

TOXOPLASMOSIS

SUMMARY

Description of disease: *Toxoplasmosis is a zoonotic infection of animals caused by the protozoan parasite Toxoplasma gondii. It has the capacity to infect all warm-blooded animals and, while infection does not cause clinical illness in the majority of animal species, in some it causes acute life-threatening disease and in others, particularly sheep and goats, it may manifest itself as a disease of pregnancy by multiplying in the placenta and fetus. In these latter animals it can result in the abortion or the birth of weak lambs/kids, which may be accompanied by a mummified fetus. Characteristically, in these cases, the placental intercotyledonary membranes are normal, but white foci of necrosis, approximately 1–3 mm in diameter, may be visible in the cotyledons. Microscopically, these foci appear as areas of coagulative necrosis that are relatively free of inflammation. Inflammation, when present, is nonsuppurative. Toxoplasma tachyzoites are seen only rarely in association with these foci, usually at the periphery of the lesion. Examination of the brain may reveal focal microgliosis. The lesions often have a small central focus of necrosis that might be mineralised. Focal leukomalacia in cerebral white matter, due to anoxia arising from placental pathology, is often present. Focal microgliosis is more specific, as leukomalacia reflects placental damage but may occur in other pathological conditions where the placenta is compromised, including, though rarely, ovine chlamydiosis. Infection in pigs may cause severe fetal losses in pregnant sows, but more usually is mild and unnoticed. Acute fatal infections affect New World monkeys, marsupials and certain other animals.*

Identification of the agent: *Toxoplasma gondii is an obligate intracellular parasite that has a sexual cycle in Felidae and a two-stage asexual cycle in all warm-blooded animals. It predominantly comprises three molecular lineages (I, II and III). In the acute phase of infection, tachyzoites multiply in cells to cause varying degrees of tissue destruction and, in fatal cases, tachyzoites may be demonstrated in ascitic fluid or in lung impression smears. With the onset of an immune response, tachyzoites are transformed into bradyzoites that multiply slowly in cells to produce tissue cysts. In aborting sheep, goats and pigs, T. gondii is often difficult to find in tissue sections, but is more likely to be seen in sections of brain and placenta. Its identity can be confirmed by immunohistochemistry, while the polymerase chain reaction may be used to identify parasite DNA in tissues. Isolation of T. gondii from samples is expensive and slow but, if required, is best achieved by inoculation of mice with tissue homogenate derived from fetal brain or placenta. The sexual life-cycle of the parasite takes place exclusively in epithelial cells of the feline intestine and can result in the excretion of large numbers of oocysts in the faeces. Oocysts may remain viable in the environment for many months.*

Serological tests: *The dye test is the longest established serological method, and in many ways represents the 'gold standard', at least in humans. The dye test uses live, virulent Toxoplasma tachyzoites, a complement-like 'accessory-factor' and test serum. When specific antibody acts on the tachyzoites, the latter do not stain uniformly with alkaline methylene blue. The test has proven unreliable in some species. In addition, as live Toxoplasma is used, the test carries a potential risk of human infection as well as being expensive to conduct. The dye test is used in only a few laboratories in the world. The indirect fluorescent antibody (IFA) test is safer, gives titres comparable with the dye test and can be used to differentiate IgM and IgG antibodies. The direct agglutination test and the latex agglutination test are both relatively rapid and neither requires complex laboratory facilities. The enzyme-linked immunosorbent assay requires more sophisticated laboratory equipment but can process large numbers of samples and does not rely on human interpretation for the result.*

Requirements for vaccines: A vaccine composed of live *T. gondii* tachyzoites is available commercially for use in sheep in certain European countries and New Zealand. The vaccine is supplied as a concentrated suspension of tachyzoites with an approved diluent and delivery system. The vaccine must be maintained and handled strictly according to the manufacturers' instructions as it has a very short shelf life.

A. INTRODUCTION

Toxoplasma gondii is a zoonotic, obligate intracellular protozoan parasite that has the capacity to infect all warm-blooded animals including birds. While infection does not cause clinical illness in the majority of animal species, in some it causes acute life-threatening disease and in others, particularly sheep and goats, but also pigs, it manifests itself as a disease of pregnancy by multiplying in the placenta and fetus. Acute, potentially fatal, infections are recorded in New World monkeys (Cunningham *et al.*, 1992), marsupials (Canfield *et al.*, 1990) and certain other animals (see below). Worldwide reports of clinical and subclinical infections in domestic and wild animals were summarised in two books (Dubey, 2010; Dubey & Beattie, 1988). In acute cases clinical signs may include lymphadenopathy, hepatomegaly, interstitial pneumonia and neural signs. At post-mortem examination lymph nodes, spleen and liver may be enlarged and the latter may have pale foci. In sheep, goats and pigs a primary infection established during pregnancy may result in apparent infertility or in stillbirths and abortion, according to the stage of pregnancy at which infection was initiated. In a typical case of abortion, a ewe or doe infected in mid-gestation produces a stillborn lamb/kid a few days earlier than the predicted end of pregnancy. The aborted fetus is often accompanied by either a weak sibling or a 'mummified' fetus (Buxton, 2000). The ewe/doe remains clinically normal. In such cases, placental cotyledons are typically speckled with white foci around 2–3 mm in diameter while the intercotyledonary membranes appear normal. Infection in early pregnancy, when the fetus has only a rudimentary immune system, results in fetal death and resorption. In this case the mother may present as barren, which in turn can mimic a flock/herd infertility problem. Mothers that become infected in late pregnancy would be expected to produce infected but clinically normal offspring. Following an infection, either during or outside of pregnancy, the parasite would not be expected to cause abortion in any subsequent pregnancy. While recent research has questioned this conclusion (Duncanson *et al.*, 2001; Williams *et al.*, 2005) the majority of current thinking tends towards the view that recrudescence of a persistent infection during pregnancy leading to repeat abortions is not normally a significant occurrence (Dubey, 2010; Rodger *et al.*, 2006). Infection in pigs may cause severe fetal losses in pregnant sows but under modern intensive farming conditions, when contamination of the housing and feed by *T. gondii* oocysts is minimal or absent, infection would generally be expected to be at a very low incidence and cause only mild or unnoticed signs of infection (Lind & Buxton, 2000). However when pigs are maintained outdoors under extensive systems, they are much more likely to encounter oocysts, and so infection would be expected to be more common (Dubey, 2010; Kijlstra *et al.*, 2004).

Toxoplasma gondii is an obligate intracellular parasite that has a two-stage asexual cycle in warm-blooded animals and a sexual cycle in felidae. The parasite comprises three genetic lineages (I, II and III) in the main, with type II and III being associated with disease in animals while type II is the predominant form identified in human disease (Howe & Sibley, 1995; Khan *et al.*, 2006). In the asexual cycle, the two developmental stages are the rapidly multiplying tachyzoite and the slowly multiplying bradyzoite. In acute infection, tachyzoites actively penetrate host cells where they multiply causing the cell to rupture and release organisms locally and into the bloodstream. As the host develops immunity, the parasite retains its overall size and shape but transforms into the bradyzoite stage and multiplies more slowly within tissue cysts to establish a persistent infection. These microscopic tissue cysts are present most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host. Viable tissue cysts within muscle (meat) are a significant source of human infection. In animals that succumb to acute infection tachyzoites may be demonstrated in ascitic fluid or in lung impression smears as well as in tissue sections of the liver and other affected organs.

The sexual cycle occurs in enteroepithelial cells of the feline definitive host and results in the production of *Toxoplasma* oocysts. Following a primary infection of a cat, oocysts may be shed in the faeces for several days. The oocysts sporulate in the environment over the next 1–5 days (depending on aeration, humidity and temperature), at which time they become infective. They are very resistant to environmental conditions and may remain infective for a year or more. Sporulated oocysts are 11 × 13 µm in diameter and each contains four sporozoites in each of two sporocysts (Dubey & Beattie, 1988). When a susceptible animal ingests sporulated oocysts the sporozoites are released to penetrate the intestinal lining, become tachyzoites and establish an infection.

In sheep, goats, pigs, horses and humans, tissue cysts may remain for the rest of the life of the individual (Dubey & Beattie, 1988). *Toxoplasma* does not usually cause clinical illness in cattle, camelids or deer but, as noted, can cause fatal disease in New World monkeys, marsupials and certain other animals including hares (*Lepus europaeus*; *L. timidus*) (Gustafsson & Uggla, 1994), the Pallas cat (Brown *et al.*, 2005), the arctic fox (Sørensen *et al.*, 2005), some birds and marine mammals (Dubey, 2010). It is suggested that these and other similarly affected animals have had minimal exposure to *T. gondii* in their natural habitat through the ages, making them particularly vulnerable to the parasite.

Abortion in sheep and goats due to *T. gondii* must be differentiated from that caused by other infectious agents, including infections with *Chlamydophila abortus* (see Chapter 3.8.5 *Enzootic abortion of ewes*), *Coxiella burnetii* (see Chapter 3.1.16 *Q fever*), *Brucella melitensis* (see Chapter 3.1.4 *Brucellosis* [*Brucella abortus*, *B. melitensis* and *B. suis*]), *Campylobacter fetus* (see Chapter 3.4.4 *Bovine genital campylobacteriosis*), *Salmonella* spp. (see Chapter 3.10.7 *Salmonellosis*), border disease (see Chapter 3.8.1 *Border disease*), and the viruses that cause bluetongue, Wesselsbron's disease and Akabane disease. In pigs, *Brucella suis* (see chapter 3.1.4) may also cause fetal death, mummification and abortion.

1. Human health risks

Toxoplasma gondii readily infects human beings and while infection is relatively common (approximately 30% of the population depending on age and environment), clinical illness is relatively uncommon. Those particularly at risk of developing clinical illness include pregnant women, as the parasite can pose a serious threat to the unborn child if the mother becomes infected for the first time while pregnant, and individuals who are immunosuppressed, such as tissue transplant patients, AIDS patients, patients with certain types of cancer and those undergoing certain forms of cancer therapy. These individuals are at risk of developing acute lethal infection if left untreated. The very young and very old may also be more susceptible. On occasions, people with no apparent immune deficiency may develop an illness characterised by general malaise, fever and lymphadenopathy. The most likely sources of human infection are ingestion of raw or lightly cooked meat containing live *T. gondii* tissue cysts, ingestion of raw or lightly cooked vegetables contaminated with oocysts or exposure to oocysts derived from cat faeces, such as may be encountered in gardens and children's sand pits. Toxoplasmosis is now also recognised to be a water-borne zoonosis (Dubey, 2010). This method of transmission occurs where water treatment is ineffective or non-existent and there is a sizeable local felid population that contaminates surface water with oocysts (Bowie *et al.*, 1997; Dubey, 2004). Linked to this there is now also an appreciation that marine mammals are becoming infected by waters from contaminated land and from untreated urban sewage.

All laboratory manipulations with live organisms should be handled with appropriate measures determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of toxoplasmosis 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 |
| Identification of the agent | | | | | | |
| PCR | – | – | – | ++ | – | – |
| Histopathology | – | – | – | + | – | – |
| Detection of immune response | | | | | | |
| IFAT | – | – | – | ++ | ++ | – |
| ELISA | – | – | – | ++ | ++ | – |
| DAT/MAT | – | – | – | ++ | ++ | – |

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;

ELISA = enzyme-linked immunosorbent assay; DAT = direct agglutination test; MAT = modified agglutination test.

1. Identification of the agent

1.1. Histopathology

In animals that die with acute toxoplasmosis, focal mononuclear inflammation with or without focal necrosis may be seen in a number of tissues, including the liver, heart and lungs. The latter may be oedematous. Lymph nodes may have undergone expansion and there may or may not be focal necrosis with or without haemorrhage. Typically *Toxoplasma* tachyzoites may be demonstrable in association with necrosis and inflammation.

In cases of abortion and stillbirth in sheep and goats, affected placental cotyledons typically contain large foci of coagulative necrosis that may have become mineralised with time. Any associated inflammation is characteristically slight and nonsuppurative. Well preserved samples of placental cotyledons may show moderate oedema of the mesenchyme of the fetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. Sometimes small numbers of intracellular and extracellular toxoplasms are visible, usually on the periphery of a necrotic area or in a villus that is in the early stages of infection. The *Toxoplasma* tachyzoites appear ovoid, 2–6 µm long, with nuclei that are moderately basophilic and located centrally or towards the posterior end.

In the fetal brain primary and secondary lesions may develop. Microglial foci, typically with a necrotic and sometimes mineralised centre and often associated with a mild focal lymphoid meningitis, represent a fetal immune response following direct damage by local parasite multiplication. *Toxoplasma* tissue cysts are only rarely found, usually at the periphery of these lesions. Focal leukomalacia is also common and is considered to be due to fetal anoxia in late gestation caused by advanced lesions in the placentome preventing sufficient oxygen transfer from mother to fetus. Such foci occur most commonly in the cerebral white matter cores, but sometimes also in the cerebellar white matter. Focal leukomalacia on its own suggests placental disease or acute insufficiency but the two types of neuropathological change seen together are characteristic of *Toxoplasma* infection. Confirmation of the identity of *T. gondii*-like structures in tissue sections from such cases, as well as from instances of acute toxoplasmosis, may be achieved by immunohistochemistry that labels intact *T. gondii* or antigenic debris. The method is both convenient and sensitive and is used with fixed tissues (including archive tissues) that may also exhibit a degree of decomposition, where isolation would not be appropriate or possible. The ABC indirect immunoperoxidase method and the peroxidase–antiperoxidase (PAP) technique (Ugglå *et al.*, 1987) are equally good, and details of the procedure can be found in Dubey (2010).

1.2. Nucleic acid recognition methods

Several polymerase chain reaction (PCR)-based assays have been developed for the detection of nucleic acids from *T. gondii* (Table 1). The main target regions are the B1 repetitive sequence, and the 300-copy 529-bp element the P30 (SAG1) gene and 18S ribosomal RNA (rRNA) (Burg *et al.*, 1989; Dubey, 2010; Ellis, 1998; Savva *et al.*, 1990). The sensitivity of the PCR is dependent on the copy number of the target sequence (P30: 1 copy; B1: 35 copies; rRNA: 110 repeat units). Customised synthetic DNA oligonucleotides are commercially available (e.g. www.sigma-genosys.co.uk). Recently, the method for amplification of the B1 repetitive sequence has been used to analyse the lens aspirates of congenitally infected human cataract patients (Mahalakshmi *et al.*, 2007) and was found to be more sensitive than the conventional method used (enzyme-linked immunosorbent assay [ELISA]). However, although the PCR is extremely sensitive, care should be taken if it is the only test available, as in many situations a more reliable diagnosis will be gained if it is used in combination with other diagnostic data.

Recently, a real-time PCR has been developed to allow simultaneous quantification and amplification of DNA. It is very similar to existing PCR methods and can be carried out on 96-well microtitre plates. After each round of amplification, fluorogenic dyes intercalate with the double-stranded DNA and the results, shown on an amplification plot, allow quantification of the parasite DNA in the sample. Real-time PCR has been used to amplify and quantify DNA from the *T. gondii* B1 gene (Costa *et al.*, 2000; Lin *et al.*, 2000). This quantification of parasite DNA can be used to determine the number of parasites in tissues and fluids, such as the amniotic fluid of patients suspected of being congenitally infected with *T. gondii*. (Nagy *et al.*, 2007). The real-time PCR is a highly sensitive and specific method, however it is expensive and requires specialised detection systems and therefore may only be cost-effective in laboratories where analysis of large numbers of samples is carried out.

The following method is a nested form of the PCR, amplifying the B1 repetitive sequence of DNA (Wastling *et al.*, 1993). Parasite DNA can be extracted and purified from several tissues, including placenta, the central nervous system, heart and skeletal muscle.

Contaminating red blood cells in tissues are removed by washing in 10 mM Tris/NH₄Cl lysis buffer, pH 7.6, followed by centrifugation at 2000 *g* for 15 minutes. DNA is then extracted from the resultant pellet and resuspended in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ containing proteinase K 100 µg/ml and 0.5% Tween 20.

Samples are incubated at 55°C for at least 1 hour, then the proteinase K is inactivated by boiling. The PCR procedure is performed in 50 µl volumes. Amplification of the B1 gene is performed by modifying the procedure described in ref. 1. The reaction mixture contains 10 mM Tris, pH 8.3, 2.5 mM MgCl₂, 40 mM KCl, 0.01% gelatin, 0.1 mM dNTPs, 0.2 µM of each primer (oligonucleotide primers are those described in Bowie *et al.*, 1997), two sense primers P1 and P2 and two antisense primers P3 and P4) and 2.5 units of Taq polymerase.

Primary amplification is performed with primers 1 and 4 to give a 193 bp product over 25 cycles of 93°C for 1 minute, 50°C for 1.5 minutes and 72°C for 3 minutes. The amplified product is then diluted 1/20 in distilled water to reduce amplification of non-specific products.

Secondary amplification using nested primers 2 and 3 and the same reaction conditions, is carried out over 15 cycles to give a 94 bp product. The final product is then visualised on 1% agarose gels. Southern blotting, using a labelled probe, can be used to confirm the identity of the B1 PCR products and to distinguish them from non-specific products.

1.3. Oocyst detection in drinking water

Toxoplasma gondii oocysts have been detected in drinking water using the method for the detection of *Cryptosporidium* oocysts (Issac-Renton *et al.*, 1998). The method relies on the collection of a large-volume sample of water and passing it through a cartridge filter. Identification of *Toxoplasma* oocysts was by means of inoculation of rodents. However, unlike *Cryptosporidium*, the number of *T. gondii* oocysts in water is very low.

From a public health viewpoint, it is necessary to distinguish *T. gondii* oocysts from oocysts of a related coccidium, *Hammondia hammondi*, also present in cat faeces. *Hammondia hammondi* is non-pathogenic. Bioassays, currently the only definitive way to detect viable oocysts of these parasites, are expensive and only a few laboratories in the world have the facilities to do them. Although DNA detection is considered highly specific, cross reactivity has been observed between *T. gondii* and *H. hammondi*. Recently, *H. hammondi*-specific primers have been published (Walzer *et al.*, 2014).

Detection of DNA from *T. gondii* oocysts may present additional problems because of inhibitors in faecal matter, and difficulty of releasing DNA from the oocysts. A method is detailed below for preparation of oocysts and extraction of DNA.

1.3.1. Reagents

- i) PBS: 300 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.7 mM NaH₂PO₄.
- ii) Sodium hypochlorite, aqueous solution, ≥ 4% as active chlorine.
- iii) OOC-lysis buffer (pH 9.5): 600 mM EDTA (ethylene diamine tetra-acetic acid), 1.3% (v/v) N-lauroylsarcosine, 2 mg/ml proteinase K.
- iv) OOC-CTAB buffer: 2% (w/v) cetyl-trimethyl ammonium bromide, 1.4 M NaCl, 0.2 % (v/v) mercapthoethanol, 20 mM EDTA, 100 mM tris(hydroxymethyl)aminomethane.
- v) phenol/chloroform/isoamyl alcohol (25/24/1).
- vi) 4 M NaCl.
- vii) 96% (v/v) ethanol. I. 70% (v/v) ethanol.

1.3.2. Procedure

- i) Wash oocysts four times by centrifugation (1100 *g* for 7 minutes without the use of the brake) in 15 ml PBS in a 15 ml centrifugation tube.
- ii) Incubate oocyst pellet and the remaining contaminants (up to 0.5 ml) in 2 ml 5.75 % sodium hypochlorite (30 minutes at 37°C).
- iii) Add double-distilled H₂O up to 15 ml.

- iv) Centrifuge supernatant in a 15 ml tube (1100 **g** for 7 minutes without the use of the brake) and mix the pellet with PBS. Wash the pellet three times with PBS (1100 **g** for 7 minutes without brake).
- v) After a last centrifugation, re-suspend the pellet in 1 ml PBS, transfer into a 1.5 ml reaction tube and spin down (1100 **g** for 7 minutes without brake).
- vi) Carefully remove as much of the supernatant as possible and apply three freeze–thaw cycles (10 minutes at –20°C followed by 2 minutes at room temperature) to the pellet.
- vii) Re-suspend the pellet in 100 µl OOC lysis buffer (45 minutes, 65°C).
- viii) Add 400 µl OOC-CTAB buffer (60 minutes, 60°C).
- ix) Mix with 500 µl phenol/chloroform/isoamylalcohol (25/24/1) by inverting 50 times. Centrifuge for 7 minutes at 13,000 **g**.
- x) Transfer the supernatant to a fresh tube and mix again with 500 µl phenol/chloroform/isoamylalcohol (25/24/1) by inverting 50 times. Centrifuge for 7 minutes at 13,000 **g**.
- xi) Transfer the supernatant to a fresh tube and add 0.04 volumes of 4 M NaCl and 2–3 volumes of –20°C cold 96% (v/v) ethanol to precipitate DNA (keep at least 20–30 minutes at –20°C).
- xii) Centrifuge for 15 minutes at 13,000 **g**. Decant the supernatant.
- xiii) Wash the pellet using 70% (v/v) ethanol and centrifuge for 15 minutes at 13,000 **g**.
- xiv) Discard the ethanol solution and air dry the pellet.
- xv) Resolve DNA in double-distilled water for at least 12 hours at 4°C.
- xvi) Use 2.5–10 µl aliquots for PCR (see Section 1.2 above).

2. Serological tests

There are several serological tests available for the detection of *T. gondii* antibodies (Table 1). In one type of test the observer judges the given colour of tachyzoites under a microscope, such as with the dye test (DT) and IFA test. Another depends on the principle of agglutination of *Toxoplasma* tachyzoites, red blood cells or latex particles, as with the direct agglutination test (DAT and modified agglutination test [MAT]) and indirect haemagglutination test (IHA) and latex agglutination (LA) test, respectively. With the ELISA, the degree of colour change defines the quantity of specific antibody in a given solution. The DT, IFA test, DAT and ELISA are outlined below and the IFA test is given in more detail.

The DT (Sabin & Feldman, 1948) is the so-called ‘gold standard’ serological test for *Toxoplasma* antibody in humans. Live *Toxoplasma* tachyzoites are incubated with a complement-like accessory factor and the test serum at 37°C for 1 hour before methylene blue is added. Specific antibody induces membrane permeability in the parasite so that the cytoplasm is able to leak out and the tachyzoite does not incorporate the dye and so appears colourless. Tachyzoites not exposed to specific antibody (i.e. a negative serum sample) take up the dye and appear blue. The DT is both specific and sensitive in humans, but may be unreliable in other species. In addition, it is potentially hazardous as live parasite is used. It is expensive and requires a high degree of technical expertise. It should be noted that on animal welfare grounds, tachyzoites should be grown in tissue culture rather than in mouse peritoneum whenever possible.

The IFA test (Munday & Corbould, 1971) is a simple and widely used method. Whole, killed *Toxoplasma* tachyzoites are incubated with diluted test serum, the appropriate fluorescent antispecies serum is added, and the result is then viewed with a fluorescence microscope. Fluorescent-labelled antibodies are available commercially for a variety of animal species, the method is relatively inexpensive and kits are commercially available. However, the method requires a fluorescence microscope and the results are read by eye, so individual variation may occur. It may be difficult to find some species-specific conjugates and there is a risk of possible cross-reactivity with rheumatoid factor and anti-nuclear antibodies.

The DAT (Desmonts & Remington, 1980) is both sensitive and specific. Formalinised *Toxoplasma* tachyzoites are added to U-shaped well microtitre plates and dilutions of test sera are then applied. Positive samples will produce agglutination that can be graded, while negative samples will produce a ‘button’ of precipitated tachyzoites at the bottom of the well. The test is simple and easy to perform although relatively large amounts of antigen are required. Kits are commercially available. It is important to treat sera with mercaptoethanol to avoid false positives due to non-specific IgM. The procedure was modified by Dubey & Desmonts (1987), who called it the modified agglutination test (MAT). The MAT has been used extensively for the detection of *T. gondii* animals in sera of all species of animals and the procedure is detailed below. The MAT may give false negative results in the early

stages of infection, or when performed on canine sera. A commercially available latex agglutination test (LAT) is also available but this test is relatively insensitive compared with MAT or IFA.

The original ELISA (Voller *et al.*, 1976) uses a soluble antigen preparation made from *Toxoplasma* RH strain tachyzoites (as described below) and layered into wells in a microtitre plate. Test sera (e.g. ovine in origin) are added, followed by an anti-species enzyme-labelled conjugate such as horseradish peroxidase-labelled anti-ovine-IgG. Any attached conjugate causes a colour change in the substrate that is directly related to the amount of bound antibody, and which can be read with a spectrophotometer at the absorbance specific to the substrate used. The assay is simple, can readily test a large number of samples and is easy to perform with the chosen anti-species conjugate. Defined anti-species conjugates, substrates and whole kits are commercially available. However, the assay does require a spectrophotometer. The ELISA is well suited to laboratories required to analyse large numbers of samples.

Recently, a kinetics ELISA (KELA) has been developed (Werre *et al.*, 2002). The KELA system measures the rate of reaction between bound enzyme and the substrate solution that leads to development of colour. Three optical densities (OD) are read at 45-second intervals (using the KELA data management program) and the results are reported in terms of slopes. The correlation between the ELISA and the KELA is very high, and therefore, the two tests are very good diagnostic tools, differing only in their convenience of application.

To improve the specificity of the conventional ELISA, assays that use recombinant antigens (Johnson & Illana, 1991) and affinity purified *Toxoplasma*-specific antigens (Lekutis *et al.*, 2001) have been developed for use in sheep (Sager *et al.*, 2003; Tenter *et al.*, 1992) but these tests are not yet routinely used.

Clinically, there is a need to distinguish recent (acute) infections from longstanding (chronic) infections. With the conventional ELISA the detection of *Toxoplasma*-specific IgG and IgM antibodies along with IgA may permit a degree of discrimination between acute and chronic toxoplasmosis. An assay to define avidity of IgG for the P30 antigen of *T. gondii* in sheep has been developed. The avidity was shown to increase over a period of 10 weeks post-infection (Sager *et al.*, 2003). This test is a good diagnostic tool for discriminating relatively recent from more established infections.

2.1. Preparation of antisera and antigens

Antisera to *T. gondii* and conjugated antisera for use in the IFA test and ELISA, to allow screening of a variety of animal species, may be obtained commercially. International standards for animal sera are not available.

Below are protocols for the preparation of tachyzoite antigen for use in the IFA test and ELISA. Tachyzoites may be grown in tissue culture and retained as whole parasites for the IFA test, or prepared as soluble antigen for the ELISA.

2.2. Preparation of aliquots of a frozen stabilate of *T. gondii* tachyzoites

2.2.1. Test procedure

- i) Produce tachyzoites in tissue culture as described.
- ii) Centrifuge at 500 **g** for 5 minutes and resuspend in Iscove's modified Dulbecco's medium (IMDM) approximately three times.
- iii) Add the following solutions to give these concentrations: 10% dimethyl sulphoxide; 20% normal horse serum (free from antibody to *T. gondii*); 70% resuspended tachyzoites to give a final concentration of 1×10^8 tachyzoites/ml.
- iv) Allow the preparation to stand on the bench for 1 hour.
- v) Dispense into 1-ml aliquots using screw-topped tubes appropriate for liquid nitrogen storage.
- vi) Put the tubes into a small container, wrap in thick insulating material and place in -70°C freezer to allow the tachyzoites to freeze gradually.
- vii) The next day transfer to liquid nitrogen, keeping well insulated while transferring.
- viii) This stabilate may then be used for mouse inoculation or tissue culture growth of the parasite. When removing from storage thaw the sample rapidly in warm water.

2.3. Production of *Toxoplasma* tachyzoites in tissue culture

2.3.1. Test procedure

- i) *Toxoplasma gondii* can be grown and maintained in tissue culture by twice-weekly passage in African green monkey kidney (Vero) cells.
- ii) Cells and parasite are grown in IMDM supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2% fetal bovine serum.
- iii) Tachyzoites are harvested from tissue culture flasks by scraping the cell monolayer using a sterile cell scraper.
- iv) Using 25 cm² vented tissue culture flasks that have each been seeded with 1 × 10⁵ Vero cells, add tachyzoites at the rate of two tachyzoites per monolayer cell and incubate at 37°C in a 5% CO₂ humidified chamber. Harvest after 3–4 days.

2.4. Preparation of whole tachyzoites for use in the IFA test

2.4.1. Test procedure

- i) Produce 4 × 10⁷/ml suspension of RH strain *T. gondii* tachyzoites in PBS.
- ii) Add formaldehyde (40%) to give a final concentration of 0.2% (v/v).
- iii) Incubate at 4°C overnight and divide into aliquots in suitable sealed tubes and store frozen until required.

2.5. Production of soluble antigen for ELISA

2.5.1. Test procedure

- i) Produce a suspension of RH strain *T. gondii* tachyzoites in PBS.
- ii) Centrifuge at 2000 **g** for 15 minutes, retain the pellet and resuspend it in nine times its volume of distilled water.
- iii) Rupture the tachyzoites by freezing and thawing three times.
- iv) The antigen preparation is then sonicated for 20 seconds at 4°C at an amplitude of 20 microns.
- v) Remove any cellular debris by centrifugation at 10,000 **g** for 30 minutes at 4°C.
- vi) Retain the supernatant and store at –20°C until required. (Protein estimation might be expected to give a value of between 2 and 4 µg/ml.)

2.6. IFA test

The following is a protocol for carrying out an IFA test for anti-*Toxoplasma* IgG antibodies in sheep serum. It only requires minor modifications for testing different species or for measuring IgM antibody.

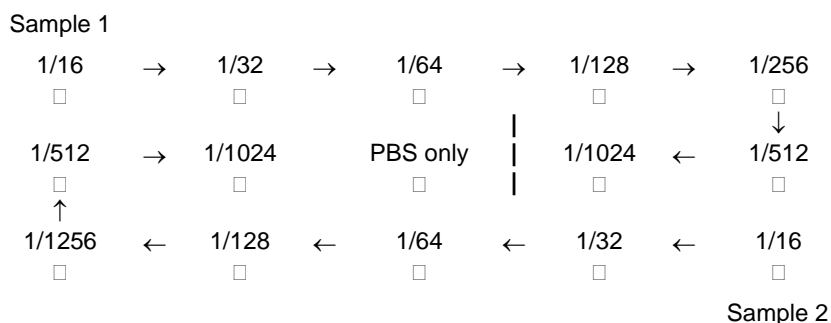
2.6.1. Test procedure

- i) Clean the required number of tissue culture 15-well multitest slides (Flow laboratories) and allow to dry.
- ii) Layer 5 µl of a whole tachyzoite preparation on to each well and allow to dry.
- iii) Fix in methanol for 10 minutes.
- iv) Wash twice for 10 minutes each time in 0.3 M PBS, pH 7.4.
- v) Add 5 µl of the given test sheep serum (diluted in PBS) to each well. (Prepare serial dilutions of the test sera, e.g. 1/16, 1/32, etc. up to 1/1024.) Ensure that positive and negative control sera are included in each test as well as a 'PBS-only' sample. Incubate for 30 minutes at room temperature.
- vi) Wash twice for 10 minutes each time in PBS.

- vii) Add 5 µl of an appropriate dilution of rabbit-anti-sheep IgG conjugated to fluorescein isothiocyanate, diluted in 0.2% filtered Evan's blue dye in PBS, to each well and incubate for 30 minutes at room temperature.
- viii) Wash three times for 10 minutes each time in PBS.
- ix) Mount the slides under cover-slips with buffered glycerol (nine parts PBS one part glycerol).
- x) Examine using a fluorescence microscope, fitted with x20 and x40 objective lenses.

With a negative test serum result the whole parasites will appear red due to the autofluorescence of the Evan's blue dye. They may also present with a green fluorescent cap at the parasite pole (nonspecific polar fluorescence). With a positive test serum the parasites will fluoresce red and at least 80% of them within a given well will be surrounded by an unbroken band of green fluorescence. In an adult sheep/goat a positive titre could be defined as $\geq 1/64$ and a negative titre as $\leq 1/32$. For lamb/kid and fetal sera, respective titres could be defined as $\geq 1/32$ and $\leq 1/16$.

An example slide set-up is shown below:



2.7. Procedure for the modified agglutination test

2.7.1. Serum diluting buffer

- i) Dissolve 42.5 g NaCl, 1.54 g NaH₂PO₄ (M.W. 120), and 5.4 g Na₂HPO₄ (M.W. 142) in 900 ml deionised water.
- ii) Adjust the pH to 7.2. Bring the volume to 1 litre with deionised water.
- iii) Store in a refrigerator. This is the 5× stock solution.
- iv) Dilute this stock solution 1/5 to give 0.01 M PBS (1 part stock and 4 parts deionised water). PBS should be filtered just before use through a 0.22 µm membrane.

2.7.2. Antigen diluting buffer

- i) Dissolve 7.01 g sodium chloride, 3.09 g boric acid, 2.0 g sodium azide in 900 ml deionised water.
- ii) Add 24 ml 1 N NaOH and adjust the pH to 8.95.
- iii) Bring the volume to 1 litre. This is the stock solution and can be stored at room temperature.
- iv) For the working antigen diluting buffer, dissolve 0.4 g bovine serum albumin (BSA) in 100 ml borate buffer. Store at 4°C.

2.7.3. Serum dilutions

- i) Dilute serum samples with serum diluting buffer (Section B.2.7.1 above) in small test tubes (1.2 ml in strips of 8 or 12) with a multichannel pipette, starting at 1/25.
- ii) Microtitre plates may also be used for making serum dilutions.

2.7.4. Preparation of antigen mixture

- i) For each plate, mix 2.5 ml antigen diluting buffer (see Section B.2.7.2 above), 35 µl 2-mercaptoethanol, 50 µl Evans blue dye solution (2 mg/ml water) and 0.15 ml antigen (formalin-fixed whole parasites).

2.7.5. Agglutination procedure

Agglutination is done in U bottom 96 well microtitre plates.

- i) Pipette 25 µl antigen mixture to each well immediately after mixing.
- ii) Pipette 25 µl serum dilutions into the wells and mix gently with the antigen by repeated pipetting action.
- iii) A positive control should be included in each plate. The control should have a titre of 1/200, and two-fold dilutions from 1/25 to 1/3200 should be used.
- iv) Cover the plates with sealing tape and incubate at 37°C over night.
- v) Read results using a magnifying mirror. A blue button at the bottom of the well means negative. A clear bottom means positive.

C. REQUIREMENTS FOR VACCINES

The only available vaccine is a commercially produced live preparation for sheep, currently licensed for use in the UK, Ireland, France, Portugal and Spain and New Zealand. It consists of tissue culture grown S48 *T. gondii* tachyzoites attenuated by over 3000 passages in mice. The vaccine stimulates effective protective immunity for at least 18 months following a single subcutaneous injection, but as it is unable to produce tissue cysts, sheep are not left with a persistent vaccinal infection. The vaccine has a short shelf life and is a potential risk to immunosuppressed and pregnant female operatives (Buxton, 1993). The vaccine should be stored and used strictly according to the manufacturer's instruction, thus it should not be frozen at any time, should be maintained cool (around 4°C) and not exposed to direct sunlight. The diluent provided should be added to the concentrated suspension of tachyzoites immediately prior to use.

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NB: At the time of publication (2017) there were no OIE Reference Laboratories for toxoplasmosis (please consult the OIE Web site: <https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2017.