**Annex 18. Item 5.1. – Chapter 3.10.8. Toxoplasmosis**

2 MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

3 **Paris, 4–8 September 2023**

4

5 **CHAP T E R 3 . 1 0 . 8 .**

# 6 T OXOPL A SMOSI S

7 **SUMMARY**

1. *Toxoplasmosis is a zoonotic infection of animals caused by the protozoan parasite* Toxoplasma gondii*. This*
2. *parasite has the potential to infect all warm-blooded animals. Although infection does not result in clinical*
3. *illness in the majority of animal species, in some it causes acute life-threatening disease. In some animals,*
4. *particularly small ruminants,* Toxoplasma *infection may manifest itself as a disease of pregnancy by*
5. *multiplying in the placenta and fetus. In these animals it can result in abortion or the birth of weak offspring.*
6. *Human infections are generally asymptomatic, but they can cause abortion in pregnant women, ocular*
7. *disease, hydrocephalus or intracranial calcifications in congenitally infected children, ocular toxoplasmosis*
8. *in immunocompetent individuals, and serious symptoms and even death in severely immunocompromised*
9. *patients.*
10. Toxoplasma gondii *is an obligate intracellular parasite that has a sexual cycle in Felidae and a two-stage*
11. *asexual cycle in all warm-blooded animals. Globally the* T. gondii *population structure is diverse and the*
12. *various genotypes are associated with the extent of virulence in particular hosts. In the acute phase of*
13. *infection, tachyzoites multiply in host cells to cause varying degrees of tissue destruction. With the onset of*
14. *an immune response, tachyzoites transform into bradyzoites that multiply slowly in cells to produce tissue*
15. *cysts.*
16. ***Detection of the agent:*** *In aborted fetuses and placenta,* T. gondii *is often difficult to find histologically, but*
17. *is more likely to be seen in tissue sections of brain and placenta. Parasitic stages can be identified by*
18. *immunohistochemistry, while nucleic acid-based assays might be used to confirm presence of parasite DNA*
19. *in tissues and may allow genotyping of the parasite in biological specimens.* In-vitro *isolation of* T. gondii
20. *from host samples is expensive, time consuming and rarely used.*
21. *The sexual part of the life-cycle of* T. gondii *takes place exclusively in epithelial cells of the feline intestine*
22. *and can result in the excretion of large numbers of oocysts in the faeces. Oocysts may remain viable in the*
23. *environment for many months. Oocysts of* T. gondii *~~morphologically~~ resemble those from* Hammondia
24. hammondi*, a related but non-virulent parasite that also uses cats as definitive hosts. Nucleic acid-based*
25. *molecular tests are available to distinguish between these related parasites.*
26. ***Serological tests:*** *Among the easy-to-perform serological tests, the indirect fluorescent antibody test* (*IFAT*)
27. *and the direct agglutination test* (*DAT*) *allow the titration of sera and the establishment of appropriate cut-*
28. *offs to ensure diagnostic sensitivity and specificity. The IFAT can be used to differentiate IgM and IgG*
29. *antibodies. The DAT is fast and requires no complex laboratory facilities. Enzyme-linked immunosorbent*
30. *assays* (*ELISA*) *require more sophisticated laboratory equipment but can process large numbers of samples*
31. *and are easier to standardise.*
32. ***Requirements for vaccines:*** *A vaccine composed of live* T. gondii *tachyzoites is available commercially for*
33. *use in sheep in certain countries. The vaccine is supplied as a concentrated suspension of tachyzoites with*
34. *an approved diluent and delivery system. The vaccine must be handled strictly according to the*
35. *manufacturer’s instructions as it can be hazardous to the user and has a very short shelf life.*

## 43 A. INTRODUCTION

1. *Toxoplasma gondii* is a zoonotic, obligate intracellular protozoan parasite with the capacity to infect all warm-blooded
2. animals, including birds. Although clinical toxoplasmosis seldom occurs in the majority of animal species, acute life-
3. threatening disease has been reported in some animals. In small ruminants, in particular, it manifests itself as a disease
4. of pregnancy by multiplying in the placenta and fetus. Acute, potentially fatal, infections have been recorded from a range
5. of wild or zoo animals (Dubey, 2022)*.* Infected humans often show no symptoms, but congenital toxoplasmosis, postnatally
6. acquired ocular toxoplasmosis in immunocompetent individuals, or toxoplasmosis in severely immunocompromised
7. patients represent serious threats (EFSA, 2018).
8. *Toxoplasma gondii* has a two-stage asexual cycle in warm-blooded animals and a sexual cycle in Felidae. A systematic
9. review and meta-analysis have reported a global seroprevalence of 38% in domestic cats and 64% wild felids (Hatam-
10. Nahavandi *et al.*, 2021). The genetic diversity of *T. gondii* is complicated; three archetypal clonal lineages (I, II, and III)
11. prevail in Europe and North America; in South America, Asia, and Africa, much greater genetic diversity is apparent and,
12. furthermore, fewer clonal and non-clonal lineages have been genotyped (Lorenzi *et al.*, 2016). Transport between
13. continents via animal migration, including birds, and human activity such as trade may have contributed to the genetic
14. population structures of *T. gondii* in different geographical regions (Shwab *et al.*, 2018).
15. In the asexual part of the lifecycle, the two developmental stages are the rapidly multiplying tachyzoite and the slowly
16. multiplying bradyzoite. In acute infection, tachyzoites actively penetrate host cells where they multiply, causing the cell to
17. rupture and release organisms locally and into the circulation. As the host develops immunity, the parasite retains its overall
18. size and shape, but transforms into the bradyzoite stage and multiplies more slowly within tissue cysts to establish a
19. persistent infection. These microscopic tissue cysts occur most frequently in brain and skeletal muscle and represent the
20. quiescent stage of the parasite within the host. Viable tissue cysts within muscle (meat) are a significant source of human
21. infection, and ingestion of bradyzoites in prey is probably the main route of infection to predators, including the felid
22. definitive host. In animals that succumb to acute infection, tachyzoites may be demonstrated in ascitic fluid or in lung
23. impression smears, as well as in tissue sections of the liver and other affected organs.
24. Abortions in sheep and goats due to *T. gondii* are of particular veterinary importance. Toxoplasmosis in small ruminants
25. must be differentiated from diseases caused by other infectious agents, including infections with *Chlamydophila abortus*
26. (see Chapter 3.8.5 *Enzootic abortion of ewes*), *Coxiella burnetii* (see Chapter 3.1.17 *Q fever*), *Brucella melitensis* (see
27. Chapter 3.1.4 *Brucellosis* [*Brucella abortus*, *B. melitensis* and *B. suis*]), *Campylobacter foetus* (see Chapter 3.4.4 *Bovine*
28. *genital campylobacteriosis*), *Salmonella* spp. (see Chapter 3.10.7 *Salmonellosis*), and the viruses that cause border
29. disease (see Chapter 3.8.1 *Border disease*), bluetongue (see Chapter 3.1.3), Wesselsbron’s disease, and Akabane
30. disease (see Chapter 3.10.1). In pigs, *Brucella suis* (see Chapter 3.1.4) may also cause fetal death, mummification, and
31. abortion.
32. The sexual part of the lifecycle occurs in enteroepithelial cells of the feline definitive host, and results in the production of
33. *T. gondii* oocysts. Following primary infection of a cat, oocysts may be shed in the faeces for several days, with large
34. numbers contaminating the environment; up to one billion oocysts from domestic cats, and probably similar numbers from
35. wild felids (Shapiro *et al.*, 2019). The oocysts sporulate in the environment over the next 1–5 days (depending on aeration,
36. humidity, and temperature), at which time they become infective. The structure of *T. gondii* oocysts results in extreme
37. resistance to environmental conditions, with the polymeric structure of the walls ~~giving~~ providing strength against
38. mechanical forces and protection against chemical agents (Shapiro *et al.*, 2019). This results in prolonged survival, up to
39. 18 months in water at 4°C and, once sporulated, can persist in damp soil for as long, at temperatures ranging from −20°C
40. to 35°C. Sporulated oocysts are 11 × 13 µm in diameter and each contains eight sporozoites, four in each of two sporocysts
41. (Dubey, 2022). When a susceptible animal ingests sporulated oocysts, the sporozoites are released to penetrate the
42. intestinal lining, become tachyzoites, and establish an infection.

#### 1. Human health risks

1. *Toxoplasma gondii* is a zoonotic parasite and readily infects people. While human infection, as determined by seropositivity,
2. is moderately common globally (local prevalence ranges from under 10% to over 90% [Pappas *et al.*, 2009]), clinical illness
3. is relatively uncommon. The immunosuppressed are particularly at risk of developing clinical illness. In patients being
4. treated with immunosuppressive drugs, toxoplasmosis may occur due to new infection or activation of chronic infection. In
5. addition, the parasite can pose a serious threat to an unborn child if the mother becomes infected for the first time while
6. pregnant. The *T. gondii* genotype is also relevant, and outbreaks of clinical infection with some non-archetypal exotic
7. strains have occurred in people with no apparent immune deficiency. Toxoplasmosis usually manifests as general malaise,
8. fever, and lymphadenopathy. However, more severe symptoms may occur, including ocular problems, such as
9. retinochoroiditis, potentially resulting in loss of vision, pneumonitis, and also toxoplasmic encephalitis. The main burden of
10. human disease, based on disability-adjusted life years (DALYs), ranks as having a high contribution to disease burden
11. globally (Torgerson *et al.,* 2015).
12. As with animal infections, people may be infected by ingestion of bradyzoites in raw or lightly cooked meat containing live
13. *T. gondii* tissue cysts or by ingestion of sporulated oocysts. These may be as contaminants of water or of raw or lightly
14. cooked fresh produce; less commonly, people can be infected by ingestion of tachyzoites in non-heat-treated milk. In
15. addition, transmission via blood transfusion or organ transplantation is also possible. Outbreaks of both waterborne and
16. foodborne toxoplasmosis have been described (EFSA, 2018; Shapiro *et al.,* 2019). The largest outbreak to date occurred
17. in Santa Maria, Brazil in 2018, and is considered to be due to contamination of the water supply with oocysts of a virulent
18. strain of *T. gondii*; at least 930 confirmed cases occurred, among which 8% required hospitalisation, with three fetal deaths,
19. 10 abortions, and 29 cases of congenital transmission, with 19 infants with ocular lesions (Dubey, 2021).
20. Clearly, *T. gondii* represents a human health risk and all laboratory manipulations with live organisms should be handled
21. with appropriate measures determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard*
22. *for managing biological risk in the veterinary laboratory and animal facilities.*

## 109 B. DIAGNOSTIC TECHNIQUES

110 ***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 |
| **Detection and identification of the agent** |
| **PCR (including nested and real- time PCR)** | – | – | – | ++ | + | – |
| **LAMP** | – | – | – | ++ | + | – |
| **Histopathology** | – | – | – | + | – | – |
| **Immmuno- histochemistry** | – | – | – | + | – | – |
| **Detection of immune response** |
| **IFAT(a)** | + | + | + | ++ | ++ | + |
| **ELISA(a)** | + | + | + | +++(a) | +++ | ++ |
| **DAT/MAT** | + | + | + | + | ++ | + |

111 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

112 + = suitable in very limited circumstances; – = not appropriate for this purpose.

1. PCR = polymerase chain reaction; LAMP = loop mediated isothermal amplification; IFAT = indirect fluorescent antibody test;
2. ELISA = enzyme-linked immunosorbent assay; DAT = direct agglutination test; MAT = modified agglutination test.
3. (a)In IFAT and ELISA, detection of *Toxoplasma*-specific IgG and IgM antibodies may permit some discrimination between acute and
4. chronic cases of infection. In ELISA, assays assessing the avidity of an IgG response to *T. gondii* may provide information regarding
5. how recently the tested animals have experienced a primary *T. gondii* infection.

#### 1. Detection of the agent

##### 1.1. Histopathology

1. In animals that die with acute toxoplasmosis, focal mononuclear inflammation, with or without focal necrosis, may be
2. seen in a number of tissues, including the liver, heart, and lungs. The latter may be oedematous. Lymph nodes may
3. have undergone expansion and there may or may not be focal necrosis with or without haemorrhage. Typically, *T.*
4. *gondii* tachyzoites may be demonstrable in association with necrosis and inflammation. In fatal cases, tachyzoites
5. may be demonstrated in ascitic fluid or in lung impression smears.
6. In cases of abortion and stillbirth in small ruminants, affected placental cotyledons typically contain large foci of
7. coagulative necrosis that may have become mineralised with time. Any associated inflammation is characteristically
8. slight and nonsuppurative. Well-preserved samples of placental cotyledons may show moderate oedema of the
9. mesenchyme of the foetal villi, with a diffuse hypercellularity due to the presence of large mononuclear cells. Small
10. numbers of intracellular and extracellular stages are sometimes visible, usually on the periphery of a necrotic area or
11. in a villus that is in the early stages of infection. The *T. gondii* tachyzoites appear ovoid, 2–6 µm long, with nuclei that
12. are moderately basophilic and located centrally or towards the posterior end.
13. In the fetal brain, primary and secondary lesions may develop. Microglial foci, typically with a necrotic and sometimes
14. mineralised centre, and often associated with a mild focal lymphoid meningitis, represent a fetal immune response
15. following direct damage by local parasite multiplication. *Toxoplasma gondii* tissue cysts are only rarely found, usually
16. at the periphery of these lesions. Focal leukomalacia is also common and is considered to be due to fetal anoxia in
17. late gestation caused by advanced lesions in the placentome preventing sufficient oxygen transfer from mother to
18. fetus. Such foci most commonly occur in the cerebral white matter cores, but sometimes also in the cerebellar white
19. matter. Focal leukomalacia on its own suggests placental disease or acute insufficiency, but the two types of
20. neuropathological change seen together are characteristic of *T. gondii* infection.

##### 1.2. Immunohistochemistry

1. Confirmation of the identity of *T. gondii*-like structures in tissue sections from such cases, as well as from instances
2. of acute toxoplasmosis, may be achieved by immunohistochemistry that labels intact *T. gondii* or antigenic debris
3. using polyclonal or monoclonal *T. gondii* specific antibodies (Dubey, 2022). The antigen-antibody reaction can be
4. visualized by avidin-biotin-complex (ABC) or indirect immune-peroxidase and the peroxidase–antiperoxidase (PAP)
5. technique. The method is both convenient and sensitive and is used with fixed tissues (including archived tissues)
6. that may also exhibit a degree of decomposition, where isolation would not be appropriate or possible. However,
7. cross-reactions with related parasites like *Neospora caninum* are possible.

##### 1.3. Detection of oocysts

1. *Toxoplasma gondii* oocysts can be detected in stools of felids, as well as contaminating different environmental
2. matrices, such as soil and water, or food, such as molluscs and fresh produce. The low quantity or sparse distribution
3. of oocysts in the contaminated matrix, as for water and fresh produce, means that an initial procedure to concentrate
4. the oocysts from a large volume of sample is needed. Chemical flocculation (e.g. using ferric or aluminium sulphate
5. or calcium carbonate), filtration by cellulose acetate or polycarbonate membranes or cartridge filters and flotation with
6. sucrose or caesium chloride gradient have been widely used for water samples. Washing with appropriate buffer(s)
7. and pelleting by centrifugation is often used for fresh produce (Shapiro *et al.*, 2019; Slana *et al.*, 2021).
8. Although the autofluorescence of *T. gondii* oocysts, pale blue under UV light, facilitates detection by microscopy, this
9. property is shared with oocysts and sporocysts of other related coccidian parasites (e.g. *Hammondia hammondi*)
10. (Lindquist *et al.*, 2003). As a commercially available antibody specific for *T. gondii* oocysts for microscopy detection
11. is currently lacking, molecular assays are usually used to confirm *T. gondii* identification in field samples. Molecular
12. methods are needed to assess or confirm the identity of oocysts ~~observed~~.

##### 1.4. Molecular methods – detection of nucleic acids

1. The presence of *T. gondii* (tachyzoites, tissue cysts, oocysts) can be assessed by detecting the parasite genomic
2. DNA using several molecular techniques, including conventional polymerase chain reaction (PCR), nested PCR and
3. loop-mediated isothermal amplification (LAMP) (Table 1). No standard methods are available and many of the
4. published protocols are not yet sufficiently validated (for details refer to Chapter 1.1.6. *Principles and methods of*
5. *validation of diagnostic assays for infectious disease* and Chapter 2.2.3. *Development and optimisation of nucleic*
6. *acid detection assays*).
7. Appropriate protocols may allow detection of *T. gondii* DNA from circulating tachyzoites (acute infection) or
8. bradyzoites in tissue cysts (latent infection) and in different biological samples, including animal and human
9. tissues (e.g. heart and skeletal muscle, placenta, brain) and body fluids (e.g. blood, urine, aqueous humour,
10. cerebrospinal fluid, amniotic fluid, milk). In addition, DNA from oocysts in stool (only felids), food and
11. environmental samples (fresh produce, water, soil) can be detected (Slana *et al.*, 2021). No standard method
12. for extracting *T. gondii* DNA exists, but suitable DNA extraction protocols, based on both in-house protocols and
13. commercial kits have been developed (Dzib Paredes *et al.*, 2016). Sample preparation and DNA extraction
14. procedures are likely to have a considerable impact on the sensitivity of the test. Sensitivity is generally higher
15. in DNA-poor matrices than DNA-rich ones (e.g. tap water vs meat samples). Moreover, inhibitors of DNA
16. amplification differ and are related to sample type. Inhibition of DNA amplification can be avoided by DNA
17. extraction optimised for sample type or by using appropriate additives (e.g. bovine serum albumin) during DNA
18. amplification. A specific concentration of the parasite stage (e.g. oocysts) or its DNA (e.g. by magnetic capture)
19. from the matrix might be required prior to DNA extraction as reported for oocysts from water, fresh produce, or
20. faeces (Slana *et al.*, 2021). A validated protocol for DNA extraction from meat and meat products using a
21. commercial kit is available at the website of the European Union Reference Laboratory for Parasites (EURLP) [48](#_bookmark135).
22. An example of in-house method for DNA extraction from pig tissue (Jauregui *et al.*, 2001) is reported below.
23. 1.4.1. DNA extraction from animal tissue
24. **Procedure**
25. i) Homogenate sample (e.g. 50 g of brain or tongue, or 1 g of muscle) in a blender with 5 volumes
26. of sterile saline solution (phosphate-buffered saline [PBS]: 300 mM NaCl, 2.7 mM KCl, 10 mM
27. Na2HPO4, 1.7 mM NaH2PO4).
28. ii) Digest sample with an equal volume of warm (37°C) pepsin-HCl (1.4 mg of pepsin and 10 mg
29. of NaCl per ml in 0.1 N HCl) for 1 hour at 37°C in a shaking water bath.
30. iii) Neutralise mixture by two washes with 0.1 M Tris buffer (pH 8.0).
31. iv) Centrifuge mixture aliquots for 10 minutes at 1180 ***g***.
32. v) For each aliquot, digest the post centrifugation pellet overnight at 55°C with DNA digestion
33. buffer (0.5% sodium dodecyl sulphate [SDS], 25 mM ethylene diamine tetra-acetic acid [EDTA],
34. 100 mM NaCl, 20 mM Tris-HCl [pH 8.0], and proteinase K [0.1 mg/ml final concentration]).
35. vi) Extract with one volume of phenol-chloroform-isoamyl alcohol (25:24:1).
36. vii) Precipitate DNA in 0.3 M sodium acetate (final concentration) with 2.5 volumes of 100% ethanol.
37. viii) Resuspend DNA pellets in TE buffer (10 mM Tris-HCl, 1 mM EDTA). Store DNA at –20°C until
38. use.
39. PCR-based assays are commonly applied for the molecular detection of *T. gondii* genomic DNA (Dzib
40. Paredes *et al.*, 2016; Robert *et al.*, 2021; Slana *et al.*, 2021).
41. 1.4.2. DNA extraction from oocysts
42. Although DNA detection is considered highly specific, cross reactivity has been observed between
43. *T. gondii* and *H. hammondi*, a non-zoonotic coccidian that also uses felids as definitive hosts and
44. cannot be differentiated based on oocyst morphology. A real-time PCR targeting a repetitive element
45. of *H. hammondi* (HhamREP-529) has been demonstrated to be highly sensitive and efficient in
46. distinguishing between the two parasites (Schares *et al.*, 2021).
47. Detection of DNA from *T. gondii* oocysts may present additional challenges because of inhibitors in
48. faecal matter, vegetable or water sediment, and difficulty of extracting DNA from the oocysts. Options
49. for ~~an~~ efficient breaking of oocyst~~s~~ walls include bead-beating, freeze–thaw cycles, heating or
50. chemical/enzymatic treatments (Slana *et al.*, 2021). An in-house method is detailed below for
51. preparation of oocysts and extraction of DNA. An example of a validated method using a commercial
52. kit and a bead-beating-based DNA extraction is available (Lalle *et al.*, 2018).
53. **Procedure**
54. i) Wash oocysts four times in 15 ml PBS (300 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.7 mM
55. NaH2PO4) in a 15 ml centrifugation tube, with centrifugation between washes (1100 g for
56. 7 minutes without braking).

1. <https://www.iss.it/documents/5430402/5722370/MI_12_rev._1.pdf/a82a4078-f511-affe-8f90-cabc397bc8ce?t=1620381672663>
2. ii) Incubate pellet (up to 0.5 ml) in 2 ml 5.75 % sodium hypochlorite (sodium hypochlorite, aqueous
3. solution, ≥ 4% as active chlorine) for 30 minutes at 37°C.
4. iii) Add double-distilled H2O up to 15 ml.
5. iv) Centrifuge supernatant (1100 g for 7 minutes without use of brake) and mix the pellet with PBS.
6. Wash the pellet three times with PBS (1100 ***g*** for 7 minutes without brake).
7. v) After final centrifugation, re-suspend pellet in 1 ml PBS, transfer into a 1.5 ml reaction tube and
8. spin down (1100 ***g*** for 7 minutes without brake).
9. vi) Remove as much supernatant as possible and apply three freeze–thaw cycles (10 minutes at
10. –20°C followed by 2 minutes at room temperature) to the pellet.
11. vii) Re-suspend pellet in 100 µl OOC lysis buffer (600 mM EDTA, 1.3% [v/v] N-lauroylsarcosine, 2
12. mg/ml proteinase K, pH 9.5) for 45 minutes, at 65°C.
13. viii) Add 400 µl OOC-CTAB buffer (2% [v/v] cetyl-trimethyl ammonium bromide, 1.4 M NaCl, 0.2 %
14. [v/v] mercaptoethanol, 20 mM EDTA, 100 mM tris[hydroxymethyl]aminomethane) for 60
15. minutes at 60°C.
16. ix) Mix with 500 µl phenol/chloroform/isoamyl alcohol (25/24/1) by inverting 50 times. Centrifuge
17. for 7 minutes at 13,000 ***g***.
18. x) Transfer supernatant to fresh tube and mix with 500 µl phenol/chloroform/isoamyl alcohol
19. (25/24/1) by inverting 50 times. Centrifuge for 7 minutes at 13,000 ***g***.
20. xi) Transfer supernatant to a fresh tube and add 0.04 volumes of 4 M NaCl and 2–3 volumes of –
21. 20°C cold 96% (v/v) ethanol to precipitate DNA (keep at least 20–30 minutes at –20°C).
22. xii) Centrifuge for 15 minutes at 13,000 ***g***. Decant the supernatant.
23. xiii) Wash the pellet using 70% (v/v) ethanol and centrifuge for 15 minutes at 13,000 ***g***.
24. xiv) Discard the ethanol solution and air dry the pellet.
25. xv) Solve DNA in double-distilled water for at least 12 hours at 4°C.
26. xvi) Use 2.5–10 µl aliquots for PCR (see Section B.1.2 above).
27. The same PCR-based and LAMP assays detailed in Section B.1.2 have been also used for oocyst
28. detection, with B1 gene and the 529RE being targets of choice (Slana *et al.*, 2021).
29. Another important issue is the possibility of combining detection with information on oocyst viability.
30. Bioassays, currently the only definitive way to detect viable oocysts, are expensive and relatively few
31. laboratories have the necessary facilities. Reverse transcription (RT) real-time PCR (real-time RT-
32. PCR) or propidium monoazide-based real-time PCR have shown some promise for assessing oocyst
33. viability in complex sample matrices (Kim *et al.*, 2021; Rousseau *et al.*, 2018).
34. 1.4.3. Nucleic acid detection methods
35. Although single copy genes (e.g. SAG1, SAG2, SAG3, GRA6, and GRA7) have been used, multi-
36. copy genes or genetic elements (e.g. 18S rDNA, B1, ITS1, 529RE) are preferred as they provide a
37. higher sensitivity (Dzib Paredes *et al.*, 2016; Slana *et al.*, 2021). For instance, 35 copies of the B1
38. gene and 200–300 copies of the 529 bp repetitive element (529RE) are present in the *T. gondii*
39. genome, and 10 to 100-fold higher sensitivity is generally observed in amplification targeting the
40. 529RE compared with B1, although this also reflects the type of assay and sample being analysed
41. (Belaz *et al.*, 2015). In addition, some strains may have partially lost, or have a mutated, 529RE, and
42. this could compromise diagnostic sensitivity (Wahab *et al.*, 2010).
43. Conventional PCR targeting B1 was the first to be used in clinical diagnostics in people (Burg *et al.*,
44. 1989). To obtain details on *T. gondii* genotype (e.g. for outbreak investigation, infection source
45. tracing) the methods of choice are multi-locus PCR combined with restriction fragment length
46. polymorphism (PCR-RFLP) or sequencing and multi-locus microsatellite typing (Ajzenberg *et al.*,
47. 2010; Su *et al.*, 2006).
48. To increase sensitivity, several nested PCR assays have been implemented (Dzib Paredes *et al.*,
49. 2016). The reaction consists of two successive rounds of amplification. The product of the first
50. amplification serves as template for the second amplification, using one or two internal primers. The
51. risk of cross- and carry-over contamination and false positives is increased with nested PCR, and
52. precautions should be taken to mitigate the risk (Dzib Paredes *et al.*, 2016).
53. There are several real-time PCR protocols and real-time PCR in combination with a hydrolysis probe
54. is the most frequently applied (Slana *et al.*, 2021). This has largely improved both sensitivity and
55. specificity of detection of *T. gondii* DNA, with the advantage of avoiding post-amplification
56. manipulations and thus limiting the risk of carry-over contamination. Although sensitivity can be
57. satisfactory with both conventional and real-time PCR using pure genomic *T. gondii* DNA, assay
58. specificity might be affected when testing field samples. Conventional PCR can result in non-specific
59. amplification, whereas this is not detected by real-time PCR due to the probe detection, despite the
60. amplified target being the same. Furthermore, real-time PCR can be multiplexed and simultaneous
61. amplification of an internal amplification control (a heterologous DNA fragment) can be used to
62. monitor for the presence of inhibitors. In addition, amplifying two *T. gondii*-specific targets at once
63. may increase sensitivity. Another advantage of real-time PCR is the possibility of quantification of *T.*
64. *gondii* DNA.
65. A selective enrichment of target DNA combined with real-time PCR (i.e., magnetic capture PCR) has
66. been reported to increase *T. gondii* detectability in animal samples (Gisbert Algaba *et al.*, 2017). The
67. principle relies on separating and concentrating *T. gondii* DNA from sample DNA by specific DNA
68. probes, complementary to the targeted parasite 529RE genomic region, which are conjugated to
69. magnetic beads and followed by real-time PCR (Gisbert Algaba *et al.*, 2017). However, this method
70. is expensive, time consuming, and requires further expertise, so might be not suitable for routine
71. analysis or large surveys.
72. As an alternative to PCR, LAMP has been considered for *T. gondii* DNA detection, and diagnostic
73. purposes, in environmental, veterinary, and human samples. LAMP takes advantage of a DNA
74. polymerase (originally Bst) having both high strand displacement and replication activities. Nucleic
75. acid amplification is performed under isothermal conditions (60–65°C), without the need of a DNA
76. denaturation step. Both, B1 and 529RE have been widely used as targets in different LAMP assays,
77. and LAMP is reported as comparable to real-time PCR for the detection of *T. gondii* in blood and
78. animal tissues (Robert *et al.*, 2021). Although LAMP provides an opportunity for development of point-
79. of-care testing or implementation of molecular tests in settings with limited facilities, there are several
80. drawbacks including design of appropriate primers and the high risk of carry-over contamination.
81. Commercial assays for both real-time PCR and LAMP are available for clinical diagnosis of
82. toxoplasmosis.
83. Overall, the reported sensitivity of published molecular methods can be as low as one (or even less)
84. genome equivalent per reaction. However, this largely depends on sample type, DNA extraction,
85. copies of the targeted gene or sequence, amplification and detection reagents, procedures and
86. platforms. The lack of accepted standard methods prevents robust comparison of sensitivity and
87. specificity of the currently applied molecular tests.

#### 2. Serological tests

1. There are several serological tests available for the detection of *T. gondii* antibodies (Table 1). All serological tests have
2. limitations in diagnostic sensitivity and specificity and need proper validation to ensure confidence in results (refer to
3. Chapter 1.1.6. *Principles and methods of validation of diagnostic assays for infectious disease* and Chapter 2.2.1.
4. *Development and optimisation of antibody detection assays*).
5. The Sabin-Feldman dye test (DT) is a reference serological test for *T. gondii* antibody in humans (Dubey, 2022). Although
6. the DT appears both specific and sensitive in humans, it is not extensively validated in other species. In addition, it is
7. potentially hazardous as live parasite is used, is expensive, and requires a high degree of technical expertise.

##### 2.1. Preparation of antisera and antigens

1. Antisera to *T. gondii* and conjugated antisera for use in IFAT and ELISA, to allow screening of a variety of animal
2. species, may be obtained commercially. International standards for animal sera are not available.
3. Below are protocols for the preparation of tachyzoite antigen for use in the IFAT and ELISA. Tachyzoites may be
4. grown in tissue culture and retained as whole parasites for the IFAT, or prepared as soluble antigen for the ELISA.

##### 2.2. Preparation of frozen stabilates of *T. gondii* tachyzoites

1. 2.2.1. Test procedure
2. i) Produce tachyzoites in tissue cell culture as described. Suitable *T. gondii* strains, able to multiply
3. in cell culture, like the RH strain are available in a number of repositories; e.g. at the American
4. Tissue Cell Culture Collection (ATCC [49](#_bookmark136)).
5. ii) Centrifuge three times at 500 ***g*** for 5 minutes and resuspend tachyzoites in Iscove’s modified
6. Dulbecco’s medium (IMDM) or any other cell culture medium suitable to cultivate *T. gondii.* Final
7. concentration of the tachyzoite suspension should be approximately 1.5 × 108 tachyzoites/ml.
8. iii) Combine dimethyl sulphoxide (DMSO), normal horse serum (free from antibody to *T. gondii*)
9. and the tachyzoite suspension to give these final concentrations: 10% DMSO, 20% normal
10. horse serum, 70% tachyzoite suspension; this gives a final concentration of approximately 1 ×
11. 108 tachyzoites/ml.
12. iv) Allow the preparation to stand on the bench for 1 hour (4-10°C; optimally on ice).
13. v) Dispense into 1-ml aliquots using screw-topped tubes appropriate for liquid nitrogen storage.
14. vi) Put the tubes into a small container, wrap in thick insulating material (e.g. paper towels) and
15. place in –70°C freezer to allow the tachyzoites to freeze gradually.
16. vii) The next day transfer to liquid nitrogen, keeping well insulated while transferring.
17. viii) These stabilates may then be used for tissue culture growth of the parasite. When removing
18. from storage, thaw the sample rapidly in a water bath (37°C).
19. ix) Centrifuge three times at 500 ***g*** for 5 minutes and resuspend tachyzoites in Iscove’s modified
20. Dulbecco’s medium (IMDM) or any other cell culture medium suitable to cultivate *T. gondii* and
21. add suspension to cell culture.

##### 2.3. Production of *Toxoplasma* tachyzoites in cell culture

1. 2.3.1. Test procedure
2. i) *Toxoplasma gondii* can be grown and maintained in tissue culture by twice-weekly passage in
3. African green monkey kidney (Vero) cells. Other cell lines (e.g. MARC145 cells) are also
4. suitable. Cell lines are available from repositories (e.g. ATCC).
5. ii) Cells and parasite are grown in IMDM supplemented with 50 IU/ml penicillin, 50 µg/ml
6. streptomycin, and 2% fetal bovine serum; there are other cell culture media suitable as well.
7. iii) Tachyzoites are harvested from tissue culture flasks by scraping the cell monolayer using a
8. sterile cell scraper.
9. iv) Using 25 cm2 vented tissue culture flasks that have each been seeded with 1 × 105 Vero cells,
10. add tachyzoites at the rate of two tachyzoites per monolayer cell and incubate at 37°C in a 5%
11. CO2 humidified chamber. Harvest, when 2/3 of the cell layer was destroyed by tachyzoite
12. multiplication, usually after 3–4 days.

##### 2.4. Preparation of whole tachyzoites for IFAT and agglutination

1. 2.4.1. Test procedure
2. i) Produce 4 × 107/ml suspension of *T. gondii* tachyzoites in PBS.
3. ii) Add formaldehyde (40%) to give a final concentration of 0.2% (v/v).
4. iii) Incubate at 4°C overnight and divide into aliquots in suitable tubes and store frozen until
5. required (–20°C).

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1. American Type Culture Collection, P.O. Box 1549, Manassas, Virginia 20108, United States of America.

##### 2.5. Production of soluble antigen for ELISA

1. 2.5.1. Test procedure
2. i) Produce a suspension of *T. gondii* tachyzoites in PBS.
3. ii) Centrifuge at 2000 ***g*** for 15 minutes, retain the pellet and resuspend it in nine times its volume
4. of distilled water.
5. iii) Lyse the tachyzoites by freezing and thawing three times.
6. iv) Sonicate the antigen preparation for 20 seconds at 4°C at an amplitude of 20 microns.
7. v) Remove any cellular debris by centrifugation at 10,000 ***g*** for 30 minutes at 4°C.
8. vi) Retain the supernatant and store at –20°C until required; protein estimation should be between
9. 2 and 4 µg/ml.

##### 2.6. Indirect fluorescent antibody test

1. The indirect fluorescent antibody test (IFAT) (Dubey, 2022) is a simple and widely used method. Whole, killed
2. *Toxoplasma* tachyzoites are incubated with diluted test serum, the appropriate fluorescent labelled secondary
3. antibody is added, and the result is then viewed with a fluorescence microscope. Fluorescent-labelled species-
4. specific secondary antibodies are available commercially, the method is relatively inexpensive, and kits are
5. commercially available. However, the results are read by eye, so subjective variation may occur. It may be difficult to
6. find some species-specific conjugates and there is a risk of possible cross-reactivity with rheumatoid factor and anti-
7. nuclear antibodies. The following is a protocol for carrying out an IFAT for anti-*Toxoplasma* IgG antibodies in sheep
8. serum. It only requires minor modifications for testing different species or for measuring IgM antibody.
9. 2.6.1. Test procedure
10. i) Clean the required number of multi-well immunofluorescence assay slides (e.g. 10–21 well-
11. slides with wells of 4–6 mm in diameter are suitable) and allow to dry.
12. ii) Apply 5 µl of a whole tachyzoite preparation (Section B.2.4.1 above) on to each well and allow
13. to air dry.
14. iii) Fix in methanol for 10 minutes.
15. iv) Wash twice (10 minutes for each wash) in 0.3 M PBS, pH 7.4.
16. v) Prepare serial dilutions of the test sera in PBS (e.g. 1/16, 1/32, etc. up to 1/1024).
17. vi) Add 5 µl of the given test sheep serum (diluted in PBS) to each well. Ensure that positive and
18. negative control sera are included in each test as well as a ‘PBS-only’ sample. Incubate for
19. 30 minutes at room temperature.
20. vii) Wash twice (10 minutes each time) in PBS.
21. viii) Add 5 µl of an appropriate dilution of rabbit-anti-sheep IgG conjugated to fluorescein
22. isothiocyanate, diluted in 0.2% filtered Evans blue dye in PBS (filtered through a 0.45 µm sterile
23. filter), to each well and incubate for 30 minutes at room temperature.
24. ix) Wash three times for 10 minutes each time in PBS.
25. x) Mount the slides under cover-slips with buffered glycerol (nine parts PBS, one part glycerol).
26. xi) Examine using a fluorescence microscope, fitted with ×20 and ×40 objective lenses.
27. With a negative test serum result, the whole parasites will appear red due to the autofluorescence of
28. the Evans blue dye. They may also present with a green fluorescent cap at the parasite pole
29. (nonspecific polar fluorescence). With a positive test serum, the parasites will fluoresce red and at
30. least 80% of them within a given well will be surrounded by an unbroken band of green fluorescence.
31. In an adult sheep/goat a positive titre could be defined as ≥1/64 and a negative titre as ≤1/32. For
32. lamb/kid and fetal sera, respective titres could be defined as ≥1/32 and ≤1/16. These cut off values
33. should be validated locally as results may vary between laboratories, depending on, e.g., the
34. fluorescence microscope and the operator. Optimally, each slide should include positive control and
35. negative controls.

##### 2.7. Modified agglutination test

1. The modified agglutination test (MAT) (Dubey, 2022) is both sensitive and specific. Formalinised *Toxoplasma*
2. tachyzoites are added to U-shaped well microtiter plates and dilutions of test sera are then applied. Positive samples
3. will produce agglutination that can be graded, whereas negative samples will produce a ‘button’ of precipitated
4. tachyzoites at the bottom of the well. The test is simple and easy to perform although relatively large amounts of
5. antigen are required. Kits are commercially available. It is important to treat sera with 0.2 2-mercaptoethanol to avoid
6. false positives due to non-specific IgM. The MAT has been used extensively for detection of *T. gondii* antibodies in
7. sera of many animal species and the procedure is detailed below. A commercially available latex agglutination test
8. is also available, but this test is regarded relatively insensitive compared with MAT or IFAT.
9. 2.7.1. Serum-diluting buffer
10. i) Dissolve 42.5 g NaCl, 1.54 g NaH2PO4, and 5.4 g Na2HPO4 in 900 ml deionised water.
11. ii) Adjust the pH to 7.2. Bring the volume to 1 litre with deionised water.
12. iii) Store in a refrigerator. This is the 5× stock solution.
13. iv) Dilute this stock solution 1/5 to give 0.01 M PBS (working serum-diluting buffer: 1 part stock and
14. 4 parts deionised water). PBS should be filtered through a 0.22 µM filter immediately prior to
15. use.
16. 2.7.2. Antigen-diluting buffer
17. i) Prepare a stock of borate buffer: dissolve 7.01 g sodium chloride, 3.09 g boric acid, 2.0 g sodium
18. azide in 900 ml deionised water.
19. ii) Add 24 ml 1 N NaOH and adjust the pH to 8.9.
20. iii) Bring the volume to 1 litre. This is the stock solution and can be stored at room temperature.
21. iv) For the working antigen-diluting buffer, dissolve 0.4 g bovine serum albumin in 100 ml borate
22. buffer. Store at 4°C.
23. 2.7.3. Serum dilutions
24. i) Dilute serum samples with working serum-diluting buffer (Section B.2.7.1 above) in small test
25. tubes (1.2 ml in strips of 8 or 12) with a multichannel pipette, starting at 1/25.
26. Note: Microtiter plates may also be used for making serum dilutions.
27. 2.7.4. Preparation of antigen mixture
28. i) For each plate, mix 2.5 ml working antigen-diluting buffer (see Sections B.2.4.1 and B.2.7.2
29. above), 35 µl 2-mercaptoethanol, 50 µl Evans blue dye solution (2 mg/ml water) and 0.15 ml
30. antigen (formalin-fixed whole parasites).
31. 2.7.5. Agglutination procedure
32. Agglutination is done in U-bottom 96-well microtiter plates.
33. i) Pipette 25 µl antigen mixture to each well immediately after mixing.
34. ii) Pipette 25 µl serum dilutions into the wells and mix gently with the antigen by repeated pipetting
35. action.
36. iii) A positive control should be included in each plate. The control should have a titre of 1/200, and
37. two-fold dilutions from 1/25 to 1/3200 should be used.
38. iv) Cover the plates with sealing tape and incubate overnight at 37°C.
39. v) Read results using a magnifying mirror. A blue button at the bottom of the well means negative.
40. A clear bottom means positive.

##### 2.8. Enzyme-linked immunosorbent assay

1. One of the first *T. gondii* enzyme-linked immunosorbent assay (ELISA) (Voller *et al*., 1976) used a soluble antigen
2. preparation made from *T. gondii* RH strain tachyzoites (as described below) and layered into wells in an ELISA
3. microtiter plate. Test sera are added, followed by a species-specific secondary antibody conjugated with a reactive
4. enzyme, such as horseradish peroxidase. Protein A/G conjugates were used to replace species-specific antibody
5. conjugates, making ELISAs applicable to more than one animal species. Any conjugated enzyme causes a colour
6. change in the substrate that is directly related to the amount of bound antibody, and which can be read with a
7. spectrophotometer at the absorbance wavelength specific to the substrate used. The assay is simple, can readily test
8. a large number of samples, and is easy to perform with the chosen anti-species conjugate. Defined anti-species
9. conjugates, substrates, and whole kits are commercially available. The ELISA is well suited for analysing large
10. numbers of samples. A large number of species-specific or multi-species ELISAs are commercially available to detect
11. *T. gondii* antibodies.
12. To improve the specificity of the conventional ELISA, native purified *T. gondii*-specific antigens have been used
13. (Basso *et al.*, 2013). In addition, severam recombinant antigens have been established, and these seem suitable for
14. replacing native antigens for serological diagnostic tests. For many of these recombinant antigen ELISAs, thorough
15. validation is lacking.
16. Clinically, there is a need to distinguish between recent (acute) and long-standing (chronic) infections. With the
17. conventional ELISA, detection of *Toxoplasma*-specific IgG and IgM antibodies, along with IgA, may permit some
18. discrimination between acute and chronic *T. gondii* infection. Assays assessing the avidity of an IgG response to
19. *T. gondii* have been applied in sheep and pigs. However, such avidity tests were used for research purpose only.

## 466 C. REQUIREMENTS FOR VACCINES

1. Currently there is only one commercially available live vaccine, which is licensed for use in breeding sheep in some regions
2. (Europe and New Zealand) to reduce the effects of *T. gondii* infection (e.g. early embryonic death, abortion). It consists of
3. ≥ 105 tachyzoites of the S48 strain of *T. gondii* that has been attenuated by multiple passages in mice. The vaccine
4. stimulates effective protective immunity for at least 18 months following a single intramuscular injection given at least 4
5. weeks prior to mating and only for use in healthy, non-pregnant female sheep. Despite the acknowledged importance of
6. human toxoplasmosis, human vaccines are currently unavailable, and the vaccine for sheep has disadvantages, such as
7. a short shelf-life (10 days), strict storage conditions, and, as a live vaccine, potential risk to operators. Information on the
8. production details of this vaccine and QC requirements are not available.
9. Although the importance of a killed or non-live vaccine is acknowledged – for vulnerable humans (e.g. women before they
10. are pregnant), for reducing abortions in sheep, for reducing tissue cysts in meat animals (pigs, cattle, chickens, etc.), and
11. for limiting oocyst shedding from kittens – to date this remains elusive (Innes *et al.,* 2019). However, with recent scientific
12. advances, including availability of genetic, transcriptomic, and metabolomic data, the potential for developing knockout
13. variants, and other new technologies suggest new possibilities for development of such a vaccine (Mevelec *et al.,* 2020;
14. Zhang *et al.*, 2022).

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557 **NB:** At the time of publication (2024) there were no WOAH Reference Laboratories

558 for toxoplasmosis (please consult the WOAH Web site:

559 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

560 **NB**: First adopted in 2004. Most recent updates adopted in 2017.