CHAPTER 3.6.8.

EQUINE PIROPLASMOSIS

SUMMARY

Equine piroplasmosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents are blood parasites named Theileria equi *and* Babesia caballi*. Infected animals may remain carriers of these parasites for long periods and act as sources of infection for ticks, which act as vectors. These parasites are also easily spread by blood contaminated instruments.*

The introduction of carrier animals into areas where tick vectors are prevalent can lead to an epizootic spread of the disease.

Detection of the agent: *Infected horses can be identified by demonstrating the parasites in stained blood or organ smears during the acute phase of the disease. Romanovsky-type staining methods, such as Giemsa, give the best results. In carrier animals, low parasitaemias make it extremely difficult to detect parasites, especially in the case of* B. caballi *infections, although they may sometimes be demonstrated by using a thick blood smear technique.*

Paired merozoites joined at their posterior ends are a diagnostic feature of B. caballi *infection. The parasites in the erythrocytes measure 2 × 5 µm. The merozoites of* T. equi *are less than 2–3 µm long, and are pyriform, round or ovoid. A characteristic of* T. equi *is the arrangement of four pear-shaped merozoites forming a tetrad known as a 'Maltese cross'.*

Molecular techniques for the detection of T. equi *and* B. caballi *based on species-specific polymerase chain reaction (PCR) assays, targeting the 18S rRNA gene as well as BC48* (B. caballi) *and EMA-1* (T. equi) *genes, have been developed and continue to expand. These tests have been shown to be highly specific and sensitive and promise to play an increasing role in the diagnosis of infections. Importantly, the specificity of PCR can be defined beyond evaluation of the molecular mass of amplicons. Hybridisation with specific probes, restriction endonuclease analysis and sequencing of amplicons are also available.*

Serological tests*: Currently, the indirect fluorescent antibody test (IFAT) and the competitive enzyme-linked immunosorbent assay (C-ELISA) are the primary tests used for qualifying horses for importation. The complement fixation test (CFT), for many years the primary test, has been replaced by the IFA and C-ELISA; animals may be CF negative but still be infected. The IFAT and C-ELISA have been shown to be highly specific for each of the two species of piroplasmosis agents involved. One challenge with the IFAT is the need to dilute sera to reduce nonspecific binding and subsequent background, which may preclude binding to the intra-erythrocytic parasites. Sera dilutions to enhance specificity lead to a decrease in sensitivity of the IFAT and a specific cut-off should be determined. Indirect ELISAs using recombinant* T. equi *and* B. caballi *merozoite proteins in diagnostic assays appear to be very promising in the accurate determination of equine piroplasmosis infection.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

Equine piroplasmosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents of equine piroplasmosis are *Theileria equi* and *Babesia caballi*. Approximately fourteen species of Ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified as transstadial vectors of *B. caballi* and *T. equi*, while eight of these species were also able to transmit *B. caballi* infections transovarially (De Waal, 1992). Other genera such as *Ambloyomma* have also been identified as competent vectors (Scoles *et al.*, 2011). Infected animals may remain carriers of these blood parasites for long periods and act as sources of infection for tick vectors. DNA of some of these parasites has also been detected in camels and dogs without clinical disease (Onyiche *et al.,* 2019). The role of non-equid species in the epidemiology of the disease is unclear.

The parasites occur in southern Europe, Asia, countries of the Commonwealth of Independent States, Africa, Cuba, South and Central America, and certain parts of the southern United States of America. *Theileria equi* has also been reported from Australia (but never established itself in this region), and is now believed to have a wider general distribution than *B. caballi*.

During the life cycle of *Babesia*, sporozoites initially invade red blood cells (RBCs) where they transform into trophozoites. In this situation the trophozoites grow and divide into two round, oval or pear-shaped merozoites. The mature merozoites are capable of infecting new RBCs and the division process is then repeated.

For *Babesia caballi,* the merozoites in the RBCs are pear-shaped, 2–5 µm long and 1.3–3.0 µm in diameter (Levine, 1985). The paired merozoites joined at their posterior ends are considered to be a diagnostic feature of *B. caballi* infection.

For *Theileria equi,* the merozoites are relatively small, less than 2–3 µm long (Levine, 1985), and are pyriform, round or ovoid. A characteristic of *T. equi* is the arrangement of four pear-shaped merozoites, measuring about 2 µm in length, forming a tetrad known as the 'Maltese cross' arrangement (Holbrook *et al*., 1968).

In *T. equi* infection, it has been shown that sporozoites inoculated into horses via a tick bite invade the lymphocytes (Schein *et al*., 1981). The sporozoites undergo development in the cytoplasm of these lymphocytes and eventually form *Theileria*-like schizonts. Merozoites released from these schizonts enter RBCs. Vertical transmission of *T. equi* from mare to foal has also been reported (Allsopp *et al*., 2007). In experimental infection, *T. equi* was detected not only in the blood but also in the other tissues such as livers, spleens, lungs, and bone marrows (Alhassan *et al*., 2007).

The taxonomic position of *T. equi* has been controversial and only relatively recently it has been redescribed as a *Theileria* (Mehlhorn & Schein, 1998). Further support for the close relation with *Theileria* spp. also comes from the homology found between 30 and 34 kDa *T. equi* surface proteins and similar sized proteins of various *Theileria* spp. (Knowles *et al*., 1997). However, the position of *T. equi* in phylogenetic trees based on the small subunit ribosomal RNA genes is variable and mostly appear as a sister clade of the Theilerids (Criado-Fornelio *et al*., 2003) leading some to suggest that *T. equi* is ancestral to the Theilerids (Criado-Fornelio *et al*., 2003) or a different group altogether (Allsopp *et al*., 1994). Completion of the *T. equi* genome supported its phylogenetic position as a sister taxon to *Theileria* spp. (Kappmeyer *et al.,* 2012). Sequence heterogeneity exists within both *B. caballi and T. equi*. In particular, the unusual high sequence diversity of *T. equi* 18S rDNA and the recent discovery of a new *Theileria* species, *Theileria haneyi*, closely related to *T. equi,* strongly indicate that various cryptic species are now collectively referred to as *T. equi* (Knowles *et al*., 2018). These sequence heterogenicities and cryptic species could potentially impact the interpretation of molecular diagnostic tests.

The clinical signs of equine piroplasmosis are often nonspecific, and the disease can easily be confused with other conditions. Piroplasmosis can occur in peracute, acute and chronic forms. The acute cases are more common, and are characterised by fever that usually exceeds 40°C, reduced appetite and malaise, elevated respiratory and pulse rates, congestion of mucous membranes, and faecal balls that are smaller and drier than normal.

Clinical signs in subacute cases are similar. In addition, affected animals show loss of weight, and fever is sometimes intermittent. The mucous membranes vary from pale pink to pink, or pale yellow to bright yellow. Petechiae and/or ecchymoses may also be visible on the mucous membranes. Normal bowel movements may be slightly depressed and the animals may show signs of mild colic. Mild oedematous swelling of the distal part of the limbs sometimes occurs.

Chronic cases usually present nonspecific clinical signs such as mild inappetence, poor performance and a drop in body mass. The spleen is usually found to be enlarged on rectal examination.

A rare peracute form where horses are found either dead or moribund has been reported.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of equine piroplasmosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	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 examination	–	+	–	++	+	–
PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
IFAT	++	++	++	–	++	–
C-ELISA	+++	++	+++	–	+++	–

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

Negative results in agent identification or serological tests do not necessarily mean that the animals are free from infections. In persistently infected carrier animals, the parasites may be sequestered in organs such as spleen and bone marrow, while the parasites and their genetic materials are undetectable in the general circulation (Pitel *et al*., 2010; Ribeiro *et al*., 2013). Similarly, during the early stage of the infections, horses may be seronegative until the antibodies reach the levels detectable by the serodiagnostic tools, while such animals may be positive by PCR assays (Abedi *et al*., 2014; Posada-Guzman *et al*., 2015). Therefore, PCR and serological tests are essential to determine whether an individual animal is free from infection. On the other hand, microscopy and PCR, which may be used in combination, are essential for confirming clinical cases associated with current infection. Treatment with antiparasitic drugs may mask infection and give rise to false negative results.

1. Detection of the agent

1.1. Microscopic examination

Infected horses may be identified by demonstrating the parasites in stained blood, optimally collected from superficial skin capillaries, or organ smears during the acute phase of the disease. Romanovsky-type staining methods, such as the Giemsa method, usually give the best results. However, even in acute clinical cases of *B. caballi* infection, the parasitaemia is very low and difficult to detect. Experienced workers sometimes use a thick blood smear technique to detect very low parasitaemia. Thick films are made by placing a small drop (approximately 50 µl) of blood on to a clean glass slide, which is then air-dried, heat-fixed at 80°C for 5 minutes, and stained in 5% Giemsa for 20–30 minutes.

An accurate identification of the parasite species is sometimes desirable, as mixed infections of *T. equi* and *B. caballi* probably occur frequently.

Identification of equine piroplasmosis in carrier animals by blood smear examination is not only very difficult but also inaccurate and therefore serological methods are preferred (see below). Serological tests however, may give false-negative or false-positive reactions (Tenter & Freidhoff, 1986).

1.2. *In-vitro* culture

Success in the establishment of *in-vitro* cultures of *T. equi* and *B. caballi* may be one alternative to supplement the methods described above, in order to identify carriers of the parasites. *Babesia caballi* parasites were successfully cultured from the blood of two horses that tested negative by the complement fixation test (CFT) (Holman *et al*., 1993). Similarly, *T. equi* could be cultured from horses that did not show any patent parasitaemia at the time of the initiation of the cultures (Zweygarth *et al*., 1997). This technique is largely superseded by molecular methods.

1.3. Molecular methods

Molecular techniques for the detection of *T. equi* and *B. caballi* have been described. These methods are based on species-specific polymerase chain reaction (PCR) assays, which mainly target the 18S rRNA gene. PCR assays for the specific detection of *T. equi* (forward primer: CAT-CGT-TGC-GGC-TTG-GTT-GG; reverse primer: CCA-AGT-CTC-ACA-CCC-TAT-TT) and *B. caballi* (forward primer: TTC-GCT-TCG-CTT-TTT-GTT-TTT-ACT; reverse primer: GTC-CCT-CTA-AGA-AGC-AAA-CCC-AA) based on the 18S rRNA gene have previously been described (Bashiruddin *et al.,* 1999). In one 18S rRNA-based multiplex PCR, 3 primers including a common forward primer for both *T. equi* and *B. caballi* (TCG-AAG-ACG-ATC-AGA-TAC-CGT-CG), a *T. equi*-specific reverse primer (TGC-CTT-AAA-CTT-CCT-TGC-GAT), and a *B. caballi*-specific reverse primer (CTC-GTT-CAT-GAT-TTA-GAA-TTG-CT) were used for simultaneous detection and identification of *T. equi* and *B. caballi* (Alhassan *et al.,* 2005). In addition to the PCR assays, other molecular diagnostic tests such as the highly sensitive loop-mediated isothermal amplification (LAMP) have been reported (Alhassan *et al*., 2007). The acquisition of the *T. equi* genome provides additional opportunities to improve and broaden diagnostic modalities for this parasite (Kappmeyer *et al.,* 2012). As mentioned above, there is significant sequence heterogenicity within both *T. equi* and *B. caballi.* As a result, molecular assays designed to detect some isolates may have reduced sensitivity for the detection of heterogenous isolates.

2. Serological tests

It is extremely difficult to diagnose the organisms in carrier animals by means of the microscopic examination of blood smears. Furthermore, it is by no means practical on a large scale. The serological testing of animals is therefore recommended as a preferred method of diagnosis, especially when horses are destined to be imported into countries where the disease does not occur, but the vector is present.

Sera should be collected and dispatched to diagnostic laboratories in accordance with the specifications of that laboratory. Horses for export that have been subjected to serological tests and shown to be free from infection, should be kept free of ticks to prevent accidental infections.

A number of serological techniques have been used in the diagnosis of piroplasmosis, such as the CFT, the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA).

2.1. Indirect fluorescent antibody test

The IFAT has been successfully applied to the differential diagnosis of *T. equi* and *B. caballi* infections (Madden & Holbrook, 1968). The recognition of a strong positive reaction is relatively simple, but any differentiation between weak positive and negative reactions requires considerable experience in interpretation. A detailed description of the protocol of the IFAT has been given (Madden & Holbrook, 1968). One challenge with the IFAT is the need to dilute sera to reduce non-specific binding and subsequent background, which may preclude binding of the intra-erythrocytic parasites. Sera dilutions to enhance specificity lead to a decrease in sensitivity of the IFAT. An example of an IFA protocol is given below.

2.1.1. Antigen production

Blood for antigen is obtained from horses with a rising parasitaemia, ideally 2–5%. Carrier animals that have already produced antibodies are not suitable for antigen production. Alternatively, parasites cultured *in vitro* can be used for the preparation of slide antigens to avoid contamination of antibodies to infected RBCs and for constant supply of infected RBCs, especially for *B. cabalii*.

Blood (about 15 ml) is collected into 235 ml of phosphate-buffered saline (PBS), pH 7.2. The RBCs are washed three times in cold PBS (1000 ***g*** for 10 minutes at 4°C). The supernatant fluid and the white cell layer are removed after each wash. After the last wash, the packed RBCs are reconstituted to the initial volume with 4% bovine serum albumin fraction V made up in PBS, i.e. the original packed cell volume = 30% so that one-third consists of RBCs. If the original RBC volume is 15 ml, then 5 ml of packed RBCs + 10 ml of 4% bovine albumin in PBS constitutes the antigen. After thorough mixing, the antigen is placed on to prepared wells on a glass slide using a template or a syringe. Alternatively, the cells can be spread smoothly on to microscope slides, covering the entire slide with an even, moderately thick film. These slides are allowed to dry, wrapped in soft paper and sealed in plastic bags or wrapped in aluminium foil, and stored at –20°C for up to 1 year.

2.1.2. Test procedure

i) Each serum sample is tested against an antigen of *B. caballi* and of *T. equi*.

ii) Prior to use, the frozen antigen slides are removed from storage at –20°C and incubated at 37°C for 10 minutes.

iii) The antigen smears are then removed from their protective covering and fixed in cold dry acetone (–20°C) for 1 minute. Commercially produced slides are available that are pre-fixed.

iv) If smears were prepared on the whole slide surface, squares (14–21 in number, i.e. 2–3 rows of 7 each) are formed on the antigen smears with nail varnish or rapidly drying mounting medium.

v) Test, positive and negative control sera are diluted from 1/80 to 1/1280 in PBS. Negative and positive control sera are included in each test.

vi) Sera are applied (10 µl each) at appropriate dilutions to the different wells or squares on the antigen smear, incubated at 37°C for 30 minutes, and washed several times in PBS and once in water.

vii) An anti-horse immunoglobulin prepared in rabbits and conjugated with fluorescein isothiocyanate (this conjugate is available commercially) is diluted in PBS and applied to the smear, which is then incubated and washed as before.

viii) After the final wash, two drops of a solution containing equal parts of glycerin and PBS are placed on each smear and mounted with a cover-slip.

ix) The smear is then examined under the microscope for the fluorescing parasites. Sera diluted 1/80 or more that show strong fluorescence are usually considered to be positive, although due consideration is also given to the patterns of fluorescence of the positive and negative controls.

2.2. Competitive enzyme-linked immunosorbent assay

A number of recombinant antigens for the use in ELISAs have been described. Recombinant *T. equi* (EMA-1; EMA-2) and *B. caballi* proteins (RAP-1; Bc48) have been produced in *Escherichia coli* (Huang *et al*., 2003; Kappmeyer *et al*., 1999; Knowles *et al*., 1992) or in insect cells by baculovirus (Xuan *et al*., 2001). Recombinant antigens produced in *E. coli* or by baculovirus have the obvious advantage of avoiding the need to infect horses for antigen production, and of eliminating the cross-reactions that have been experienced in the past with the crude ELISA antigens. They also provide a consistent source of antigen for international distribution and standardisation.

Indirect ELISAs using EMA-2 and BC48 have shown high sensitivity and specificity in detecting antibodies in infected horses (Huang *et al*., 2003; Ikadai *et al*., 1999; Kumar *et al.*, 2013). Initial results from these tests are promising and further validation of the assays is underway.

A competitive inhibition ELISA (C-ELISA) using EMA-1 protein and a specific monoclonal antibody (MAb) that defines this merozoite surface protein epitope, have been used in a C-ELISA for *T. equi* (Knowles *et al*., 1992). This C-ELISA overcomes the problem of antigen purity, as the specificity of this assay depends only on the specificity defined by the MAb *T. equi* epitope. A 94% correlation was shown between the C-ELISA and the CFT in detecting antibodies to *T. equi*. Sera that gave discrepant results were evaluated for their ability to immunoprecipitate 35S-methionine-labelled *in-vitro* translated products of *T. equi* merozoite mRNA. Samples that were C-ELISA positive and CFT negative clearly precipitated multiple *T. equi* proteins. However, immunoprecipitation results with serum samples that were C-ELISA negative and CFT positive were inconclusive (Knowles *et al*., 1991). This C-ELISA for *T. equi* was also validated in Morocco and Israel, giving a concordance of 91% and 95.7% with the IFAT, respectively (Rhalem *et al*., 2001; Shkap *et al*., 1998). A similar C-ELISA has been developed using the recombinant *B. caballi* rhoptry-associated protein 1 (RAP-1) and a MAb reactive with a peptide epitope of a 60 kDa *B. caballi* antigen (Kappmeyer *et al*., 1999). The results of 302 serum samples tested with this C-ELISA and the CFT showed a 73% concordance. Of the 72 samples that were CFT negative and C-ELISA positive, 48 (67%) were shown to be positive on the IFAT, while four of the five samples that tested CFT positive and C-ELISA negative were positive on the IFAT (Kappmeyer *et al*., 1999).

A test protocol for an equine piroplasmosis C-ELISA has been described and used for additional validation studies (United States Department of Agriculture [USDA], 2005). The apparent specificity of the *T. equi* and *B. caballi* C-ELISAs lay between 99.2% and 99.5% using sera from 1000 horses presumed to be piroplasmosis free. One thousand foreign-origin horses of unknown infection status were tested by the C-ELISA and the CFT with an apparent greater sensitivity of the C-ELISA. The results were 1.1% (*T. equi*) and 1.3% (*B. caballi*) more seropositive animals detected by C-ELISA than by the CFT; the additional positive results were confirmed by IFAT. A similar study of 645 foreign-origin horses tested for import and pre-import purposes used heat-treated sera (58°C for 30 minutes), and resulted in 3.6% (*T. equi*) and 2.1% (*B. caballi*) more seropositive animals detected by the C-ELISA than by the CFT. Both C-ELISAs were highly reproducible well-to-well, plate-to-plate, and day-to-day, with overall variances of ± 1.2% and ±1.6% for the *T. equi* and *B. caballi* tests, respectively.

The C-ELISA protocol is given below.

A detailed description of antigen production and a test protocol has been given by the National Veterinary Services Laboratories (NVSL) of the USDA (2005). A commercial kit is now available that is based on the same antigens and monoclonal antibodies.

2.2.1. Solutions

i) Antigen-coating buffer

Prepare the volume of antigen-coating buffer required using the following amounts of ingredients per litre: 2.93 g sodium bicarbonate; 1.59 g sodium carbonate; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Adjust to pH 9.6.

ii) C-ELISA wash solution (high salt diluent)

Prepare the volume of C-ELISA wash solution required by using the following amounts of ingredients per litre: 29.5 g sodium chloride; 0.22 g monobasic sodium phosphate; 1.19 g dibasic sodium phosphate; 2.0 ml Tween 20; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Mix well. Adjust pH to 7.4. Sterilise by autoclaving at 121°C.

iii) Chromogenic substrate

0.1% (w/v) stock solution of C-ELISA substrate is prepared by dissolving 3,3',5,5'-tetramethylbenzidine (TMB) in dimethyl sulphide at 1 mg/ml. 10% (v/v) working solution is prepared by diluting TMB stock solution with phosphate–citrate buffer at pH 5.0. Fresh 30% hydrogen peroxide is added to TMB working solution at 0.02% (v/v) just before use.

2.2.2. Antigen production

Frozen transformed *E. coli* culture is inoculated at a 1/10,000 dilution into any standard non-selective bacterial growth broth (e.g. Luria broth) containing added carbenicillin (100 µg/ml) and isopropyl-thiogalactoside (IPTG, 1 mM). Cultures are incubated on an orbital shaker set at 200 rpm at 37°C overnight. Cells grown overnight are harvested by centrifugation (5000 ***g*** for

10 minutes), washed in 50 mM Tris/HCl and 5 mM ethylene diamine tetra-acetic acid (EDTA) buffer, pH 8.0, and harvested again as before[1].

Cells are resuspended to 10% of the original volume in the Tris/EDTA buffer to which 1 mg/ml of lysozyme has been added, and incubated on ice for 20 minutes. Nonidet P-40 detergent (NP-40) is then added to a final 1% concentration (v/v), vortexed, and the mixture is incubated on ice for 10 minutes. The material is next sonicated four times for 30 seconds each time at 100 watts, on ice, allowing 2 minutes between sonications for the material to remain cool. The sonicate is centrifuged at 10,000 ***g*** for 20 minutes. The resulting supernatant is dispensed in 0.5 ml aliquots in microcentrifuge tubes and may then be stored at –70°C for several years. The presence of heterologous host bacterial antigens does not interfere with the binding of specific equine anti-piroplasma antibodies or the binding of the paired MAbs to their respective expressed recombinant antigen epitopes, and is confirmed by the following procedures. The antigen-containing supernatants are quality controlled by titrating them with their paired MAbs and with reference monospecific equine antisera to verify both an adequate level of expression and complete specificity for the homologous species of piroplasmosis agent. Normal serum (negative serum) controls must not interfere with binding of the MAbs or positive equine reference sera to the expressed antigen preparation.

2.2.3. Test procedure

i) Microtitration plates are prepared by coating the wells with 50 µl of either *T. equi* antigen or *B. caballi* antigen diluted in antigen-coating buffer. The dilution used is determined by standard serological titration techniques. The plate is sealed with sealing tape, stored overnight at 4°C, and frozen at –70°C. Plates can be stored at –70°C for up to 6 months.

ii) The primary anti-*T. equi* or anti-*B. caballi* MAb and secondary antibody-peroxidase conjugate is diluted as directed by the manufacturer at the time of use in the C-ELISA, with antibody-diluting buffer (supplied with the test kit).

iii) Plates are thawed at room temperature, the coating solution is decanted, and the plates are washed twice with C-ELISA wash solution.

iv) The serum controls and test serum samples are diluted 1/2 with serum-diluting buffer before 50 µl of sera is added to wells. Each unknown serum sample is tested in single or duplicate wells. Positive control sera and blanks are tested in duplicate while negative controls are tested in triplicate on different parts of the plate. Plates are incubated covered, at room temperature (21–25°C) for 30 minutes in a humid chamber, and then washed three times in C-ELISA wash solution.

v) All wells then receive 50 µl/well of diluted primary anti-*T. equi* or anti-*B. caballi* MAb. (The MAb is produced in a cell culture bioreactor and is available from the NVSL, P.O. Box 844, Ames, Iowa 50010, USA.) Plates are incubated covered for 30 minutes at room temperature (21–25°C) in a humid chamber, and then washed three times in C-ELISA wash solution.

vi) Diluted secondary peroxidase anti-murine IgG (50 µl/well) conjugate is added to each well. Plates are incubated covered for 30 minutes at room temperature (21–25°C) in a humid chamber, and then washed three times in C-ELISA wash solution.

vii) Chromogenic enzyme substrate (50 µl/well) is added to all wells, and plates are incubated for 15 minutes at room temperature (21–25°C) during colour development.

viii) The colour development is stopped by adding 50 µl of stop solution to all wells and the plates are read immediately on a plate reader.

ix) The plates are read at 620, 630 or 650 nm wavelength (OD). The average OD is calculated for the duplicate wells for all control sera and blank wells. For a valid test, the mean of the negative controls must produce an OD >0.300 and <2.000. The mean positive control sera must produce an inhibition of ≥40%.

1 Antigen is available from the NVSL, P.O. Box 844, Ames, Iowa 50010, USA.

x) Per cent inhibition [%I] is calculated as follows: %I = 100 – [(Sample OD × 100) ÷ (Mean negative control OD)].

xi) If a test samples produces ≥40% inhibition it is considered positive. If the test sample produces <40% inhibition it is considered negative.

2.3. Complement fixation test

The CFT has been used in the past by some countries and is still widely used in some regions, but is no longer recommended to qualify horses for importation. The CFT is accurate for detection of early (acute) infections only, for which purpose it shows good sensitivity and specificity, but it may not identify all infected animals, especially those that have been drug-treated or that produce anti-complementary reactions, or because of the inability of IgG(T) (the major immunoglobulin isotype of equids) to fix guinea-pig complement. Antigen for the CFT is prepared by the experimental infection of horses, which raises animal welfare concerns. Therefore, it is likely that the CFT will be discontinued in the future; the IFAT and C-ELISA have replaced it as the tests that are most suitable for certifying individual animals prior to movement, including international trade.

C. REQUIREMENTS FOR VACCINES

No commercial vaccines are available currently.

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

NB: FIRST ADOPTED IN IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.