CHAPTER 3.5.3.

DOURINE

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 (*Doflein, 1901*).

Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. Trypanosoma equiperdum *differs from other trypanosomes in that it 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 it was claimed that spontaneous recoveries do occur and latent carriers do exist as well. Subclinical infections can occur. Donkeys and mules are more resistant than horses and may remain unapparent carriers. Infection is not always transmitted by an infected animal at every copulation. Although adaptation to other hosts is not always possible, laboratory rodents, such as rabbits, rats and mice, can be infected experimentally and can be used to 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 were also found in equids infected with T. evansi *occasionally.*

Identification of the agent: *Definitive diagnosis depends on the recognition of clinical signs and identification of the parasite. As this is rarely possible, diagnosis is usually based on clinical signs, together with serological evidence from complement fixation* (*CF*) *tests.*

Serological tests: *Humoral antibodies are present in infected animals whether or not they display clinical signs. The CF test 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 CF test results. Enzyme-linked immunosorbent assays are also used.*

Molecular tests: *Genetic markers which will allow differentiating* T. equiperdum *from* T. evansi *within the subgenus* Trypanozoon *are under investigation.*

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 transmitted directly from animal to animal during coitus. The causal organism is *Trypanosoma equiperdum* (Doflein, 1901). 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. Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. *Trypanosoma equiperdum* differs from other trypanosomes in that it 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.

Infection 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. 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 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 its semen and vaginal secretions contain infective trypanosomes. In 2011, an outbreak of dourine was reported in Italy, 16 years after the previous officially reported dourine case in that country (Pascucci *et al*., 2013; Scacchia *et al*., 2011).

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 at every copulation involving an infected animal. Transmission of infection from mare to foal can occur via the mucosa, such as the conjunctiva. 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). Animals other than equids can hardly be infected experimentally, e.g. rabbits, rats and mice. Thus the isolation of new strains is quite difficult. It has been reported that adaptation to rats is possible after isolation in rabbits by intratestis inoculation (Heisch *et al.*, 1970; Schneider & Buffard, 1900; Soldini, 1939). Rodent-adapted strains can be maintained indefinitely; infected rodent blood can be satisfactorily cryopreserved. Antigens for serological tests are commonly produced from infected laboratory rodents.

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 of this disease 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 lips paralysis, ocular lesions, anaemia, and emaciation. A pathognomonic sign is the oedematous plaque consisting of an elevated lesion 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. They are not a constant feature.

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, with an anterior extension 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	Confirmation of clinical cases	Prevalence of infection – surveillance
Agent identification[1]				
Microscopy	–	+	+++	–
PCR	–	+	++	+
Detection of immune response				
CFT	+	+++	+++	+++
IFAT	++	+	+	++
ELISA	++	+	+	++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
PCR = polymerase chain reaction; CFT = complement fixation test;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. Overview of parasitological methods

A definitive diagnosis depends on the recognition of the clinical signs and the demonstration of the parasite. 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 (moreover, in some countries [e.g. in South America], *T. evansi* infections give rise to similar clinical signs; (b) the trypanosomes are only sparsely present and are extremely difficult to find, even in oedematous areas; and (c) the trypanosomes are only fleetingly present in the blood, and in small numbers that defy detection. Illustrating the challenges to isolate *T. equiperdum*, no parasite strain widely accepted to be *T. equiperdum* has been isolated in any country of the world since 1979 and most of the strains currently available in national veterinary diagnostic laboratories are related to *T. evansi* (Claes *et al.*, 2003; Lun *et al*., 1992).

Recently, new putative *T. equiperdum* strains have been isolated in Ethiopia (Dodola), in Italy and Venezuela (TeAp-N/D1), although these isolates still have to be further characterised (Hagos *et al*., 2010; Perrone *et al*. 2009; Pascucci *et al.,* 2013). In practice, diagnosis is based on clinical evidence supported by the mode of transmission, serology and histopathology. Recently, other approaches have been studied and reported on (Claes *et al.*, 2003).

In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the external genitalia, in the vaginal mucus (Parkin, 1948) and exudates of plaques and mammary gland exudates (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. Later, parasites may be found in the fluid contents of oedemas and plaques, especially shortly after their eruption. 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 that lesions should be examined at intervals. As the parasite is rarely found in thick blood films, the use of concentration techniques is recommended, such as capillary tube

1 A combination of agent identification methods applied on the same clinical sample is recommended.

centrifugation, and mini anion exchange centrifugation technique (Büscher *et al*., 2009; Lanham & Godfrey, 1970; Woo 1970).

As dourine is the only trypanosome to affect horses in areas free from Nagana or Surra diseases, the observation of trypanosomes in thick blood films is sufficient for a positive diagnosis. In countries where Nagana or Surra occur, it is difficult to distinguish *T. equiperdum* microscopically (morphology, motility) from other members of the subgenus *Trypanozoon* (*T. evansi, T. brucei*). 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 pleomorphic, stumpy forms are recognised. Typical strains of the parasite 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, akinetoplast strains of *T. evansi* (no kinetoplast was stained with Giemsa) were found in infected animals, but this situation was not observed in *T. equiperdum*. More relevant for distinction is the presence of maxi-circles in *T. equiperdum* and the absence in *T. evansi,* which provides a possible differentiation between these two parasites (Li *et al*., 2007). In addition, other publications suggest that absence of the VSG RoTat 1.2 could be a molecular marker to differentiate *T. equiperdum* from *T. evansi* (Claes *et al*., 2003; 2004), however VSG RoTat 1.2 is absent from some *T. evansi* stains (Ngaira *et al.,* 2005). Although no *T. equiperdum*-specific polymerase chain reaction (PCR) method is available, subgenus *Trypanozoon*-specific PCR can be used for detection of *T. equiperdum* DNA (see Chapter 3.1.21 Trypanosoma evansi *infections* [*including surra*]). Recently, a highly sensitive real-time PCR for *Trypanozoon* subgenus was applied on tissues and fluid samples from a naturally dourine-infected horse, enabling the detection of low numbers of parasites (Pascucci *et al*., 2013; Scacchia *et al.,* 2011).

PCR and other related DNA amplification methods have been used to examine exudates or tissue samples, taking into account their failure on blood samples after the initial phase of the infection (Calistri *et al*., 2013).

2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation (CF) test (UK Ministry of Agriculture, Fisheries and Food [MAFF], 1986) 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, the indirect fluorescent antibody (IFA) test is of advantage. There is no internationally adopted protocol. Cross-reactions are possible due to the presence in some countries of other trypanosomes, for example, *T. cruzi* and *T. evansi*. Enzyme-linked immunosorbent assays (ELISAs) are also used. *Trypanosoma equiperdum* is closely related to other Old World trypanosomes, including *T. brucei* and *T. evansi*. Members of this genus 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 (Herr *et al.*, 1985). 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 *T. evansi*, careful attention has to be paid to which *T. equiperdum* strain is used for the antigen preparation. According to recent progresses for differentiation of the two closely related species (Claes *et al*., 2003, 2004), true *T. equiperdum* seems to be differentiated from *T. evansi* type A by absence of the VSG RoTat 1.2 gene. With this in mind, *T. equiperdum* OVI and BoTat 1.1 strains could be the suitable strains for antigen sources.

i) A specific-pathogen free rat is inoculated with *T. equiperdum* cryopreserved stock. Adult rats receive 0.5–1.0 ml of rapidly thawed frozen stabilate intraperitoneally. At maximum parasitaemia, blood is collected into an anticoagulant, such as heparin, which will serve as a stock culture for the inoculation of additional rats.

ii) Twenty large rats are inoculated intraperitoneally with 0.5–1.0 ml of this stock culture. All rats are 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 from the tail for thin wet blood films and examined microscopically. When parasitaemia is maximal, the rat is killed and blood is collected 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. Although parasitemia is usually synchronous, if not, blood can be collected and held in Alsever's or ACD saline solution at 4°C until blood has been collected from all the rats. The blood is filtered through muslin gauze and centrifuged at 800 ***g*** for 4 minutes. The RBCs are mostly deposited while the trypanosomes remain in suspension.

iv) The supernatant fluid is transferred to a fresh tube; the upper layer of RBCs is mixed with trypanosomes to a second tube, and the next layer to a third. Alsever's or ACD saline solution is added to tubes 2 and 3 to prevent clotting of cells. All tubes are mixed and centrifuged at 1500 ***g*** for 5 minutes.

v) The supernatant fluid is discarded and the upper white layer of trypanosomes is transferred from all tubes into a clean tube. The next pink layer is transferred into a second tube, and the lower layer to a third tube.

vi) Physiological saline is added and mixed and the tubes are centrifuged again at 1500 ***g*** for 5 minutes to separate the trypanosomes. The washing step is repeated until all the trypanosomes are collected as a pure white mass. Ten rats should produce 3–5 g of antigen. The concentrated trypanosomes are diluted with two volumes of veronal buffer and 5% polyvinylpyrrolidone as a cryopreservative. Before use in CF tests, 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 at pH 8.0 and thus lysed by hypotonic shock during 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], 2006). 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 must 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 rated as positive. If the results are any closer, a fresh sample of serum must be requested. Dilution of the serum 1/2 and heat

inactivation at 60–63°C for 30 minutes may result in reduction or removal of the anticomplementary effect.

2.1.4. Buffers and reagents

0.15 M veronal buffered saline, pH 7.4, is used for diluting reagents and for washing sheep RBCs. Antigen is pretested by checkerboard 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 with confirmation in a tube test with 3% RBC (USDA, 2006). 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 five-times 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 IFA test for dourine can also be used (MAFF, 1986) as a confirmatory test or to resolve inconclusive results obtained by the CF test. The test is performed as follows:

2.2.1. Antigen

(For method, see preparation of CF test 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 is added to the packed RBCs, the mixture is agitated, and smears are made that evenly cover the whole slide.

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

Fluorescence-labelled sheep anti-horse immunoglobulins.

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 (Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, 1995; 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 phosphate buffered saline (PBS) (KH_2PO_4 (0.2 g); $Na_2HPO_4 \times 12\ H_2O$ (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. Citric phosphate buffer

Citric acid monohydrate (4.2 g in 200 ml distilled water); $Na_2HPO_4 \times 12\ H_2O$ (in 200 ml distilled water). Both components are mixed at equal volumes.

2.3.5. Substrate indicator system

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

2.3.6. Conjugate

Rabbit anti-horse IgG (H+L) PO or IgY anti-horse Ig-PO.

2.3.7. Antigen

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

2.3.8. 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 300 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, 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 415 nm.

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

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

A competitive ELISA has also been described for detecting antibody against *T. equiperdum* (Katz *et al.*, 2000).

2.4. Other serological tests

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion (AGID) tests (Caporale *et al.*, 1981; Hagebock *et al.*, 1993). The AGID has been used to confirm positive tests and to test anticomplementary sera. A seven-well pattern in 0.8% agarose in Tris buffer is used, with the CF test antigen in the centre well and positive control sera and unknown sera in alternate peripheral wells. A method has been published for diagnosing equine piroplasmosis, glanders and dourine at the same time, using immunoblotting (Katz *et al.*, 1999). A card agglutination test has been developed that compares favourably with the CF test (Claes *et al.*, 2005).

3. Confirmed case of dourine

In case of a serologically positive result and after clinical examination: repeat serological tests two times at 15- to 20-day intervals and perform an accurate epidemiological investigation.

A confirmed case of dourine is defined as an animal having a positive result with CFT or IFA or PCR and (i) showing clinical signs compatible with dourine or (ii) showing an increase in serological CFT titre in two consecutive tests or (iii) epidemiologically linked with a confirmed case of dourine (Calistri *et al*., 2013).

C. REQUIREMENTS FOR VACCINES

No vaccines are available for this disease.

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NB: FIRST ADOPTED IN 1990; MOST RECENT UPDATES ADOPTED IN 2013.