CHAPTER 2.5.3.

DOURINE

SUMMARY

Dourine is a chronic or acute contagious disease of breeding solipeds that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma 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 rarely invades 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, but spontaneous recoveries do occur but latent carriers do exist. Subclinical infections occur, and donkeys and mules are more resistant than horses and may remain inapparent carriers. Infection is not always transmitted by an infected animal at every copulation. Rats 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. Fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial paralysis, ocular lesions, anaemia, and emaciation may all be observed. Oedematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are pathognomonic.

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. Noninfected 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.

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

A. INTRODUCTION

Dourine is a chronic or acute contagious disease of breeding solipeds 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, el dourin, morbo coitale maligno, Beschälseuche, slapsiekte, sluchnaya bolyezn, and covering disease (1, 8).

Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in infected Algerian horses. 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 for several years (6). 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.

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. Mares’ milk has
been shown to be infectious. Animals other than equids can be infected experimentally. Rat-adapted strains can
be maintained indefinitely; infected rat blood can be satisfactorily cryopreserved. Antigens for serological tests are
commonly produced from infected laboratory rats.

The disease 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 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 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
semitransparent patches. Folds of swollen membrane may protrude through the vulva. It is not uncommon 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

1. Identification of the agent

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. For unknown reasons, no parasite strain of
T. equiperdum has been isolated in any country of the world since 1982 and most of the strains currently available in national
veterinary diagnostic laboratories are related to T. evansi (3).

In practice, diagnosis is based on clinical evidence supported by serology. Recently, other approaches have been
studied and reported on (3).

In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the
external genitalia, in the vaginal mucus, and fluid contents of plaques. 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. The parasite is rarely found in thick blood films, but is sometimes detectable after centrifuging blood and examining the recentrifuged plasma.

As dourine is the only trypanosome to affect horses in temperate climates, 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 trypanostigotes with a free flagellum, although pleomorphic, stumpy, proteonuclear forms are recognised. However, in kinetoplastic strains, the presence of maxicircles in *T. equiperdum* and the absence in *T. evansi* provides a possible differentiation (14).

Typical strains of the parasite range in length from 15.6 to 31.3 μm.

2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation (CF) test (12) 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 (monoclonal and polyclonal) currently available for use in serodiagnostic testing contain these conserved elements or antibodies to them, and therefore none of the serological procedures described below is specific for dourine. The diagnosis of dourine must include history, clinical, and pathological findings as well as serology. Significant improvements in dourine serodiagnosis will require development of more trypanosome-specific subunit antigens and antibodies to them.

a) Complement fixation test (the prescribed test for international trade)

Standard or microplate techniques may be used (7). 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.

- Antigen production

i) A rat is inoculated with *T. equiperdum* cryopreserved stock. The rat must be free from *T. lewisi*, which could be achieved by injection with neoarsphenamine, but is better accomplished by using specific-pathogen-free rats. A stock of rapidly thawed frozen stabilate, intramuscularly or 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 intramuscularly or 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 thick smears and examined microscopically. When parasitaemia is maximal, the rat is killed and blood is collected in Alsevers or acid–citrate–dextrose (ACD) saline solution. If parasitaemia is not synchronous, blood can be collected and held in Alsevers or ACD saline at 4°C until blood has been collected from all the rats.

iii) 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. Alsevers or ACD saline 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. This purification procedure can also be carried out using a column of DEAE (diethylaminoethyl) cellulose in a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (11).

vii) 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 (15). This antigen may be divided into aliquots, frozen and lyophilised.

The antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

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 (13). 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.

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.

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 (13). 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.

- **Primary dilutions**
  i) 100 µl of test serum is diluted with 400 µl of veronal buffer (1/5).
  ii) 100 µl of both positive and negative control sera is diluted with 400 µl of veronal buffer (1/5).
  iii) The solutions are incubated in a water bath at 58°C for 30 minutes to inactivate complement and destroy anticomplementary factors.

- **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 two units is placed in the first well only for each serum.
  iv) 25 µl of complement diluted to contain two 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.

b) **Indirect fluorescent antibody test**

An IFA test for dourine can also be used (12) as a confirmatory test or to resolve inconclusive results obtained by the CF test. The test is performed as follows:

**Antigen:** (For method, see preparation of CF test antigen in Section B.2.a) 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 (about ten parasites per 10× 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 −76°C.

iv) Slides stored at −20°C should retain their activity for about 1 year, at −76°C they should remain useable for longer.

**Acid–citrate–dextrose solution:** Use 15 ml per 100 ml of blood.

**Conjugate:** Fluorescein-labelled sheep anti-horse immunoglobulins.

**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 in a water bath at 37°C for 30 minutes.

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

v) Fluorescein-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 in a water bath at 37°C 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) or immersion oil (commercially available, non-fluorescing grade).

viii) The slides are then examined under UV illumination. Incident light illumination is used with barrier filter K 530 and exciter filter BG 12. 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.

c) **Enzyme-linked immunosorbent assay**

The ELISA has been developed and compared with other serological tests for dourine (14, 16).

**Carbonate buffer, pH 9.6, for antigen coating on to microtitre plates:** \( \text{Na}_2\text{CO}_3 \) (1.59 g); \( \text{NaHCO}_3 \) (2.93g); and distilled water (1 litre).
Chapter 2.5.3. - Dourine

Blocking buffer: Carbonate buffer + 3% fetal calf serum (FCS).

PBS, pH 7.4, with Tween 20 (PBST) for washing: KH$_2$PO$_4$ (0.2 g); Na$_2$HPO$_4$ × 12 H$_2$O (2.94 g); NaCl (8.0 g); KCl (0.2 g in 1 litre distilled water), and Tween 20 (0.5 ml).

Sample and conjugate buffer: PBST + 6% FCS.

Citric phosphate buffer: Citric acid monohydrate (4.2 g in 200 ml distilled water); Na$_2$HPO$_4$ × 12 H$_2$O (in 200 ml distilled water). Both components are mixed at equal volumes.

Substrate indicator system: ABTS (2,2'-Azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) (40 mg) is dissolved in citric phosphate buffer (100 ml), and stored at 4°C in the dark. Just before use, 100 µl of 1/40 H$_2$O$_2$ is added to 10 ml of ABTS.

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

Antigen: Lyophilised T. equiperdum antigen (0.5 ml) is reconstituted with coating buffer (5 ml), sonicated twice for 10 seconds each at 12 µm peak to peak, and centrifuged at 10,000 g for 4 minutes. The supernatant is further diluted to a pretested working dilution (e.g. 1/500).

Test procedure

i) Wells in columns 2, 4, 6, etc., are charged with 50 µl of antigen, columns 1, 3, 5, etc., are charged with the same amount of carbonate buffer. The plate is incubated for 40 minutes at 37°C in a humid chamber, washed in tap water, and 50 µl of blocking buffer is added to each well. The plate is incubated for 20 minutes, washed in tap water followed by three wash cycles with PBST, with soaking times of 3 minutes/cycle.

ii) 50 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 30 minutes, washed in tap water, followed by three wash cycles with PBST.

iii) Properly diluted conjugate in sample/conjugate buffer is added in volumes of 50 µl to all wells. The plate is incubated for 30 minutes with subsequent washing as above.

iv) 100 µl of substrate indicator system is added to all wells.

v) The reaction is stopped after 15 minutes at room temperature by the addition of 25 µl of 37 mM NaCN. Alternatively, commercially available detergents can be used after pretesting. The results are read photometrically at a wavelength of 405 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.

A competitive ELISA has also been described for detecting antibody against Trypanosoma equiperdum (10).

d) Other serological tests

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion (AGID) tests (2, 5). 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 (9). A card agglutination test has been developed that compares favourably with the CF test (4).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available. Control of the disease depends on compulsory notification and slaughter of infected animals. Good hygiene at assisted matings is also essential.

REFERENCES

Chapter 2.5.3. – Dourine


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NB: There is an OIE Reference Laboratory for Dourine (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).