanosoma equiperdum* is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids.

Dourine is transmitted during copulation, more commonly from stallion to mare, but also from mare to stallion, due to the presence of the parasite in the seminal fluid and mucous exudate of the penis and sheath of the infected male, and in the vaginal mucus of the infected female. Initially, parasites are found free on the surface of the mucosa or between the epithelial cells of a newly infected animal. Invasion of the tissues takes place, and oedematous patches appear in the genital tract. Parasites then may pass into the blood, where they are carried to other parts of the body such as the central nervous system. In typical cases, this metastatic invasion gives rise to characteristic cutaneous plaques.

The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild, and may persist from 6 months to 2 years (Henning, 1955). In Ethiopia and Mongolia, dourine appears to be endemic rather than epidemic (Davaasuren *et al*., 2017; Hagos *et al*., 2010). In other areas, such as Northern Africa and South America, the disease tends to be more acute, often lasting only 1–2 months or, exceptionally, 1 week. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery can occur. Subclinical infections are recognised. Donkeys and mules are more resistant than horses. In donkeys, the disease passes often unperceived whereas their semen and vaginal secretions contain infective trypanosomes.

As trypanosomes are not continually present in the genital tract throughout the course of the disease, transmission of the infection does not necessarily take place during every copulation involving an infected animal. Transmission of infection from mare to foal can occur via the mucosa, such as the conjunctiva (Hoare, 1972). Trypanosomes were found in the mammary gland of a non-lactating mare (Parkin, 1948) and in skin samples after examination by immunohistochemistry (Pascucci *et al*., 2013; Scacchia *et al*., 2011). Not all *T. equiperdum* strains can be readily adapted to laboratory animals such as rodents or rabbits. It has been reported that adaptation to rats is possible after isolation in rabbits by intratestis inoculation (Schneider & Buffard, 1900; Soldini, 1939). Direct *in-vitro* isolation from the urethral tract has also been achieved (Suganuma *et al*., 2016). Rodent- and *in-vitro*-adapted strains can be maintained indefinitely and cryopreserved. Antigens for serological tests are commonly produced from infected laboratory rodents or *in-vitro* propagated parasites.

Dourine is marked by stages of exacerbation, tolerance or relapse, which vary in duration and which may occur once or several times before death or recovery. The clinical signs most frequently noted are: pyrexia, tumefaction and local oedema of the genitalia and mammary glands, oedematous cutaneous eruptions, knuckling of the joints, incoordination, facial and lip paralysis, ocular lesions, anaemia, and emaciation. A frequent but not constant sign is the formation of oedematous plaques consisting of raised lesions in the skin, up to 5–8 cm in diameter and 1 cm thick. The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days. Oedematous plaques have long been considered pathognomonic, but have also been described in *T. evansi* infections (Van den Bossche *et al*., 2009).

Generally, the oedema disappears and returns at irregular intervals. During each recess, an increasing extent of permanently thickened and indurated tissue can be seen. The vaginal mucosa may show raised and thickened semi-transparent patches. Folds of swollen membrane may protrude through the vulva. It is common to find oedema of the mammary glands and adjacent tissues. Depigmentation of the genital area, perineum, and udder may occur. In the stallion, the first clinical sign is a variable swelling involving the glans, penis and prepuce. The oedema extends posteriorly to the scrotum, inguinal lymph nodes, and perineum, and anteriorly along the inferior abdomen. In stallions of heavy breeds, the oedema may extend over the whole floor of the abdomen.

Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears, and throat. Facial paralysis is usually unilateral. In fatal cases, the disease is usually slow and progressive, with increasing anaemia and emaciation, although the appetite remains good almost throughout.

At post-mortem examination, gelatinous exudates are present under the skin. In the stallion, the scrotum, sheath, and testicular tunica are thickened and infiltrated. In some cases, the testes are embedded in a tough mass of sclerotic tissue and may be unrecognisable. In the mare, the vulva, vaginal mucosa, uterus, bladder, and mammary glands may be thickened with gelatinous infiltration. The lymph nodes, particularly in the abdominal cavity, are hypertrophied, softened and, in some cases, haemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discoloured, particularly in the lumbar and sacral regions.

## B. DIAGNOSTIC TECHNIQUES

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

| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
| | Population freedom from infection | Individual animal freedom from infection prior to movement | Contribute to eradication policies | Confirmation of clinical cases | Prevalence of infection – surveillance | Immune status in individual animals or populations post-vaccination |
| **Detection of the agent[(a)]** | | | | | | |
| Microscopic observation | – | + | + | +++ | – | – |
| PCR/ real-time PCR | – | + | + | +++ | + | – |
| **Detection of immune response** | | | | | | |
| CFT | ++ | +++ | +++ | +++ | +++ | – |
| IFAT | ++ | + | ++ | + | ++ | – |
| ELISA | +++ | + | +++ | + | +++ | – |
| ICT | + | + | + | + | + | – |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; CFT = complement fixation test; IFAT = indirect fluorescent antibody test;
ELISA = enzyme-linked immunosorbent assay; ICT = immunochromatographic test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended

### 1.1. Overview of parasitological methods

A confirmed diagnosis depends on the recognition of clinical signs, demonstration of the parasite and evidence of infection via coitus. This is rarely possible because: (a) although the clinical signs and gross lesions in the developed disease may be pathognomonic, they cannot always be identified with certainty, especially in the early stages or in latent cases; they can be confused with other conditions, such as coital exanthema, or infections with other trypanosomes such as *T. evansi* (Büscher *et al*., 2019); (b) the trypanosomes are usually sparsely present and are extremely difficult to find, even in oedematous areas; and (c) knowledge about the transmission route is often lacking.

Recently, new *T. equiperdum* strains have been isolated in Ethiopia (Dodola), Italy (ICT 2011), Venezuela (TeAp-N/D1) and Mongolia (IVM-t1), although these isolates still have to be further characterised (Hagos *et al*., 2010; Pascucci *et al.,* 2013; Sánchez *et al*., 2015a, 2015b; Suganuma *et al*., 2016). In practice, diagnosis is based on clinical evidence supported by the mode of transmission, serology and histopathology.

In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the external genitalia, in the urethral and vaginal mucus (Parkin, 1948) and in exudates of plaques and mammary glands (Pascucci *et al*., 2013; Scacchia *et al*., 2011). They are usually undetectable in the blood, but may be found in the urethral or vaginal mucus collected from preputial or vaginal washings or scrapings 4–5 days after infection. The skin of the area over the plaque should be washed, shaved and dried, and the fluid contents aspirated by syringe. Blood vessels should be avoided. The fresh aspirate is examined microscopically for motile trypanosomes. These are present for a few days only, so lesions should be examined at regular intervals. As the parasite is rarely found in thick blood films, the use of concentration techniques is recommended, such as capillary tube centrifugation, or mini-anion exchange centrifugation technique (Lanham & Godfrey, 1970; Woo 1970).

*Trypanosoma equiperdum* is relatively easy to distinguish morphologically from *T. congolense* and *T. vivax,* both of which can infect horses. However, in countries where *T. evansi* or *T. brucei* occur, it is difficult to distinguish *T. equiperdum* microscopically (morphology, motility) from these other members of the subgenus *Trypanozoon*. In particular, *T. equiperdum* and *T. evansi* cannot be differentiated on the basis of morphological criteria. Both are monomorphic, slender trypomastigotes with a free flagellum, although stumpy forms have occasionally been reported. For typical strains the parasites range in length from 15.6 to 31.3 μm.

### 1.2. Detection of trypanosomal DNA and differential diagnosis

Kinetoplast DNA in the mitochondrion is the most remarkable characteristic in the order Kinetoplastida. In field situations, akinetoplastic strains of *T. evansi* (no kinetoplast visible when stained with Giemsa) were found in infected animals, but this situation was not observed in *T. equiperdum*. The suggestion by Li *et al.* (2007) that *T. equiperdum* can be distinguished from *T. evansi* by the apparent presence of intact maxi-circles in *T. equiperdum* remains controversial. On the other hand, the absence of the RoTat 1.2 variant surface glycoprotein (VSG) could be a molecular marker to differentiate *T. equiperdum* from *T. evansi* type A infections in horses (Claes *et al*., 2003). Although no *T. equiperdum*-specific polymerase chain reaction (PCR) method is available, subgenus *Trypanozoon*-specific PCR and real-time PCR can be used for detection of *T. equiperdum* DNA (Benfodil *et al.,* 2020; Masiga *et al*., 1992) (see also Chapter 3.1.21 *Surra in all species* [Trypanosoma evansi *infection*]). A highly sensitive real-time PCR for the *Trypanozoon* subgenus has been applied on tissues and fluid samples from a naturally dourine-infected horse, enabling the detection of low numbers of parasites (Becker *et al*., 2004; Pascucci *et al*., 2013).

***Table 2. Primers and probes described for Trypanozoon-specific conventional and real-time PCR that can be used for detection of* T. equiperdum *DNA***

| Target | Primers/probe sequences (5' → 3') | Amplicon length | Reference |
|---|---|---|---|
| 177 bp repeats | TBR1: GAA-TAT-TAA-ACA-ATG-CGC-AG | 164 bp | Masiga *et al*,. 1992 |
| | TBR2: CCA-TTT-ATT-AGC-TTT-GTT-GC | | |
| 177 bp repeats | Tb177F: AAC-AAT-GCG-CAG-TTA-ACG-CTA-T | 134 bp | Becker *et al*., 2004; Pascucci *et al*., 2013 |
| | Tb177B: ACA-TTA-AAC-ACT-AAA-GAA-CAG-CGT-TG | | |
| 18S rDNA | M18SF: CGT-AGT-TGA-ACT-GTG-GGC-CAC-GT | 150 bp | Benfodil *et al*., 2020 |
| | M18SR: ATG-CAT-GAC-ATG-CGT-GAA-AGT-GAG | | |
| | M18SP: TCG-GAC-GTG-TTT-TGA-CCC-ACG-C-MGB-VIC | | |

## 2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation test (CFT) is used to confirm clinical evidence and to detect latent infections. Uninfected equids, particularly donkeys and mules, often give inconsistent or nonspecific reactions because of the anticomplementary effects of their sera. In the case of anticomplementary sera, or for clinical diagnostic purposes, the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISAs) may provide additional information. There

are no internationally adopted protocols. Cross-reactions are possible due to the presence of other trypanosomes in some countries, for example, *T. cruzi, T. vivax* and *T. evansi*. *Trypanosoma equiperdum* is closely related to other *Trypanozoon* trypanosomes, including *T. brucei* and *T. evansi*. Members of this subgenus all share conserved cytoskeletal elements that provoke a strong and cross-reactive serological response. All diagnostic antigens and antisera currently available for use in serodiagnostic testing contain these conserved elements or antibodies to them, and thus none of the serological procedures described below is specific for dourine. Therefore, the diagnosis of dourine must include history, clinical, and pathological findings as well as serology to establish the definite confirmed case of the disease (Calistri *et al*., 2013). Significant improvements in dourine serodiagnosis will require development of more *T. equiperdum*-specific subunit antigens and antibodies to them.

## 2.1. Complement fixation test

Standard or microplate techniques may be used. Guinea-pig serum (available commercially) is used as a source of complement. Other reagents are sheep red blood cells (RBCs) washed in veronal buffer, and rabbit haemolytic serum (i.e. rabbit anti-sheep RBC) (commercial) as well as known negative and positive control sera.

### 2.1.1. Antigen production

Because of lack of solid serological or molecular markers to differentiate *T. equiperdum* from the other *Trypanozoon* taxa (Büscher *et al.,* 2019; Cuypers *et al*., 2017), it is important to indicate which *T. equiperdum* strain is used for any antigen preparation. Strains that easily grow in rodents are *T. equiperdum* OVI, BoTat, Dodola and TeAp-N/D1. Strains that are adapted to *in-vitro* culture are *T. equiperdum* OVI and IVM-t1. It should be kept in mind that crude antigen preparations such as described below, are not dourine-specific and will cross-react with sera from horses infected with *T. brucei* and *T. evansi*.

#### 2.1.1.1 Antigen preparation from *in-vitro* propagated parasites

The procedure described below is based on Bassarak *et al.* (2016) with some modifications. *Trypanosoma equiperdum* OVI (ITMAS 241199C, purchased from Institute of Tropical Medicine, Antwerp, in 2008) was adapted to *in-vitro* culture conditions. Culture-adapted trypanosome stocks in liquid nitrogen are available on request[1].

i) Reagents and solutions to prepare medium

| Substance | Identification System | Number |
|---|---|---|
| MEM powder for 1 litre with Earle's salts & L-glutamine, without $NaHCO_3$ (Sigma-Aldrich M0268) | | |
| 2-Mercapto-ethanol | CAS | 60-24-2 |
| Adenosine | CAS | 58-61-7 |
| Antibiotic-antimycotic solution (100×) | | |
| Bathocuproine disulfonate | CAS | 52698-84-7 |
| Cysteine | CAS | 52-90-4 |
| D(+)-Glucose × 1 $H_2O$ | CAS | 50-99-7 |
| Glycerol | CAS | 51-81-5 |
| HEPES | CAS | 7365-45-9 |
| Isopropanol | CAS | 67-63-0 |
| Hypoxanthine | CAS | 68-94-0 |
| New-born calf serum, heat-inactivated (NCS) | | |
| Potassium chloride | CAS | 7447-40-7 |
| Magnesium sulfate × 7 $H_2O$ | CAS | 10034-99-8 |
| MEM non-essential (100×) | | |

1 From the OIE Reference Laboratory for dourine: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

| Substance | Identification System | Number |
|---|---|---|
| Sodium pyruvate | CAS | 113-24-6 |
| Sodium chloride | CAS | 7647-14-5 |
| Sodium hydrogene carbonate | CAS | 144-55-8 |
| $Na_2HPO_3 \times 12\ H_2O$ | CAS | 10039-32-4 |
| $NaH_2PO_3 \times 2\ H_2O$ | CAS | 10049-21-5 |
| Ornithine/HCl | CAS | 3184-13-2 |
| Thymidine | CAS | 50-89-5 |
| Hypoxanthine 100× stock solution | 225 ml $H_2O$, 340 mg hypoxanthine, 25 ml 1 M NaOH. Stir in water bath for 20 min at 55°C. Filter through 0.22 µm filter; store at 4°C. | |
| Cysteine/bathocuproine-disulfonate 100× stock solution | 225 ml $H_2O$, 705 mg bathocuproine disulfonate, 4550 mg cysteine, 25 ml 2 M HCl. Stir for 20 min at 55°C. Filter through 0.22 µm filter. Store at 4°C. | |

ii) Preparation of the culture medium with 15% NCS (example for 3 litre)

Using a fume hood, add 47 µl 2-mercapto-ethanol to 10 ml $H_2O$. In a 5 litre beaker with 2430 ml $H_2O$, add: 3 MEM powder packs, 6.6 g $NaHCO_3$, 17.85 g HEPES, 3 g glucose, 0.66 g sodium pyruvate, 0.15 g ornithine, 0.012 g thymidine, 0.039 g adenosine, 30 ml MEM non-ess 100× stock solution, 15 ml antibiotic-antimycotic 100× stock solution, and 10 ml of the 2-mercapto-ethanol dilution. Adjust to pH 7.4 with NaOH and HCl and stir for 10 minutes. Add 30 ml hypoxanthine 100× stock solution and 30 ml cysteine/bathocuproine-disulfonate 100× stock solution. Adjust to pH 7.4 with NaOH and HCl and add $H_2O$ up to 2550 ml.

In three 1 litre flasks, dispense 150 ml NCS. Fill the flasks with 850 ml culture medium filtered over a 0.22 µm filter. Mix gently and store at 4°C. The culture medium is stable for at least 8 weeks.

iii) Preparation of the trypanosome dilution buffer (TDB), pH 7.7

Dissolve 3.23 g $Na_2HPO_4 \times 12\ H_2O$, 0.14 g $NaH_2PO_4 \times H_2O$, 0.19 g KCl, 2.34 g NaCl, 0.13 g $MgSO_4 \times 7\ H_2O$, 1.80 g D(+)-Glucose x $H_2O$ in 450 ml $H_2O$. Adjust to pH 7.7 with NaOH & HCl. Adjust to 500 ml with $H_2O$. Filter through 0.22 µm filter. Store at 4°C (stable for at least at 8 weeks).

iv) Preparation of a 5% PVP (polyvinylpyrrolidone), 0.01% merthiolate-NaCl solution

Prepare a 1% merthiolate-NaCl solution by dissolving 4.25 mg NaCl and 5 mg sodium ethylmercurithiosalicylate in 0.5 ml $H_2O$. In a 50 ml beaker, dissolve 425 mg NaCl and 2.5 g PVP 25 in 40 ml $H_2O$. Add the 0.5 ml 1% merthiolate-NaCl solution and adjust to 50 ml with $H_2O$. Filter through 0.22 µm filter. Store at 4°C.

v) Prepare a trypanosome culture with $1 \times 10^5$ trypanosomes/ml respecting a surface-volume ratio of 3.25 $cm^2$ per ml, e.g. in three-level T-500 culture flasks filled with 154 ml culture medium, and incubate at 37°C in a $CO_2$ incubator.

vi) Harvest the trypanosomes at concentrations of $1.5–2 \times 10^6$/ml once or twice a week in batches of 400 ml cell culture medium. Keep trypanosomes on ice during the whole process. Trypanosome containing medium is filled in a set of 50 ml tubes and centrifuged (10 minutes, 4°C, 1300 ***g***). Pellets of 8 tubes are resuspended carefully with a small volume of ice-cold TDB and transferred to one new, sterile 50 ml tube. The trypanosomes are washed twice with TDB (10 minutes, 4°C, 1300 ***g***) and the supernatant is removed completely. Pellets are stored at –20°C. It is advisable to confirm sterility of preparations using blood agar plates.

vii) The total number of cells of all pellets is determined. Prepare a fresh PVP-merthiolate solution (1 ml per $1 \times 10^9$ trypanosomes). Thaw frozen pellets on ice, resuspend the pellets with 50% of the calculated volume of ice-cold 5% PVP in 0.01% merthiolate-NaCl solution and pool them in a new sterile 50 ml tube. Fill ice-cold 5% PVP in 0.01% merthiolate-NaCl

solution to 100% of the calculated volume. Fill 200 µl antigen solution each in sterile beaded rim bottles (mix thoroughly several times during process), and place them in the biosafety transport box on ice for transport to the –80°C freezer. The lyophilisation apparatus is started and after 90 minutes the frozen antigen containing bottles are placed into the lyophilisation apparatus. Lyophilisation is performed overnight. The next day, lyophilisation is completed and immediately the cap is closed tightly and the antigen stored at –20°C. Alternatively, the antigen solution can be stored in small volumes at –80°C. The working dilution of antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

**2.1.1.2 Antigen preparation from *in-vivo* propagated parasites**

Taking into account the 3Rs principle, the *in-vivo* propagation procedure described below should be considered only if *in-vitro* propagation cannot be implemented.

i) Specific-pathogen free rats are anaesthetised with either $CO_2$ or isoflurane and inoculated with *T. equiperdum* cryopreserved stock after a blood smear has indicated viability. Adult rats receive 0.3–1.0 ml of rapidly thawed frozen stabilate intraperitoneally. At maximum parasitaemia, these starter rats are anaesthetised and a cardiac puncture is used to collect blood into an anticoagulant, such as heparin or Alsever's filled syringes, which will serve as a stock culture for the inoculation of additional rats.

ii) Twenty to 100 large rats are anaesthetised immediately prior to being inoculated intraperitoneally with 0.3–1.0 ml of this stock culture. All rats need to have a heavy infection concurrently. If necessary, the dose is adjusted and additional rats are inoculated to reach maximum parasitaemia at the desired time of 72–96 hours. Rats usually die within 3–5 days; prior to this, blood is taken daily from the tail for thin wet blood films and examined microscopically. When parasitaemia is maximal, the rats are euthanised via $CO_2$ and blood is collected via cardiac puncture for separation of the trypanosomes by one of the two protocols below: differential centrifugation or anion exchange chromatography.

iii) For differential centrifugation, infected rat blood is collected in Alsever's or acid–citrate–dextrose (ACD) saline solution. The bulk blood is thoroughly mixed, and then 45 ml volumes are aliquoted into 50 ml conical centrifuge tubes. The tubes are centrifuged at 2500 ***g*** for 10 minutes at 4°C. The contents of the tube should separate out into three parts (from top to bottom): serum/anticoagulant, trypanosomes/white blood cells (WBC), and red blood cells (RBCs).

iv) The serum/anticoagulant layer is removed and discarded. The trypanosomes/WBC layer is removed from each tube and placed in a vessel large enough to "re-bulk" all trypanosomes together. In addition, approximately 1–3 ml of the RBCs below the trypanosome/WBC layer will contain trypanosomes. Several ml of RBCs below the trypanosome/WBC layer should be removed and added to the bulk.

v) To wash trypanosomes to remove RBCs, the same anticoagulant that was used in the bulk blood collection should be used, and several washing steps should be performed. Add ~25 ml bulked trypanosome layer to 50 ml conical tubes, and top up each tube to 45 ml total volume of anticoagulant. The tubes are centrifuged at 2500 ***g*** for 10 minutes at 4°C. The trypanosome-containing layer is again removed and saved. Repeat the above washing steps until the RBC have been mostly removed from the trypanosome layer.

vi) To dilute the purified trypanosomes for antigen production, the purified trypanosome layer is mixed with lyophilisation media (5% PVP). Generally, the starting dilution factor is 1:5 trypanosome layer to lyophilisation media. Before use in CFTs, the antigen must be dispersed to a fine suspension with a hand-held or motorised ground glass homogeniser chilled in ice (Watson, 1920). This antigen may be divided into aliquots, frozen and lyophilised.

vii) For anion exchange chromatography, blood is taken on heparin and loaded on a DEAE (diethylaminoethyl) cellulose gel equilibrated with a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (Lanham & Godfrey, 1970). Blood cells are retained on the gel and the eluted trypanosomes are centrifuged at 1000–1500 ***g*** for 15 minutes. One volume of sedimented trypanosomes is resuspended in ice-cold phosphate buffer (0.01 M, pH 8.0) and lysed by hypotonic shock for 15 minutes. Thereafter, the suspension is

centrifuged at 42,000 ***g*** for 1 hour and the supernatant is collected and filtered through a 0.22 μm filter. The cleared supernatant contains the hydro-soluble fraction of the trypanosomes. The protein content can be determined by UV spectrophotometry or similar method and this antigen preparation can be stored at –80°C in small volumes. The antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

#### 2.1.2. Sera

Positive and negative sera should be inactivated at 58°C for 30 minutes before being used in the tests. Mule and donkey sera are normally inactivated at 62°C for 30 minutes. The USDA complement fixation protocol calls for inactivation of sera for 35 minutes (United States Department of Agriculture [USDA], 2016). Dilutions of sera that are positive in the screening test are titrated against two units of antigen. Test sera are screened at a dilution of 1/5. Sera showing more than 50% complement fixation at this dilution are usually deemed to be positive.

#### 2.1.3. Anticomplementary sera

If the anticomplementary control shows only a trace, this may be ignored. For all other anticomplementary sera, the activity can be titrated. A duplicate series of dilutions is made and the sample is retested using *T. equiperdum* antigen in the first row and veronal buffer only in the second. The second row gives the titre of the anticomplementary reaction. Provided the first row shows an end-point that is at least three dilutions greater than the second, the anticomplementary effect may be ignored and the sample reported as positive. If the results are any closer, a fresh sample of serum must be requested.

#### 2.1.4. Buffers and reagents

i) Trypanosome dilution buffer (TDB), pH 7.7: 18 mM $Na_2HPO_4$ 12 $H_2O$, 2 mM $NaH_2PO_4$ $H_2O$, 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$ 7 $H_2O$, and 18 mM glucose.

ii) 5% PVP in 1/10,000 merthiolate-NaCl solution: 145 mM NaCl, 1% 25 mM merthiolate-145 mM NaCl solution and 5% PVP.

0.15 M veronal buffered saline, pH 7.4, or any alternative commercially available CFT buffer, is used for diluting reagents and for washing sheep RBCs. Antigen is pretested by titration, and two units are used in the test. Guinea-pig complement (C) is tested for its haemolytic activity, and diluted to provide two units for the test. Sheep RBCs in Alsever's or ACD saline solution are washed three times. A 3% solution is used for the haemolytic system. The USDA protocol calls for 2% solution in the microtitration procedure (USDA, 2016). Titrated rabbit-anti-sheep RBCs – the rabbit haemolytic serum – is taken at double the concentration of its haemolytic titre (two units). All test sera, including positive and negative control sera, are inactivated at a 1/5 dilution before testing.

#### 2.1.5. Primary dilutions

i) Test, positive and negative control sera are diluted 1 in 5 with veronal buffer.

ii) The solutions are incubated in a water bath at 58°C for 30 minutes to inactivate complement and destroy anticomplementary factors. Donkey and mule sera should be inactivated at 63°C for 30 minutes.

#### 2.1.6. Screening test procedure

i) 25 μl of inactivated test serum is placed in each of three wells.

ii) 25 μl of inactivated control serum is placed in each of three wells.

iii) 25 μl of *T. equiperdum* antigen diluted to contain 2 units is placed in the first well only for each serum.

iv) 25 μl of complement diluted to contain 2 units is added to the first two wells only for each serum.

v) 25 μl veronal buffer, pH 7.4, is added to the second well for each serum (anticomplementary well).

vi) 50 µl veronal buffer, pH 7.4, is added to the third well for each serum (lysis activity well).

vii) The complement control is prepared.

viii) The plate is shaken on a microshaker sufficiently to mix the reagents.

ix) The plate is incubated for 1 hour in a water bath, incubator or in a humid chamber at 37°C.

x) The haemolytic system is prepared. After the first 50 minutes of incubation, the sheep RBCs are sensitised by mixing equal volumes of rabbit haemolytic serum, diluted to contain two units per 50 µl, and a 3% suspension of washed RBCs; the solution is mixed well and incubated for 10 minutes at 37°C.

xi) After incubation, 50 µl of haemolytic system is added to each well.

xii) The plate is shaken on a microshaker sufficiently to mix the reagents.

xiii) The plate is incubated for 30 minutes at 37°C. To aid in reading the results, the plates can be centrifuged after incubation.

xiv) *Reading the results:* the plate is viewed from above with a light source beneath it. The fixation in every well is assessed by estimating the proportion of cells not lysed. The degree of fixation is expressed as 0, 1+, 2+, 3+, 4+ (0%, 25%, 50%, 75% or 100% cells not lysed). Reactions are interpreted as follows: 4+, 3+, 2+ = positive, 1+ = suspicious, trace = negative, complete haemolysis = negative.

xv) *End-point titration:* All sera with positive reactions at 1/5 are serially double diluted and tested according to the above procedure for end-point titration.

## 2.2. Indirect fluorescent antibody test

An IFAT for dourine can also be used as a confirmatory test or to resolve inconclusive results obtained by the CFT. The test is performed as follows:

### 2.2.1. Antigen

(For method, see preparation of CFT antigen in Section B.2.1). Blood is collected into heparinised vacutainers or into a solution of acid–citrate–dextrose from an animal in which the number of trypanosomes is still increasing (more than 10 parasites per 10×40 microscope field should be present.)

i) The blood is centrifuged for 10 minutes at 800 ***g***.

ii) One to two volumes of PBS are added to the packed RBCs, the mixture is agitated, and smears that cover the whole slide evenly are made.

iii) The smears are air-dried and then wrapped in bundles of four, with paper separating each slide. The bundles of slides are wrapped in aluminium foil, sealed in an airtight container over silica gel, and stored at –20°C or –80°C.

iv) Slides stored at –20°C should retain their activity for about 1 year, at –80°C they should remain useable for longer.

### 2.2.2. Acid–citrate–dextrose solution

Use 15 ml per 100 ml of blood.

### 2.2.3. Conjugate

Fluorophore-labelled anti-horse immunoglobulins (commercially available).

### 2.2.4. Test procedure

i) The antigen slides are allowed to reach room temperature in a desiccator. An alternative method is to remove slides directly from the freezer and fix them in acetone for 15 minutes.

ii) The slides are marked out.

iii) Separate spots of test sera diluted in PBS are applied, and the slides are incubated in a humid chamber at ambient temperature for 30 minutes.

iv) The slides are washed in PBS, pH 7.2, three times for 5 minutes each, and air-dried.

v) Fluorescence-labelled conjugate is added at the correct dilution. Individual batches of antigen and conjugate should be titrated against each other using control sera to optimise the conjugate dilution. The slides are incubated in a humid chamber at ambient temperature for 30 minutes.

vi) The slides are washed in PBS, three times for 5 minutes each, and air-dried. An alternative method, to reduce background fluorescence, is to counter-stain, using Evans Blue (0.01% in distilled water) for 1 minute, rinse in PBS and then air dry

vii) The slides are mounted in glycerol/PBS (50/50), immersion oil (commercially available, non-fluorescing grade), or mounting reagent for fluorescent staining (commercially available).

viii) The slides are then examined under UV illumination. Incident light illumination is used with a suitable filter set. Slides may be stored at 4°C for 4–5 days. Sera diluted at 1/80 and above showing strong fluorescence of the parasites are usually considered to be positive. Estimating the intensity of fluorescence demands experience on the part of the observer.

Standard positive and negative control sera should be included in each batch of tests, and due consideration should be given to the pattern of fluorescence in these controls when assessing the results of test sera.

## 2.3. Enzyme-linked immunosorbent assay (ELISA)

The ELISA has been developed and compared with other serological tests for dourine (Wassall *et al.,* 1991).

Carbonate buffer, pH 9.6, for antigen coating on to microtitre plates: $Na_2CO_3$ (1.59 g); $NaHCO_3$ (2.93 g); and distilled water (1 litre). Alternatively, PBS ($KH_2PO_4$ [0.2 g]; $Na_2HPO_4$ 12 $H_2O$ [2.94 g]; NaCl [8.0 g]; KCl [0.2 g in 1 litre distilled water]) can be used for preparation of the antigen solution.

### 2.3.1. Blocking buffer

Carbonate buffer + 3% fetal calf serum (FCS), or PBS + 1% (w/v) casein.

### 2.3.2. PBS, pH 7.4, with Tween 20 (PBST) for washing

PBS + 0.05% (v/v) Tween 20.

### 2.3.3. Sample and conjugate buffer

PBST + 6% FCS, or PBS + 1% (w/v) casein.

### 2.3.4. Substrate indicator system

ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) (commercially available).

### 2.3.5. Conjugate

Peroxidase-labelled anti-horse IgG (commercially available).

### 2.3.6. Antigen

For method, see preparation of CF test antigen in Section B.2.1.

### 2.3.7. Test procedure

i) Wells in columns 2, 4, 6, etc., are charged with 100 µl of antigen (2 µg/ml), columns 1, 3, 5, etc., are charged with the same amount of carbonate buffer or PBS. The plate is incubated for 40 minutes at 37°C (or overnight at 4°C) in a humid chamber, and 350 µl of blocking buffer

is added to each well. The plate is incubated for 1 hour at ambient temperature, washed three times with PBST, with soaking times of 3 minutes/cycle.

ii) 150 µl of test samples and equine control sera, prediluted 1/100 in sample/conjugate buffer, is added in parallel to wells with and without antigen. The plate is incubated for 1 hour and washed three times with PBST.

iii) Properly diluted conjugate in sample/conjugate buffer is added in volumes of 150 µl to all wells. The plate is incubated for 1 hour with subsequent washing as above.

iv) 150 µl of substrate indicator system is added to all wells and incubated for 1 hour.

v) The plate is shaken for 10 seconds, and the results are read photometrically at a wavelength of between 410 and 420 nm.

vi) *Calculation of results:* absorbance (with antigen) minus absorbance (without antigen) = net extinction. A reaction exceeding a net absorbance value extinction of 0.3 is regarded as a positive result.

Standard positive and negative control sera should be included in each batch of tests.

### 2.4. Other serological tests

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion but these formats are outdated. More recently, an immunochromatographic test with recombinant antigen derived from *T. evansi* and an immunoblotting test with native antigen from *T. equiperdum* OVI have been developed and deserve further evaluation (Davaasuren *et al.*, 2017; Luciani *et al*., 2018).

## C. REQUIREMENTS FOR VACCINES

No vaccines are available for this disease. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted mating because infection may be transmitted through contaminated fomites.

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**NB:** There is an OIE Reference Laboratory for dourine (please consult the OIE Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3 ). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for dourine

**NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.